Pesticide residues in food 2022

Joint FAO/WHO Meeting on pesticide residues

Evaluation

Part II – Toxicological

Food and Agriculture Organization of the United Nations

World Health Organization

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Contents

* First full evaluation

** Evaluated within the periodic review programme of the Codex Committee on Pesticide Residues

Abbreviations and acronyms

AbD	absorbed dose
AChE	acetylcholinesterase
ACTH	adrenocorticotropic hormone
AD	administered dose
ADI	acceptable daily intake
ADME	absorption, distribution, metabolism and excretion
A/G, A:G	albumin:globulin ratio
ALP	alkaline phosphatase
ALT	alanine transaminase
APTT	activated partial thromboplastin time
AR	Administered (applied) radioactivity
ARfD	acute reference dose
AST	aspartate transaminase
AUC	Area under the concentration-time curve
BMD	benchmark dose
$BMDL_{10}$	Lower confidence limit on the benchmark dose for a 10% response
BROD	benzoxyresorufin-*O*-dealkylase
BrdU	5-bromo-2′-deoxyuridine
BUN	blood urea nitrogen
bw	body weight
ca	circa (approximately)
cAMP	cyclic adenosine monophosphate
CAR	constitutive androstane receptor
CAR/PXR	constitutive androstane receptor/pregnane X receptor
CAS	Chemical Abstracts Service (No.)
CCPR	Codex Committee on Pesticide Residues
CCRVDF	Codex Committee on Residues of veterinary Drugs in Foods
CEs	cholesterol esters
CEase	cholesterol esterase
ChE	cholinesterase
CHL	cultured Chinese hamster lung
CHO	Chinese Hamster ovary
CK	creatine kinase
C_{max}	maximum concentration
CMC	carboxymethyl cellulose
CNS	central nervous system
CPA	cyclophophamide
DMSO	dimethyl sulfoxide
DMF	dimethyl formamide
DNA	deoxyribonucleic acid
DNCB	2,4-dinitrochlorobenzene
EC3	the amount of test substance needed to induce a 3-fold increase in cell proliferation in a LLNA
EC_{50}	the half-effective concentration value
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
EMS	ethyl methanesulfonate
ENU	ethylnitrosourea
EPA	Environmental Protection Agency
EROD	ethoxyresorufin-O-deethylase
$F_{(abs)}$	bioavailability (absolute)
FAO	Food and Agriculture Organization of the United Nations
FOB	functional observational battery
FSH	Follicle-stimulating hormone
GC	gas chromatography
GC–MS	gas chromatography–mass spectrometry
$GABA_A$	γ-aminobutyric acid, type A
GD	gestation day
GEF	global evaluation factor
GGTP	γ-glutamyl transpeptidase/transferase
GI	gastrointestinal
GIT	gastrointestinal tract
GLP	good laboratory practice
GSD	geometric standard deviation
hAR	human androgen receptor
HBGV	health-based guidance values
Hb	haemoglobin
HCA	α-hexylcinnamaldehyde
HCB	hexchlorobenzene
HCD	historical control data
HDL	high-density lipoproteins
HDW	haemoglobin distribution width
hERα/β	Human estrogen receptor α/β
(U)HPLC	(ultra-)high-performance liquid chromatography

HPLC-MS/MS	tandem high-performance liquid chromatography/mass spectrometry
HPRT	hypoxanthine–guanine phosphoribosyl transferase
Ht	haematocrit
HTRF	homogeneous time-resolved fluorescence (assay)
^{3}H-TdR	tritium-labelled methyl thymidine
i.v.	intravenous/intravenously
IC_{50}	half-maximal inhibitory concentration
IE:TE	Ratio of immature to total erythrocytes
i.p.	intraperitonial, intraperitonially
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
i.v.	intravenous/intravenously
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
LAH	lactic acid hydroxylase
LC	liquid chromatography
LC-MS	liquid chromatography–mass spectrometry
LC_{50}	median lethal concentration
LD	lactation day
LD_{50}	median lethal dose
LDL	low-density lipoprotein
LH	luteinizing hormone
LLNA	local lymph node assay
LOAEL	lowest-observed-adverse-effect level
LOQ	limit of quantitation
LSC	liquid scintillation counting
LUCs	large, unstained cells
MA	motor activity
mADI	microbiological ADI
mARfD	microbiological ARfD
MCH	mean corpuscular/cell haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular (cell) volume
MF	mutation frequency
MPE	mean photo effect
MPV	mean platelet volume
MMAD	mass median aerodynamic diameter
MOA	mode of action
MN-IE	micronucleated immature erythrocytes
MPCE	multinucleated polychromatic erythrocyte
MRL	maximum residue limits
MS	mass spectroscopy/spectrometry
MTD	maximum tolerated dose
NCE	normochromic erythrocyte
NMR	nuclear magnetic resonance
NIS	sodium/iodide symporter
NOAEC	no-observed-adverse-effect conc.
NOAEL	no-observed-adverse-effect level
NTE	neuropathy target esterase
OECD	Organisation for Economic Co-operation and Development
PAS	periodic acid–Schiff stain
PB	phenobarbital (sodium)
PCE	polychromatic erythrocyte
PND	postnatal day
PP(D)	postpartum (day)
PROD	pentoxyresorufin-*O*-deethylase
PT	prothrombin time
PXR	pregnane X receptor
qPCR	quantitative polymerase chain reaction
QSAR	quantitative structure–activity relationship
RBC	red blood cell (erythrocyte)
rT3	reverse triiodothyronine
S9	Rat liver supernatant obtained by centrifuging at 9000 *g*
RTG	relative total growth
SCE	sister chromatid exchange
SD	standard deviation
SDH(I)	succinate dehydrogenase (inhibitor)
SI	stimulation index
sRBC	sheep red blood cell
$t_{½}$	half-life
T3	triiodothyronine
T4	thyroxine
TFT	5-trifluorothymidine
TLC	thin-layer chromatography

T_{max}	time to reach maximum concentration
TPO	thyroid peroxidase
TRR	total radioactive residue
TSH	thyroid-stimulating hormone
TTC	threshold of toxological concern
UDP-GT	uridine diphosphate glucuronosyltransferase
UDS	unscheduled DNA synthesis
WBC	white blood cell/leucocyte count
WHO	World Health Organization
v/v	volume/volume
w/v	weight/volume
w/w	weight/weight

Introduction

Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group on Pesticide Residues

Rome, Italy, 13–22 September 2022

Sponsored jointly by FAO and WHO

A Joint Meeting of the Food and Agriculture Organization of the United Nations (FAO) Panel of Experts on Pesticide Residues in Food and the Environment and the World Health Organization (WHO) Core Assessment Group on Pesticide Residues (JMPR) was held at FAO Headquarters, Rome (Italy), from 13 to 22 September 2022. FAO Panel Members met in preparatory sessions from 8 to 12 September.

The Meeting was opened by Dr Jingyuan Xia, Director, Plant Production and Protection Division (NSP), FAO. On behalf of FAO and WHO, Dr Xia welcomed and thanked the participants for providing their expertise and for devoting significant time and effort to the work of the JMPR, noting that this was the first physical JMPR meeting since 2019 due to the impact of the COVID-19 pandemic, with 45 participants from 15 countries.

Dr Xia highlighted food safety is fundamental to healthy and sustainable food systems. The establishment of pesticide residue standards is a key and critical element in the global effort to improve food safety and agricultural development in the world. The unique role of the JMPRs work in establishing internationally acceptable MRLs for pesticide residues in food and feed which acted as global benchmarks in trade facilitation, as well as providing authoritative assessments, important in consumer protection. Dr Xia then outlined how JMPR's efforts aligned with the Divisions strategic objectives of ensuring food security and nutrition; enhancing food quality and safety; supporting farmers' livelihoods; protecting the environment and biodiversity; and facilitating safe trade and economic growth. As the establishment of global standards were a key and critical element in the global efforts to improve food safety and agricultural development in the world.

Dr Xia also took the opportunity to express his appreciation, and called on the meeting participants to express theirs, to Madam YongZhen Yang, retiring FAO JMPR Secretariat, for her dedicated commitment and outstanding contribution in fulfilling the secretariat role over the past 16 years.

Mr Soren Madsen, WHO JMPR Secretariat, took the opportunity to thank the FAO for giving priority to JMPR to allow the meeting to occur at FAO headquarters.

During the meeting, the FAO Panel of Experts was responsible for reviewing residue and analytical aspects of the pesticides under consideration, including data on their metabolism, fate in the environment and use patterns, and for estimating the maximum levels of residues that might occur as a result of use of the pesticides according to good agricultural practice (GAP). Maximum residue levels (MRLs) and supervised trials median residue (STMR) values were estimated for commodities of animal origin. The WHO Core Assessment Group was responsible for reviewing toxicological and related data in order to establish acceptable daily intakes (ADIs) and acute reference doses (ARfDs), where necessary.

The Meeting evaluated 34 pesticides, including seven new compounds and four compounds that were re-evaluated within the periodic review programme of the Codex Committee on Pesticide Residues (CCPR), for toxicity, residues, or both.

The Meeting established ADIs and ARfDs, estimated MRLs and recommended them for use by CCPR, and estimated STMR and highest residue (HR) levels as a basis for estimating dietary intake.

The Meeting also estimated the dietary exposures (both short-term and long-term) of the pesticides reviewed and, on this basis, performed a dietary risk assessment in relation to the relevant ADI and where necessary ARfD. Cases in which ADIs or ARfDs may be exceeded were clearly indicated in order to facilitate the decision-making process by CCPR.

The Meeting considered a number of current issues related to the risk assessment of chemicals, the evaluation of pesticide residues and the procedures used to recommend MRLs.

Toxicological monographs and monograph addenda

Benzovindiflupyr (addendum)

First draft prepared by
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Explanation

Benzovindiflupyr is the approved International Organization for Standardization (ISO) name for *N*-[11-(dichloromethylidene)-3-tricyclo[6.2.1.02,7]undeca-2(7),3,5-trienyl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxamide (IUPAC), for which the Chemical Abstracts Service number is 1072957-71-1. Benzovindiflupyr (SYN545192) is a broad-spectrum foliar fungicide of the pyrazole chemical class. Technical benzovindiflupyr consists of the enantiomers SYN546526 and SYN546527, at a ratio of 1:1. Both enantiomers are fungicidally active. Benzovindiflupyr is a succinate dehydrogenase inhibitor (SDHI) and thus inhibits the citric acid cycle in fungi.

Benzovindiflupyr was previously evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) held in 2013 (JMPR, 2014). That meeting established an acceptable daily intake (ADI) of 0–0.05 mg/kg body weight (bw) based on an increased incidence of minimal hepatocellular pigmentation and reduced body weight gain in a two-year study in rats. An acute reference dose (ARfD) of 0.1 mg/kg bw was also established based on a no-observed-adverse-effect level (NOAEL) of 10 mg/kg bw for decreased motor activity at one hour following oral gavage dosing in an acute neurotoxicity study in rats.

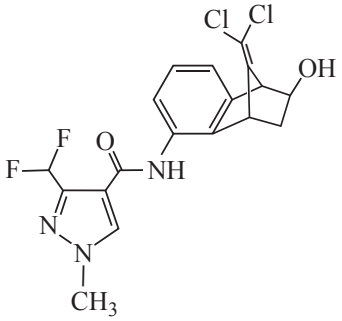
The compound was reviewed by the present meeting following a request from the Codex Committee on Pesticide Residues (CCPR) for additional maximum residue levels (MRLs), for which additional information on some of the metabolites was provided. All provided studies were compliant with good laboratory practice (GLP) unless otherwise stated, and were conducted in accordance with current test guideline requirements.

Evaluation for acceptable daily intake

1. Metabolites

1.1 Metabolite SYN546039 (CSCD695908)

Figure 1. Chemical structure of metabolite SYN546039 (CSCD695908)



Metabolite SYN546039 is a racemic mixture of:

- 3-difluoromethyl-1-methyl-*1H*-pyrazole-4- carboxylic acid (*1S,2S,4R*)-9-dichloromethylene-2-hydroxy-1,2,3,4-tetrahydro-1,4-methanonaphthalene-5-yl]-amide, and,
- [(*1R,2R,4S*)-9-dichloromethylene-2-hydroxy-1,2,3,4-tetrahydro-1,4-methano-naphthalene-5-yl]-amide))

It was previously assessed by JMPR 2013 (JMPR, 2014). Compound SYN546039 is a major rat metabolite of benzovindiflupyr, representing between ≥5% or greater and ≤19% or greater of absorbed dose and is present in plants, soil and surface water.

According to the 2013 JMPR evaluation of benzovindiflupyr:

(a) the acute oral LD_{50} of SYN546039 is greater than 2000 mg/kg bw in female rats;

(b) the 90-day dietary exposure NOAEL for SYN546039 in rats is 1000 mg/kg bw per day, the highest dose tested (doses: 0, 100, 300 and 1000 mg/kg bw per day; dietary concentrations not stated in the 2013 JMPR report);

(c) in a rabbit prenatal developmental toxicity study (doses: 0, 100, 300 and 1000 mg/kg bw per day) the maternal NOAEL for SYN546039 was 300 mg/kg bw per day, based on increased incidence of mortalities and abortions at 1000 mg/kg bw per day; the NOAEL for embryo/fetal toxicity was 300 mg/kg bw per day, based on the increased incidence of misshapen interparietal skull bone at 1000 mg/kg bw per day;

(d) SYN546039 did not induce bacterial reverse mutations.

For the current meeting, a previously evaluated oral toxicity study in rats and a previously evaluated bacterial reverse mutation study were supplied (Sieber, 2011; Sokolowski, 2011). The re-evaluation of these studies confirmed the conclusions of the 2013 JMPR evaluation. Overall, and consistent with the conclusions of the 2013 JMPR evaluation, SYN546039 is less toxic than its parent molecule: the NOAELs of benzovindiflupyr for 90-day study and developmental toxicity studies in rats were 8.2 and 15 mg/kg bw per day, for maternal and embryo/fetus, respectively . Based on the comparative data presented below in Table 1, SYN546039 is at least 10-fold less toxic than the parent molecule.

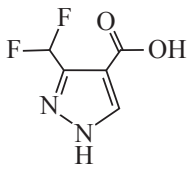
Table 1. Comparative toxicity of SNY546039 and its parent, benzovindiflupyr

Study type	SYN546039	Benzovindiflupyr
Acute oral LD_{50}	> 2000 mg/kg body weight	> 2000 mg/kg body weight
90-day/13-week dietary toxicity study	NOAEL: 1000 mg/kg bw per day (highest dose tested)	NOAEL: 8.2 mg/kg bw per day LOAEL: 58.8 mg/kg bw per day
Rabbit prenatal developmental toxicity study		
Maternal	NOAEL: 300 mg/kg bw per day LOAEL: 1000 mg/kg bw per day	NOAEL: 10 mg/kg bw per day LOAEL: 20 mg/kg bw per day
Embryo/fetal	NOAEL: 300 mg/kg bw per day LOAEL: 1000 mg/kg bw per day	NOAEL: 35 mg/kg bw per day (highest dose tested)
Genotoxicity[a]	Not genotoxic	Not genotoxic

[a] Based on an evaluation of the available genotoxicity test battery data; LOAEL: Lowest-observed-adverse-effect level

1.2 Metabolite SYN545720 (CSCD465008, R958945, DF-pyrazole acid)

Figure 2. Chemical structure of metabolite SYN545720 (CSCD465008, R958945, DF-pyrazole acid)



Metabolite SYN545720 has the formula, (difluoromethyl)-*1H*-pyrazole-4-carboxylic acid, and CAS Number 151734-02-0). It was assessed by JMPR 2013 (JMPR, 2014). Metabolite SYN545720 is present in plants, soil and water-sediment; it is not a metabolite in rats. According to the 2013 JMPR evaluation of benzovindiflupyr:

(a) the acute oral LD_{50} of SYN545720 in rats is greater than 2000 mg/kg bw;

(b) the 28-day dietary toxicity study NOAEL in rats was 1018 mg/kg bw per day, the highest dose tested (doses: 0, 2000, 6000 and 12 000 ppm, equal to 0, 175, 497 and 1018 mg/kg bw per day for males, 0, 176, 525 and 1107 mg/kg bw per day for females);

(c) SYN545720 was not genotoxic in a bacterial reverse mutation assay;

(d) SYN545720 was not genotoxic in a mouse lymphoma TK forward mutation assay;

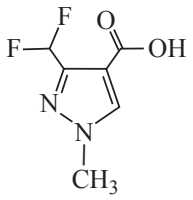
(e) SYN545720 was not genotoxic in an in vitro human lymphocyte chromosomal aberration assay.

No reproductive or developmental toxicity data was available for SYN545720.

For the current meeting an amended version of the previously evaluated 28-day dietary toxicity study in rats was provided. The study amendments made no material difference to the outcome of the study. The NOAEL for the 28-day dietary exposure study in rats remains at 12 000 ppm (equal to 1018 mg/kg bw per day, the highest dose tested; Buczynski, 2008). The conclusion of the JMPR 2013 evaluation of benzovindiflupyr was that SYN545720 was less toxic than its parent molecule. Based on the JMPR 2013 evaluation the present Meeting concluded that SYN545720 is not toxicologically relevant.

1.3 Metabolite NOA449410 (also known as CA4312, Sedaxane metabolite CSAA798670, R648993, Fluxapyroxad metabolite M700F001, DF-pyrazole acid)

Figure 3. Chemical structure of NOA449410



Metabolite NOA449410 has the formula, 3-(difluoromethyl)-1-methyl-*1H*-pyrazole-4-carboxylic acid, and the CAS Number 176969-34-9. It was assessed by JMPR in 2012 (JMPR, 2013) as part of the evaluation of fluxapyroxad. Metabolite NOA449410 is a plant, ground water sediment and groundwater metabolite of benzovindiflupyr. It is also an animal food residue derived from pydiflumetofen. A food residue evaluation of NOA449410 derived from pydiflumetofen was completed in 2018 (JMPR, 2019). A summary of these previous evaluations of NOA449410 is shown in Table 2.

Table 2. Summary of previous JMPR Findings on NOA449410

Toxicological evaluation of NOA449410			
	Study type	**Key Effects**	**NOAEL/LOAEL**
Fluxapyroxad toxicological monograph evaluation of M700F001 (NOA449410) at JMPR 2012 (JMPR, 2013)	Acute oral toxicity in rats	-	LD_{50} > 2000 mg/kg bw
	90-day dietary exposure toxicity study (dietary concentrations used in the study were not reported in the 2012 JMPR evaluation)	No adverse effects at the highest dose	NOAEL: 954 mg/kg bw per day, the highest dose tested
	In vitro and in vivo genotoxicity test battery (assays used were not reported in the 2012 JMPR evaluation)	Negative	Not genotoxic
	Rabbit prenatal developmental toxicity study (doses used in the study were not reported in the 2012 JMPR evaluation)	No adverse effects at the highest dose	Maternal and developmental NOAEL: 250 mg/kg bw per day, the highest dose tested
Food residue evaluation of NOA449410			
Pydiflumetofen food residue evaluation of NOA449410 at JMPR 2018 (JMPR, 2019)	NOA449410 was not a major food residue in hen commodities (present as < 10% of the total radioactive residue)		
	NOA449410 was not a major food residue in goat commodities except for the kidney (present at 12% of the total radioactive residue in the kidney but at < 10% in all other edible tissues)		

Some of the studies that had been previously evaluated by JMPR were resubmitted for the current meeting. Evaluation of these studies confirm the results of the previous JMPR assessments in 2012, and 2018 (JMPR, 2013, 2019), specifically:

(a) the acute oral toxicity of NOA449410 in rats was greater than 2000 mg/kg bw;

(b) NOA449410 was not genotoxic in bacterial reverse mutation assay;

(c) NOA449410 was not genotoxic in an in vitro human lymphocyte chromosomal aberration assay;

(d) NOA449410 is not genotoxic in an in vitro ($TK^{+/-}$) in mouse lymphoma L5178Y cell assay;

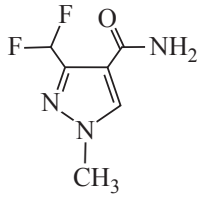
(e) in a 28-day dietary study of NOA449410 in rats the NOAEL was 1043 mg/kg bw per day, the highest dose tested (doses: 0, 2000, 6000, and 12 000 ppm, equal to 167, 511, and 1007 mg/kg per day for males, 175, 572, and 1043 mg/kg per day for females);

(f) in a 90-day feeding study in rats the NOAEL for NOA449410 was 983.1 mg/kg bw per day, the highest dose tested (dietary concentrations were adjusted weekly; achieved doses equal to 0, 94.6, 285.7, 953.6 mg/kg bw per day in males, 0, 98.8, 295.1, 983.1 mg/kg bw per day in females).

Overall, the new data supports the conclusion that NOA449410 is less toxic than its parent molecule. Based on the JMPR 2012 and 2018 evaluations the present Meeting concluded that NOA449410 is not toxicologically relevant.

1.4 Metabolite SYN508272

Figure 4. The chemical structure of metabolite SYN508272
(also known as CSCC210616, R423363, Reg. No. 5621781, Metabolite of BAS 700 F)



Metabolite SYN508272 has the formula, 3-difluoromethyl-1-methyl-*1H*-pyrazole-4-carboxylic acid amide, and the CAS Number 925689-10-7. It is a plant, animal, soil (via photolysis) and water sediment (via photolysis) metabolite of benzovindiflupyr. It is also an animal metabolite of pydiflumetofen and a downstream metabolite of SYN548263, a major metabolite of pydiflumetofen in rats, as well as a soil degradant of sedaxane and a metabolite of fluxapyroxad. Metabolite SYN508272 was not assessed as part of the 2013 JMPR feed/food residues evaluation of benzovindiflupyr (JMPR, 2014). The studies submitted for this meeting had been previously evaluated as part of the toxicological monograph from JMPR 2018 on pydiflumetofen (JMPR, 2019).

Based on the acute toxic class method, the acute oral median lethal dose (LD_{50}) of SYN508272 was greater than 500 mg/kg bw but less than 2000 mg/kg bw. Clinical observation in the 2000 mg/kg bw test group revealed impaired state and poor general condition, dyspnoea, ataxia, tremor, staggering, twitching, abdominal position and piloerection at hour 0 until hour 5 following administration. No clinical signs or findings were observed in the animals of the first 500 mg/kg bw group. In contrast, one animal of the second 500 mg/kg bw group showed impaired general state, dyspnoea, piloerection, chromodacryorrhea and reduced faeces from hour 4 until study day 1 (Cords, 2009). These conclusions were consistent with the previous JMPR evaluation (JMPR, 2019).

In a 28-day oral dietary toxicity study, four groups of five male and five female Han Wistar rats (Crl:WI(Han)) were fed diets containing SYN508272 (purity not stated) at 0, 100, 500 or 2000 ppm (males) / 4000 ppm (females), equal to 0, 7.3, 37.4 and 143.1 mg/kg bw per day for males, 0, 7.8, 42.5 and 243.5 mg/kg bw per day for females. Dietary exposure to SYN508272 resulted in no adverse effects on mortality, to clinical signs, functional observational battery results, haematology, clinical chemistry, coagulation parameters, urinalysis parameters, organ weights or anatomic pathology findings. Mean body weight, weight gain and food consumption in females receiving 4000 ppm were lower throughout the treatment period compared to controls; body weight gain in this cohort was 77.9% compared to controls at study termination ($p < 0.05$). This correlated with reduced food consumption in these animals, suggesting lower palatability. Cumulative body weight change (–40.6% compared with controls; $p < 0.05$) and food consumption in males receiving 2000 ppm were also lower than controls for the first 12 days of the treatment. However, body weight recovered and by the end of the study was not significantly ($p < 0.05$) different from controls. Food consumption was lower for the first eight days in the high dose male cohort. Consistent with the previous evaluation at JMPR 2018, the Meeting noted that these effects on body weight and food consumption may have been due to palatability issues. The NOAEL was 2000 ppm (equal to 143.1 mg/kg bw per day), the highest dose tested (Dymarkowska, 2015).

Based on the evaluation from JMPR 2013, the equivalent 28-day oral dietary exposure NOAEL for benzovindiflupyr is 400 ppm (equal to 36 mg/kg bw per day), based on lower body weights in both sexes at 1200 ppm (equal to 90 mg/kg bw per day).

Table 3. Adverse findings in the 28-day oral dietary toxicity study of SYN508272 in rats

		Dose (ppm)							
		Males				Females			
Parameter		**0**	**100**	**500**	**2000**	**0**	**100**	**500**	**4000**
Body weight gain, mean (g)	Days 0–12	37	40	49	22*	18	19	25	−2*
	Days 0–28	73	74	82	69	36	37	43	8*
Mean food intake (g/animal/day)	Days 0 – 8	24.6	24.0	24.7	21.7	17.7	17.7	17.9	12.3

* Statistically significant: $p < 0.05$

Metabolite SYN508272 was genotoxic in an in vitro human lymphocyte chromosomal aberration assay, but not genotoxic in an in vitro ($TK^{+/-}$) mouse lymphoma L5178Y cell assay and in an in vivo rat bone marrow micronucleus assay (in vivo blood level of SYN508272 was approximately 276 µg/mL between two and 24 hours following dosing; JMPR, 2019). SYN508272 is unlikely to be genotoxic.

In the JMPR 2018 evaluation of pydiflumetofen it was concluded that the toxicological profile of this metabolite was the same as that of the parent. Pydiflumetofen is not a human-relevant carcinogen, did not adversely affect reproduction or development and was not neurotoxic. As with SYN508272 the predominant effects of repeated oral exposure to pydiflumetofen were on body weight. Hence, the toxicological profile of SYN508272 was adequately characterized by the studies of systemic toxicity.

Overall, the meeting concluded that the toxicological properties of SYN508272 resemble those of benzovindiflupyr. Accordingly, human exposure to SYN508272 should be adequately covered by the health-based guidance values (HBGVs) of the parent benzovindiflupyr.

2. Microbial aspects

The possible impact of benzovindiflupyr residues on human intestinal microbiome was evaluated.

A search on literature available in the public domain did not identify information describing any direct or indirect experimental evidence to address the impact of benzovindiflupyr residues on the human intestinal microbiome. No experimental data was submitted by the sponsor in this regard.

Comments

Toxicological data on metabolites and/or degradates

SYN546039 (CSCD695908) ***see Fig. 1***

Based on the JMPR 2013 evaluation (JMPR, 2014) the present Meeting concluded that SYN546039 is at least 10-fold less toxic than its parent molecule.

SYN545720 (CSCD465008, R958945) ***see Fig.2***

Metabolite SYN545720 was assessed at JMPR 2013. Based on the JMPR 2013 evaluation (JMPR, 2014) the present Meeting concluded that SYN545720 is not toxicologically relevant.

NOA449410 (CA4312; also sedaxane metabolite CSAA798670, R648993 and fluxypyroxad metabolite M700F001) ***see Fig. 3***

For the current meeting several studies were submitted that have been previously evaluated by JMPR for other compounds: the metabolite is common to benzovindiflupyr, fluxapyroxad (JMPR 2013) and pydiflumetofen (JMPR 2019). Based on the JMPR 2012 and 2018evaluations (JMPR, 2013, 2019) the present Meeting concluded that NOA449410 is not toxicologically relevant.

SYN508272 (CSCC210616; R423363; Reg. No. 5621781; Metabolite of BAS 700 F) ***see Fig. 4***

For the current meeting several studies were submitted that have been previously evaluated by JMPR for other compounds: SYN508272 is also a metabolite of pydiflumetofen (JMPR 2019). Based on read-across from the pydiflumetofen evaluation, SYN508272 is unlikely to be carcinogenic, a reproductive and developmental toxicant or a neurotoxicant. Overall, the Meeting concluded that the toxicological properties of SYN508272 resemble those of benzovindiflupyr. Accordingly, human exposure to SYN508272 should be adequately covered by the health-based guidance values (HBGVs) of the parent benzovindiflupyr.

Toxicological evaluation

The Meeting concluded that NOA449410 and SYN545720 are not toxicologically relevant. Metabolite SYN508272 should be adequately covered by the ADI of 0–0.05 mg/kg bw and the ARfD of 0.1 mg/kg bw established for the parent, benzovindiflupyr. Metabolite SYN546039 is at least 10-fold less toxic than benzovindiflupyr.

Summary

	Value	Study	Safety factor
ADI [a]	0–0.05 mg/kg bw [b]	Two-year toxicity study (rat);	100
ARfD [a]	0.1 mg/kg bw [b]	Acute neurotoxicity study (rat)	100

[a] From JMPR 2013

[b] Applies to benzovindiflupyr and SYN508272

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Benzpyrimoxan

First draft prepared by
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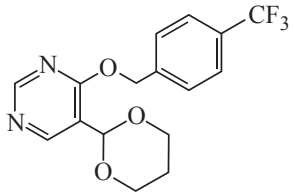
Explanation

Benzpyrimoxan is the ISO-approved common name for 5-(1,3-dioxan-2-yl)-4-[4-(trifluoromethyl) benzyloxy]pyrimidine (IUPAC), Chemical Abstracts Service number 1449021-97-9. Benzpyrimoxan is a new insecticide having biological activity towards rice plant hoppers (Hemiptera: Delphacidae). Benzpyrimoxan displays strong activity towards the nymphal stages of rice plant hoppers, but lacks activity towards the adults.

Benzpyrimoxan has not previously been evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and was reviewed by the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR). All critical studies were performed according to national or international test guidelines and contained statements of compliance with good laboratory practice (GLP) unless otherwise specified. No information was identified in the public domain from a search of the PubMed, Google Scholar and Web of Science databases, not surprisingly for such a new chemical with an unknown pesticidal mode of action (MOA).

Figure 1. Structural formula of benzpyrimoxan



Evaluation for acceptable daily intake

1. Biochemical aspects

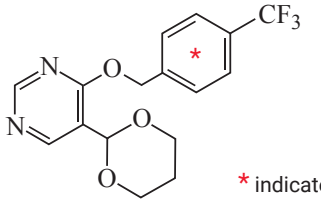
The absorption, distribution, metabolism, excretion and toxicokinetic properties of benzpyrimoxan have been investigated in the rat using two different radiolabelled test materials (on the phenyl and pyrimidinyl rings) administered orally. A study in bile duct-cannulated rats with the two labels was also available. In addition an in vitro comparative metabolism study was submitted.

1.1 Absorption, distribution and excretion

(a) Oral route

Absorption, distribution, metabolism and excretion (ADME) study with phenyl-labelled benzpyrimoxan in rats

Figure 2. Position of radiolabel in phenyl-labelled benzpyrimoxan



A study (OECD 417) was designed and conducted to investigate the absorption, distribution, metabolism and excretion of [*phenyl-U*-^{14}C]benzpyrimoxan following a single oral administration to rats. Male and female Wistar rats were given 1 or 100 mg/kg bw of [*phenyl-U*-^{14}C]benzpyrimoxan suspended in an 0.5% w/v aqueous solution of sodium carboxymethyl cellulose (CMC) also containing 0.1% w/v Tween 80. The dose group design is shown in Table 1.

Table 1. Design of absorption, distribution, metabolism and excretion study with phenyl-labelled benzpyrimoxan

Group code [a]	Dose (mg/kg bw)	Number of animals	Sex	Sampling times (hours postdose)
BML	1	4	Male	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
BMH	100	4	Male	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
BFL	1	4	Female	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
BFH	100	4	Female	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
BML-1	1	4	Male	Organs/tissues: 6
BML-2	1	4	Male	Organs/tissues: 24
BML-3	1	4	Male	Expired air: 24 Urine: 24, 48, 72, 96,120, 144, 168 Faeces: 24, 48, 72, 96,120, 144, 168 Organs/tissues: 168
BMH-1	100	4	Male	Organs/tissues: 9
BMH-2	100	4	Male	Organs/tissues: 48
BMH-3	100	4	Male	Expired air: 24 Urine: 24, 48, 72, 96,120, 144, 168 Faeces: 24, 48, 72, 96,120, 144, 168 Organs/tissues: 168
BFL-1	1	4	Female	Organs/tissues: 6
BFL-2	1	4	Female	Organs/tissues: 24
BFL-3	1	4	Female	Expired air: 24 Urine: 24, 48, 72, 96,120, 144, 168 Faeces: 24, 48, 72, 96,120, 144, 168 Organs/tissues: 168
BFH-1	100	4	Female	Organs/tissues: 9
BFH-2	100	4	Female	Organs/tissues: 48
BFH-3	100	4	Female	Expired air: 24 Urine: 24, 48, 72, 96,120, 144, 168 Faeces: 24, 48, 72, 96,120, 144, 168 Organs/tissues: 168

[a] B: Benzpyrimoxan; M: Male; F: Female; L: Low dose; H: High dose; Source: Yasunaga, 2018a

Absorption

Blood samples were collected from male and female rats after 1, 3, 6, 9, 12 and 24 hours, and every 24 hours until 168 hours post dose. Concentrations of radioactivity in blood and plasma were quantified by liquid scintillation counting.

At the low dose (1 mg/kg bw; groups BML and BFL), radioactivity in blood and plasma reached maximum concentrations (T_{max}) at 1–9 hours post dose and rapidly decreased in a biphasic pattern. After attaining their maximum, radioactivity concentrations in blood decreased with half-lives ($t_{½}$) of 0.47 (males) and 0.54 days (females), and in plasma 0.40 (males) and 0.44 days (females) in the first phase. In the second phase the corresponding values were 1.71 (males) and 2.55 days (females) for blood and 1.67 (males) and 2.63 days (females) for plasma. At the high dose (100 mg/kg bw; groups BMH and BFH), the kinetics parameters in blood and plasma showed comparable profiles to those at the low dose, except for the area under the concentration–time curve (AUC) which was almost 80 times greater, that is, slightly less than dose-proportional, and the T_{max} which was 12 hours post dosing. After attaining their maximum, radioactivity concentrations in blood decreased with half-lives of 0.58 (males) and 0.63 days (females), and in plasma 0.52 (males) and 0.54 days (females) in the first phase. In the second phase the corresponding values were 2.11 (males) and 3.30 days (females) for blood and 1.75 days for both in plasma. Dose level had no effect on $t_{½}$. No significant sex-related differences were noted.

Table 2. Concentrations of radioactivity (µg equiv. benzpyrimoxan/g) and kinetic parameters in blood and plasma following a single oral administration of [phenyl-U-^{14}C] benzpyrimoxan

Time point (hours)	Dose level (ppm)							
	Males				Females			
	1 mg/kg bw		100 mg/kg bw		1 mg/kg bw		100 mg/kg bw	
	Blood	Plasma	Blood	Plasma	Blood	Plasma	Blood	Plasma
1	0.686 ± 0.080	0.737 ± 0.109	8.3 ± 2.7	11.1 ± 4.4	0.566 ± 0.086	0.701 ± 0.127	8.8 ± 2.0	13.9 ± 5.5
3	0.560 ± 0.066	0.638 ± 0.098	13.7 ± 4.5	17.7 ± 6.7	0.461 ± 0.113	0.587 ± 0.131	18.4 ± 3.9	24.8 ± 7.2
6	0.525 ± 0.133	0.778 ± 0.232	12.9 ± 4.4	16.7 ± 6.6	0.435 ± 0.078	0.707 ± 0.165	18.4 ± 5.9	25.1 ± 10.3
9	0.458 ± 0.083	0.752 ± 0.177	19.5 ± 2.2	25.8 ± 3.9	0.434 ± 0.127	0.740 ± 0.235	24.7 ± 7.6	33.0 ± 11.1
12	0.358 ± 0.066	0.577 ± 0.127	20.6 ± 3.0	26.8 ± 5.3	0.402 ± 0.129	0.626 ± 0.158	25.5 ± 4.6	33.4 ± 5.9
24	0.074 ± 0.013	0.107 ± 0.019	13.8 ± 4.2	21.6 ± 6.1	0.124 ± 0.040	0.189 ± 0.060	17.1 ± 1.4	26.5 ± 1.8
48	0.028 ± 0.017	0.020 ± 0.007	5.3 ± 5.5	4.7 ± 5.5	0.028 ± 0.007	0.033 ± 0.011	4.4 ± 2.4	4.9 ± 2.5
72	0.010 ± 0.003	0.010 ± 0.002	1.3 ± 0.5	1.3 ± 0.4	0.018 ± 0.015	0.014 ± 0.004	1.4 ± 0.5	1.4 ± 0.4
96	0.012 ± 0.011	0.006 ± < 0.001	0.8 ± 0.2	0.7 ± 0.1	0.011 ± 0.008	0.009 ± 0.003	1.2 ± 0.6	0.8 ± 0.1
120	0.006 ± 0.002	0.005 ± < 0.001	0.9 ± 0.6	0.4 ± 0.1	0.008 ± 0.005	0.007 ± 0.003	0.7 ± 0.2	0.5 ± 0.1
144	0.005 ± 0.002	0.006 ± 0.006	0.8 ± 0.4	0.4 ± 0.1	0.005 ± 0.001	0.004 ± 0.001	0.5 ± 0.2	0.4 ± < 0.1
168	0.004 ± 0.002	0.003 ± < 0.001	0.4 ± 0.1	0.2 ± < 0.1	0.004 ± < 0.001	0.003 ± 0.001	0.4 ± 0.1	0.3 ± < 0.1
T_{max} (hours)	1	6	12	12	1	9	12	12
C_{max} (µg equiv./g)	0.686	0.778	20.6	26.8	0.566	0.740	25.5	33.4
Half-life (days)								
T_{max}–72 h	0.47	0.40	0.63	0.54	0.54	0.44	0.58	0.52
72–168 h	2.55	2.63	3.30	1.75	1.71	1.67	2.11	1.75
AUC_{0-168} (µg equiv. × hour/g)	10.909	14.699	771.5	961.7	11.594	16.590	891.6	1180.8

Source: Yasunaga, 2018a

Distribution

Following administration of [*phenyl-U-*^{14}C]benzpyrimoxan, male and female rats were terminated at six hours (around T_{max}), 24 and 168 hours post treatment at the low dose (1 mg/kg bw; groups BML-1–3 and BFL-1–3). For the high dose groups (100 mg/kg bw; BMH-1–3 and BFH-1–3) the times were nine hours (around T_{max}), 48 and 168 hours post treatment.

No significant sex- or dose-related differences were noted in the distribution profile of [*phenyl-U-*^{14}C]benzpyrimoxan. At around the T_{max} the highest radioactivity was found in the gastrointestinal (GI) contents, followed by liver, plasma, GI and kidney. By 24 hours post dose, radioactivity concentrations in all organs and tissues had significantly decreased, and by 168 hours post dose, they were nearly negligible, suggesting that no organs or tissues specifically retained [*phenyl-U-*^{14}C]benzpyrimoxan and/or its metabolites.

Table 3. Distribution of radioactivity (µg equiv. benzpyrimoxan/g) following a single oral administration of [phenyl U-^{14}C]benzpyrimoxan in selected organs

Organ/Tissue	Dose level					
	1 mg/kg bw			100 mg/kg bw		
	Males					
	6 hours	24 hours	168 hours	9 hours	48 hours	168 hours
Gastrointestinal contents	2.152 ± 0.117	0.099 ± 0.083	< 0.001 ± < 0.001	384.9 ± 120.5	1.5 ± 0.9	< 0.1 ± < 0.1
Liver	1.114 ± 0.242	0.123 ± 0.018	0.014 ± 0.003	54.0 ± 27.5	3.2 ± 1.0	0.8 ± 0.3
Plasma	0.886 ± 0.157	0.090 ± 0.066	–	23.9 ± 9.5	1.6 ± 0.8	< 0.1 ± 0.1
Kidney	0.616 ± 0.161	0.082 ± 0.022	0.006 ± 0.001	40.2 ± 18.1	2.5 ± 1.1	1.0 ± 0.9
Blood	0.585 ± 0.071	0.059 ± 0.043	0.002 ± < 0.001	20.6 ± 10.7	1.3 ± 0.6	0.3 ± 0.2
	Females					
Gastrointestinal contents	1.517 ± 0.270	0.090 ± 0.048	< 0.001 ± < 0.001	351.9 ± 46.7	3.9 ± 5.1	< 0.1 ± < 0.1
Liver	0.662 ± 0.166	0.136 ± 0.056	0.009 ± 0.001	38.8 ± 13.8	5.7 ± 1.8	1.2 ± 0.4
Plasma	0.694 ± 0.246	0.150 ± 0.068	0.001 ± < 0.001	25.8 ± 9.7	3.2 ± 1.6	0.1 ± 0.2
Kidney	0.687 ± 0.140	0.140 ± 0.030	0.007 ± 0.002	61.8 ± 25.6	7.9 ± 4.8	1.2 ± 0.3
Blood	0.399 ± 0.121	0.097 ± 0.048	0.002 ± < 0.001	19.1 ± 7.1	2.6 ± 1.3	0.3 ± 0.2

Source: Yasunaga, 2018a

Excretion

Following administration of [*phenyl-U-*^{14}C]benzpyrimoxan, excreta (urine and faeces) from male and female rats were collected every 24 hours until 168 hours post treatment at the low dose (1 mg/kg bw; groups BML-3 and BFL-3) and the high dose (100 mg/kg bw; groups BMH-3 and BFH-3). Radioactivity in the excreta was analysed by liquid scintillation counting. Expired air was collected up to 24 hours post dosing.

At both doses, recovery of radioactivity by 168 hours post-treatment was ≥ 95% of the initially administered radioactivity, and hence acceptable. Radioactivity in the carcass at the end of the study was not quantified as total recovery was ≥ 95% of the administered radioactivity. Radioactivity was rapidly excreted via urine and faeces. At the low dose, radioactivity in urine and faeces accounted for 57.6–65.4% and 33.3–41.3% of the administered radioactivity by 168 hours post-dosing, respectively. At the high dose, radioactivity in urine and faeces accounted for 45.5–46.1% and 53.9–54.2% of the administered radioactivity by 168 hours post-dosing, respectively. These data indicate that at the high dose of 100 mg/kg bw, more radioactivity was eliminated via faeces, possibly resulting from unabsorbed material. No radioactivity in expired air was detected. No significant sex-related differences in the excretion profile of [*phenyl-U-*^{14}C] benzpyrimoxan were noted.

Table 4. Excretion rates of phenyl-labelled benzpyrimoxan in urine, faeces and expired air

Dose		1 mg/kg bw		100 mg/kg bw	
Sex		Male	Female	Male	Female
Urine	0–24 hours	61.9	54.4	41.3	39.9
	0–72 hours	64.7	57.0	45.0	45.4
	0–168 hours	65.4	57.6	45.5	46.1
Faeces	0–24 hours	30.5	35.6	48.5	46.7
	0–72 hours	33.2	41.1	53.8	54.0
	0–168 hours	33.3	41.3	53.9	54.2
Expired air	0–24 hours	ND	ND	ND	ND
Cage washings[a]		0.12	0.15	0.08	0.07

[a] Collected at 168 hours post dose;

Source: Yasunaga, 2018a

Metabolism

Excretion of the administered radioactivity (AR) via urine and faeces was almost complete within 48 hours post dosing in all groups. Therefore excreta obtained by 48 hours post dosing were subjected to metabolite analysis. Additionally, plasma, liver and kidney samples obtained at around the T_{max} (six or nine hours post dosing) and plasma samples obtained at 24 hours post dosing were also subjected to metabolite analysis.

No significant sex-related or dose-related differences in the metabolic profile of [*phenyl-U*-^{14}C]benzpyrimoxan were noted.

In urine, no unchanged parent compound was detected. NNI-1501-acid-2-OH (DH-05 or M5) was the only major (greater than 10% AR) metabolite, accounting for 18–28% of the administered dose (AD) at the low dose. Other significant metabolites were NNI-1501-benzyl-N-Ac-cysteine (DH-401 or M15; 7–8% AR at the low dose), NNI-1501-benzoyl-glycine (DH-402 or M16; 4–7% AR at the low dose) and NNI-1501-benzoic acid conjugates (DH-101 conjugates or M10 conjugates; 1.5–6% AR at the low dose). In addition the following were identified as minor metabolites:

- NNI-1501-acid (DH-01 or M1),
- NNI-1501-CH_2OH (DH-02 or M2),
- NNI-1501-CH_2OH-2-OH (DH-06 or M6),
- NNI-1501-CH_2OH-oxide (DH-22 or M9),
- NNI-1501-benzoic acid (DH-101 or M10),
- NNI-1501-benzyl alcohol (DH-102),
- NNI-1501-benzaldehyde (DH-103),
- NNI-1501-CH_2OH (DH-02 or M2) conjugates,
- NNI-1501-CH_2OH-2-OH (DH-06 or M6) conjugates,
- NNI-1501-CH_2OH-oxide (DH-22 or M9) conjugates
- NNI-1501-benzyl alcohol (DH-102) conjugates.

In faeces, unchanged parent compound was detected in small amounts (0.19–0.39% AR) at the low dose but at much higher levels (24–29% AR) at the high dose. The most abundant metabolite in faeces at both dose levels was NNI-1501-acid-2-OH (DH-05 or M5), accounting for 14–29% of AR. In addition the following were identified as minor metabolites:

- NNI-1501-acid (DH-01 or M1),
- NNI-1501-CH_2OH (DH-02 or M2),
- NNI-1501-CH_2OH-2-OH (DH-06 or M6),
- NNI-1501-benzoic acid (DH-101 or M10),
- NNI-1501-benzyl alcohol (DH-102),
- NNI-1501-benzyl-N-Ac-cysteine (DH-401 or M15)
- NNI-1501-benzoyl-glycine (DH-402 or M16).

Details of this excreted metabolite study are shown in Table 5.

Table 5. Metabolites in urines and faeces following a single oral administration of [phenyl-U-^{14}C] benzpyrimoxan to male and female rats

Metabolites	Metabolites excreted (% of administered radioactivity) [a]							
	Male				Female			
	1 mg/kg bw		100 mg/kg bw		1 mg/kg bw		100 mg/kg bw	
	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces
NNI-1501	ND	0.39	ND	23.73	ND	0.19	ND	28.70
NNI-1501-acid (DH-01)	0.17	ND	0.16	ND	0.23	0.07	0.47	ND
NNI-1501-CH_2OH (DH-02)	0.05	1.12	0.22	4.02	0.17	1.70	1.26	3.85
NNI-1501-acid-2-OH (DH-05)	18.10	20.75	21.62	18.27	28.10	29.03	21.13	14.34
NNI-1501-CH_2OH-2-OH (DH-06)	0.79	0.51	1.21	1.11	1.33	0.44	1.69	0.76
NNI-1501-CH_2OH-oxide (DH-22)	4.80	ND	0.81	ND	1.84	ND	0.66	ND
NNI-1501-benzoic acid (DH-101)	2.66	0.81	1.15	0.16	1.10	0.61	1.04	ND
NNI-1501-benzyl alcohol (DH-102)	0.23	0.35	0.05	ND	0.32	ND	0.07	ND
NNI-1501-benzaldehyde (DH-103)	0.27	ND	ND	ND	ND	ND	ND	ND
NNI-1501-benzyl-*N*-Ac-cysteine (DH-401)	7.94	0.15	3.49	0.06	6.78	0.38	2.90	ND
NNI-1501-benzoyl-glycine (DH-402)	7.40	ND	1.41	ND	3.73	0.10	1.91	ND
DH-02 conjugates (glucuronide/sulfate)	0.86 (0.69/0.17)	NE	2.26 (1.60/0.66)	NE	0.91 (0.77/0.14)	NE	4.39 (3.50/0.89)	NE
DH-06 conjugates (glucuronide/sulfate)	ND (ND/ND)	NE	0.09 (ND/0.09)	NE	ND (ND/ND)	NE	0.14 (0.13/0.01)	NE
DH-22 conjugates (glucuronide/sulfate)	1.36 (1.18/0.17)	NE	0.64 (0.48/0.15)	NE	0.35 (0.24/0.12)	NE	0.50 (0.14/0.37)	NE
DH-101 conjugates (glucuronide/sulfate)	6.10 (1.65/4.45)	NE	3.93 (1.57/2.36)	NE	1.50 (0.47/1.03)	NE	2.18 (0.80/1.38)	NE
DH-102 conjugates (glucuronide/sulfate)	3.11 (2.83/0.28)	NE	0.77 (0.60/0.17)	NE	0.38 (0.34/0.04)	NE	0.48 (0.12/0.36)	NE
Polar metabolites	1.98	1.56	2.08	1.35	3.33	1.61	2.04	1.20
Others	8.25 [b]	1.60	4.74	1.07	6.41 [c]	1.43	3.85	1.29
MeCN/0.1N HCl extract [d]	-	2.06	-	1.12	-	1.99	-	1.14
MeCN/1N HCl extract [d]	-	1.51	-	1.22	-	1.67	-	1.17
Unextractable [d]	-	2.13	-	1.45	-	1.57	-	1.21
Total	64.07	32.96	44.62	53.56	56.46	40.78	44.70	53.68

ND: Not detected; Source: Yasunaga, 2018a

NE: Not examined because radioactivity retained at TLC origin in faeces was less than 5% of dose;

[a] All values are mean of four individuals. Urine and faeces obtained until 48 hours post dose were analysed

[b] No individual component exceeded 2.88% of dose in others

[c] No individual component exceeded 3.08% of dose in others

[d] In urine, all samples were analysed without extraction

In plasma, no unchanged parent compound was detected at either dose. At the low dose NNI-1501-benzoic acid (DH-101 or M10) was a major metabolite accounting for 79–86% of the total radioactive residue (TRR) in this matrix at six hours post dosing (around T_{max}). At the high dose, NNI-1501-benzoic acid (DH-101 or M10) and NNI-1501-acid (DH-01 or M1) were significant metabolites, accounting for 23–30% and 21–32% of the plasma TRR respectively at nine hours post dosing (around T_{max}). In addition the following were identified as minor metabolites:

- NNI-1501-CH_2OH (DH-02 or M2),
- NNI-1501-acid-2-OH (DH-05 or M5),
- NNI-1501-CH_2OH-2-OH (DH-06 or M6),
- NNI-1501-CH_2OH-oxide (DH-22 or M9),
- NNI-1501- benzyl alcohol (DH-102),
- NNI-1501-benzyl-N-Ac-cysteine (DH-401 or M15)
- NNI-1501-benzoyl-glycine (DH-402 or M16).

Table 6. Plasma metabolites at T_{max} following a single oral administration of [phenyl-U-^{14}C]benzpyrimoxan to male and female rats

Metabolites	Radioactivity concentration (% TRR)			
	Male		Female	
	1 mg/kg	100 mg/kg	1 mg/kg	100 mg/kg
NNI-1501	ND	ND	ND	ND
NNI-1501-acid (DH-01)	2.33	21.41	6.63	32.24
NNI-1501-CH_2OH (DH-02)	ND	7.85	0.59	4.20
NNI-1501-acid-2-OH (DH-05)	1.25	4.41	1.99	6.17
NNI-1501-CH_2OH-2-OH (DH-06)	ND	3.01	ND	1.59
NNI-1501-CH_2OH-oxide (DH-22)	3.09	6.55	1.70	1.46
NNI-1501-benzoic acid (DH-101)	85.86	29.81	79.28	22.67
NNI-1501-benzyl alcohol (DH-102)	0.70	3.03	0.16	1.19
NNI-1501-benzaldehyde (DH-103)	ND	ND	ND	ND
NNI-1501-benzyl-*N*-Ac-cysteine (DH-401)	1.92	2.71	0.20	0.91
NNI-1501-benzoyl-glycine (DH-402)	0.19	ND	ND	ND
Polar metabolites	ND	0.36	0.20	3.43
Others	ND	3.06	0.45	4.10
Unextractable	4.67	17.79	8.78	22.04
Total	100.00	100.00	100.00	100.00

ND: Not detected; Source: Yasunaga, 2018a

In liver, no unchanged parent compound was detected at either dose. At six or nine hours post dosing (around T_{max}), the most abundant metabolites were:

- NNI-1501-CH_2OH (DH-02 or M2; 20–47% liver TRR),
- NNI-1501-acid-2-OH (DH-05 or M5; 7–15% liver TRR),
- NNI-1501-CH_2OH-2-OH (DH-06 or M6; 2–11% liver TRR)
- NNI-1501-benzoic acid (DH-101 or M10; 2–14% liver TRR)

In addition the following were identified as minor metabolites:

- NNI-1501-acid (DH-01 or M1),
- NNI-1501-CH_2OH-oxide (DH-22 or M9),
- NNI-1501-benzyl-N-Ac-cysteine (DH-401 or M15),
- NNI-1501-CH_2OH (DH-02 or M2) conjugates
- NNI-1501-benzoic acid (DH-101 or M10) conjugates.

Table 7. Liver metabolites (at T_{max}) following a single oral administration of [phenyl-U-^{14}C] benzpyrimoxan to male and female rats

Metabolites	Radioactivity concentration in µg equiv. of NNI-1501/g [% TRR] [a]			
	Male		Female	
	1 mg/kg	100 mg/kg	1 mg/kg	100 mg/kg
NNI-1501	ND	ND	ND	ND
NNI-1501-acid (DH-01)	ND	0.5	ND	0.7
	-	[0.90]	-	[1.73]
NNI-1501-CH_2OH (DH-02)	0.312	26.0	0.143	10.2
	[29.00]	[46.50]	[20.21]	[26.84]
NNI-1501-acid-2-OH (DH-05)	0.098	3.4	0.097	4.2
	[9.13]	[7.37]	[14.68]	[11.15]
NNI-1501-CH_2OH-2-OH (DH-06)	0.018	5.3	0.013	4.4
	[1.61]	[9.33]	[2.11]	[11.16]
NNI-1501-CH_2OH-oxide (DH-22)	0.005	0.3	ND	ND
	[0.41]	[0.35]	-	-
NNI-1501-benzoic acid (DH-101)	0.160	1.2	0.079	1.1
	[13.82]	[2.03]	[12.39]	[3.07]
NNI-1501-benzyl alcohol (DH-102)	ND	ND	ND	ND
NNI-1501-benzaldehyde (DH-103)	ND	ND	ND	ND
NNI-1501-benzyl-*N*-Ac-cysteine (DH-401)	0.043	1.2	0.014	0.9
	[3.93]	[2.61]	[2.17]	[2.35]
NNI-1501-benzoyl-glycine (DH-402)	ND	ND	ND	ND
Polar metabolites [b]	0.239	10.6	0.148	11.4
	[20.36]	[20.19]	[23.04]	[28.37]
Others	0.044	ND	0.033	ND
	[4.49]	[ND]	[4.63]	[ND]
Unextractable	0.195	5.3	0.134	5.9
	[17.24]	[10.72]	[20.78]	[15.33]
Total	1.114	54.0	0.662	38.8
	[100.00]	[100.00]	[100.00]	[100.00]

ND: Not detected; Source: Yasunaga, 2018a

[a] All values are mean of four individuals;
the numbers in parentheses are percentage of total radioactive residue in liver (% of TRR)

[b] Polar metabolites are mainly DH-02 conjugates and DH-101 conjugates

In kidney, no unchanged parent compound was detected at either dose. At six or nine hours post dosing (around T_{max}), the most abundant metabolites were:

- NNI-1501-CH_2OH (DH-02 or M2; 9–29% kidney TRR),
- NNI-1501-acid-2-OH (DH-05 or M5; 9–33% kidney TRR),
- NNI-1501-CH_2OH-2-OH (DH-06 or M6; 1–11% kidney TRR)
- NNI-1501-benzoic acid (DH-101 or M10; 3–26% kidney TRR)

In addition the following were identified as minor metabolites:

- NNI-1501-acid (DH-01 or M1),
- NNI-1501-CH_2OH-oxide (DH-22 or M9),
- NNI-1501-benzyl-N-Ac-cysteine (DH-401 or M15)
- NNI-1501-benzoyl-glycine (DH-402 or M16).

Table 8. Kidney metabolites (at T_{max}) following a single oral administration of [phenyl-U-^{14}C] benzpyrimoxan to male and female rats

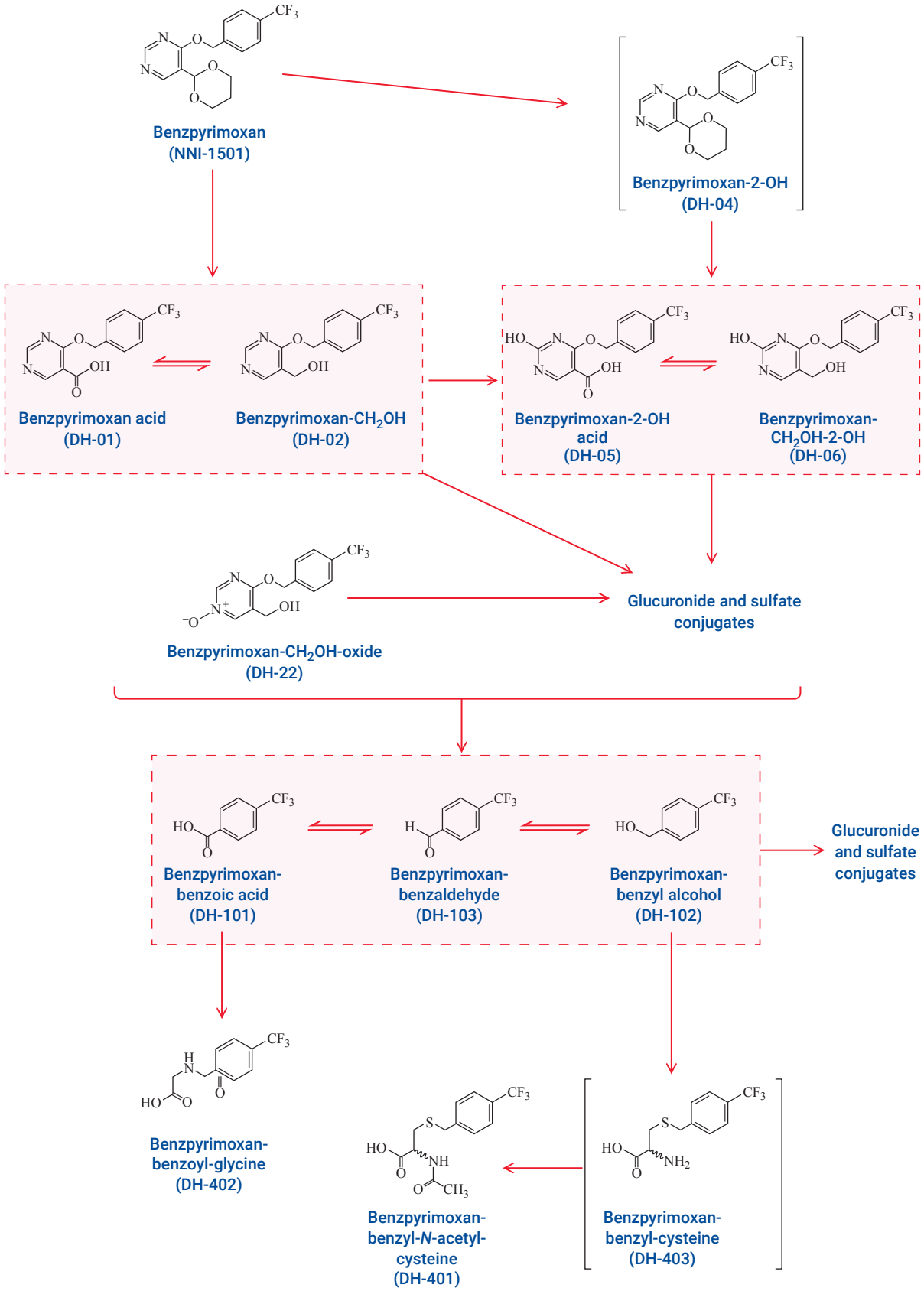
Metabolites	Radioactivity concentration in µg equiv. NNI-1501 per g [% TRR] [a]			
	Male		Female	
	1 mg/kg	100 mg/kg	1 mg/kg	100 mg/kg
NNI-1501	ND	ND	ND	ND
NNI-1501-acid (DH-01)	ND	0.8	0.008	1.4
	-	[2.01]	[1.11]	[2.48]
NNI-1501-CH_2OH (DH-02)	0.122	12.7	0.068	5.3
	[19.00]	[28.98]	[9.57]	[9.40]
NNI-1501-acid-2-OH (DH-05)	0.059	5.6	0.223	17.1
	[9.48]	[15.63]	[32.70]	[27.86]
NNI-1501-CH_2OH-2-OH (DH-06)	0.013	4.9	0.006	3.6
	[2.20]	[10.99]	[0.76]	[6.08]
NNI-1501-CH_2OH-oxide (DH-22)	0.023	2.8	0.010	0.7
	[3.58]	[6.73]	[1.29]	[1.31]
NNI-1501-benzoic acid (DH-101)	0.158	1.6	0.121	1.4
	[26.05]	[4.15]	[17.45]	[2.54]
NNI-1501-benzyl alcohol (DH-102)	ND	ND	ND	ND
NNI-1501-benzaldehyde (DH-103)	ND	ND	ND	ND
NNI-1501-benzyl-*N*-Ac-cysteine (DH-401)	0.059	2.0	0.041	3.0
	[9.34]	[5.32]	[6.00]	[4.95]
NNI-1501-benzoyl-glycine (DH-402)	0.023	ND	0.019	0.5
	[3.91]	-	[2.85]	[0.82]
Polar metabolites	0.044	4.3	0.045	7.9
	[7.36]	[11.63]	[6.65]	[12.68]
Others	0.047	0.8	0.017	5.8
	[7.70]	[2.10]	[2.42]	[7.94]
Unextractable	0.068	4.8	0.129	15.0
	[11.36]	[12.45]	[19.18]	[23.95]
Total	0.616	40.2	0.687	61.8
	[100.00]	[100.00]	[100.00]	[100.00]

ND: Not detected; Source: Yasunaga, 2018a

[a] All values are mean of four individuals;
the numbers in parentheses are percentage toward total radioactive residue in kidney (% of TRR)

From the results of the metabolite analysis, the following metabolic pathway was proposed for [*phenyl-U*-^{14}C]benzpyrimoxan. The [*phenyl-U*-^{14}C]benzpyrimoxan was mainly metabolized by hydrolysis of its acetal ring to form the carboxylic acid (DH-01 or M1, and DH-05 or M5) or the hydroxymethyl derivative (DH-02 or M2, and DH-06 or M6), followed by conjugation to form its glucuronide or sulfate. Also observed were hydroxylation (to generate DH-04 or M4) and *N*-oxidation (to generate DH-22 or M9) of the pyrimidine ring. In addition, various metabolites were also produced via loss of the pyrimidine ring (DH-101 or M10, DH-102 or M11 and DH-103 or M12) . These were further conjugated, leading to the glycine (DH-402 or M16), cysteine (DH-401 or M15), glucuronide and sulfate conjugates. More detail is shown in Fig. 3.

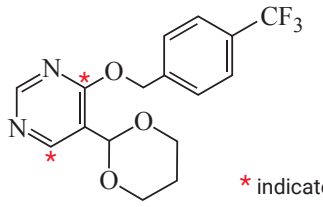
Figure 3. Proposed metabolic pathway of [phenyl-U-^{14}C] benzpyrimoxan in rats



(Redrawn from Yasunaga, 2018a)

ADME study with pyrimidinyl-labelled benzpyrimoxan in rats

Figure 4. Position of radiolabel in pyrimidinyl-labelled benzpyrimoxan



Another study (OECD 417) was conducted to investigate the absorption, distribution, metabolism, excretion and toxicokinetics of [*pyrimidinyl-4(6)*-^{14}C]benzpyrimoxan following a single oral administration to rats. The experimental plan was very similar to that of the previous study (Yasunaga, 2018a); the main difference was the position of the label. Male and female Wistar rats were administered 1 or 100 mg/kg bw of [*pyrimidinyl-4(6)*-^{14}C]benzpyrimoxan suspended in a 0.5% (w/v) aqueous solution of sodium CMC containing 0.1% (w/v) Tween 80. Since no significant sex-related differences in the kinetics of [*pyrimidinyl-4(6)*-^{14}C]benzpyrimoxan were observed in the absorption component of the study, distribution, metabolism and excretion were not investigated in the female rats of the high dose group (100 mg/kg bw). The dose group design of the study is shown in Table 9.

Table 9. Design of ADME study with pyrimidinyl-labelled benzpyrimoxan

Dose group code [a]	Dose (mg/kg bw)	Number of animals/group	Sex	Sampling time (hours post dose)
PML	1	4	Male	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
PFL	1	4	Female	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
PMH	100	4	Male	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
PFH	100	4	Female	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
PML-1	1	4	Male	Organs/tissues: 3
PML-2	1	4	Male	Organs/tissues: 24
PML-3	1	4	Male	Expired air: 24, 48, 72, 96,120, 144, 168 Urine: 24, 48, 72, 96,120, 144, 168 Faeces: 24, 48, 72, 96,120, 144, 168 Organs/tissues: 168
PFL-1	1	4	Female	Organs/tissues: 3
PFL-2	1	4	Female	Organs/tissues: 24
PFL-3	1	4	Female	Expired air: 24, 48, 72, 96,120, 144, 168 Urine: 24, 48, 72, 96,120, 144, 168 Faeces: 24, 48, 72, 96,120, 144, 168 Organs/tissues: 168
PMH-1	100	4	Male	Organs/tissues: 6
PMH-2	100	4	Male	Organs/tissues: 24
PMH-3	100	4	Male	Expired air: 24, 48, 72, 96,120, 144, 168 Urine: 24, 48, 72, 96,120, 144, 168 Faeces: 24, 48, 72, 96,120, 144, 168 Organs/tissues: 168

[a] P: Pyrimidinyl-labelled benzpyrimoxan; L: Low dose; H: High dose; Source: Yasunaga, 2018b

Absorption

Blood samples from male and female rats following administration of [pyrimidinyl-4(6)-^{14}C] benzpyrimoxan were collected after 1, 3, 6, 9, 12 and 24 hours, and thereafter every 24 hours until 168 hours post dosing. Radioactivity concentrations in blood and plasma were quantified by liquid scintillation counting.

At the low dose (1 mg/kg bw; groups PML and PFL), concentrations of radioactivity in blood

and plasma reached their T_{max} one hour post dosing and decreased in a biphasic pattern. After attaining their maximum concentrations in blood and plasma concentrations decreased in the first phase with half-lives of 0.48 (males) and 0.39 days (females) in blood. In plasma the half-lives were 0.71 (males) and 0.65 days (females) in the first phase. In the second phase the corresponding values were 4.03 (males) and 3.49 days (females) in blood, and 4.42 (males) and 3.94 days (females)in plasma.

At the high dose (100 mg/kg bw; groups PMH and PFH), radioactivity concentrations in blood and plasma reached their T_{max} at 6–9 hours post dosing. After attaining their maximum, radioactivity concentrations in blood and plasma decreased in the first phase with half-lives of 0.41 (males) and 0.34 days (females) in blood. In plasma the half-lives were 0.73 (males) and 0.65 days (females). In the second phase the corresponding values were 2.94 (males) and 2.61 (females) in blood, and 2.99 (males) and 2.91 days (females) in plasma. Dose had no effect on the first phase half-lives, while those in the second phase were slightly shorter at the high dose. Kinetic parameters in blood and plasma at the high dose were comparable to those at the low dose, except for the AUC which was almost 50 times higher, (that is, less than dose-proportional), and the T_{max} which was 6–9 times higher. There were no significant sex-related differences.

Table 10. Concentrations of radioactivity (µg equiv. benzpyrimoxan/g) and its kinetic parameters in blood and plasma following a single oral administration of [pyrimidinyl-4(6)-^{14}C] benzpyrimoxan

Time point (hours post dose)	Males				Females			
	1 mg/kg bw		100 mg/kg bw		1 mg/kg bw		100 mg/kg bw	
	Blood	Plasma	Blood	Plasma	Blood	Plasma	Blood	Plasma
1	0.479 ± 0.143	0.520 ± 0.151	11.7 ± 3.2	15.6 ± 4.8	0.551 ± 0.034	0.768 ± 0.049	11.7 ± 2.0	17.4 ± 3.3
3	0.392 ± 0.095	0.448 ± 0.094	13.6 ± 2.1	17.5 ± 4.3	0.303 ± 0.068	0.428 ± 0.095	14.9 ± 3.3	21.6 ± 4.4
6	0.277 ± 0.062	0.332 ± 0.055	17.4 ± 3.2	20.6 ± 3.5	0.213 ± 0.014	0.296 ± 0.018	14.7 ± 4.2	21.8 ± 8.4
9	0.233 ± 0.072	0.273 ± 0.062	16.2 ± 2.7	19.1 ± 2.7	0.167 ± 0.012	0.216 ± 0.015	18.2 ± 4.4	26.1 ± 8.2
12	0.210 ± 0.057	0.253 ± 0.062	15.6 ± 4.0	17.0 ± 2.5	0.139 ± 0.017	0.177 ± 0.015	15.6 ± 1.0	21.6 ± 3.3
24	0.177 ± 0.042	0.180 ± 0.042	8.7 ± 2.1	9.5 ± 2.1	0.111 ± 0.008	0.116 ± 0.008	6.4 ± 0.7	7.4 ± 1.0
48	0.139 ± 0.032	0.139 ± 0.035	6.2 ± 1.5	6.3 ± 1.5	0.088 ± 0.010	0.093 ± 0.004	3.6 ± 0.6	3.8 ± 0.9
72	0.119 ± 0.023	0.115 ± 0.022	5.0 ± 1.2	4.8 ± 1.1	0.076 ± 0.008	0.075 ± 0.007	2.9 ± 0.9	3.0 ± 0.9
96	0.097 ± 0.023	0.095 ± 0.020	4.0 ± 0.9	3.7 ± 0.9	0.063 ± 0.006	0.060 ± 0.004	2.5 ± 0.5	2.3 ± 0.7
120	0.086 ± 0.016	0.083 ± 0.018	3.3 ± 0.7	3.1 ± 0.7	0.056 ± 0.004	0.057 ± 0.006	1.8 ± 0.5	1.9 ± 0.5
144	0.071 ± 0.014	0.064 ± 0.012	2.5 ± 0.9	2.6 ± 0.6	0.049 ± 0.007	0.048 ± 0.007	1.7 ± 0.5	1.5 ± 0.4
168	0.062 ± 0.012	0.052 ± 0.010	2.1 ± 0.5	2.1 ± 0.5	0.042 ± 0.006	0.038 ± 0.003	1.3 ± 0.2	1.3 ± 0.5
T_{max} (hours)	1	1	6	6	1	1	9	9
C_{max} (µg equiv./g)	0.479	0.520	17.4	20.6	0.551	0.768	18.2	26.1
$t_{½}$ (days), T_{max}–24 h	0.71	0.65	0.73	0.65	0.48	0.39	0.41	0.34
24–168 h	4.03	3.49	2.99	2.91	4.42	3.94	2.94	2.61
$AUC_{0–168}$ (µg equiv. × hour/g)	21.022	21.403	955.5	1005.3	14.274	15.604	702.0	835.9

Source: Yasunaga, 2018b

Distribution

Following administration of [*pyrimidinyl-4(6)*-^{14}C]benzpyrimoxan, male and female rats were terminated at 3 (around T_{max}), 24 and 168 hours at the low dose (1 mg/kg bw; groups PML-1–3 and PFL-1–3), and at 6 (around T_{max}), 24 and 168 hours at the high dose (100 mg/kg bw; groups PMH-1–3). The concentration of radioactivity in major organs and tissues was quantified by liquid scintillation counting.

There were no significant sex- or dose-related differences in the distribution profile of [*pyrimidinyl-4(6)*-^{14}C]benzpyrimoxan. At around T_{max}, the highest level of radioactivity was found in the GI tract, followed by GI contents, liver, kidney and adrenals. By 168 hours post dosing, radioactivity in all organs and tissues was almost negligible and never higher than in plasma. This suggested that no organs or tissues specifically retained [*pyrimidinyl-4(6)*-^{14}C]benzpyrimoxan and/or its metabolites.

Table 11. Distribution of radioactivity (µg equiv. benzpyrimoxan/g) following a single oral administration of [pyrimidinyl-4(6)-^{14}C] benzpyrimoxan in selected organs; (Results shown as mean ± standard deviation)

	Sex, dose level								
	Males						Females		
	1 mg/kg bw			100 mg/kg bw			1 mg/kg bw		
Organ/tissue	**3 h**	**24 h**	**168 h**	**6 h**	**24 h**	**168 h**	**3 h**	**24 h**	**168 h**
Small intestine	4.751 ± 3.089	0.059 ± 0.016	0.028 ± 0.006	100.4 ± 59.7	8.2 ± 5.5	0.9 ± 0.2	2.448 ± 0.872	0.053 ± 0.010	0.022 ± 0.005
Gastrointestinal contents	2.430 ± 0.662	0.178 ± 0.031	0.036 ± 0.012	395.6 ± 56.5	38.8 ± 13.6	1.3 ± 0.4	2.873 ± 0.583	0.100 ± 0.020	0.021 ± 0.008
Liver	1.333 ± 0.098	0.206 ± 0.067	0.061 ± 0.008	54.3 ± 21.1	12.2 ± 6.1	1.4 ± 0.3	0.874 ± 0.251	0.121 ± 0.027	0.040 ± 0.009
Kidney	0.858 ± 0.083	0.114 ± 0.030	0.032 ± 0.005	42.6 ± 23.4	8.5 ± 3.6	1.2 ± 0.3	1.072 ± 0.355	0.134 ± 0.022	0.035 ± 0.007
Adrenal	0.608 ± 0.136	0.054 ± 0.016	0.010 ± < 0.001	29.9 ± 9.7	2.6 ± 1.7	0.4 ± 0.2	0.307 ± 0.045	0.034 ± 0.006	0.013 ± 0.003

h: Hours; Source: Yasunaga, 2018b

Excretion

Excreta (urine and faeces) and expired air from male and female rats following administration of [*pyrimidinyl-4(6)*-^{14}C]benzpyrimoxan were collected every 24 hours until 168 hours post treatment at the low dose (1 mg/kg bw; groups PML-3 and PFL-3), and high dose (100 mg/kg bw; group PMH-3). Radioactivity from excreta, expired air and carcass was analysed by liquid scintillation counting.

At both doses, recovery of radioactivity by 168 hours post treatment was greater than 95% of the initially administered radioactivity, hence acceptable. Radioactivity was mostly excreted via urine, faeces and expired air by 48 hours post dosing. After that, the remaining radioactivity was gradually excreted, but a significant amount of radioactivity was retained in the carcass at 168 hours post treatment. Radioactivity in expired air was confirmed as ^{14}C carbon dioxide. At the low dose, excretion in urine, faeces and expired air accounted for 39–45%, 44% and 5–6% respectively of the AR by 168 hours post treatment. At 168 hours post dosing, 3–7% of the AR was still present in the carcasses low-dose males. In high-dose males (group PMH-3), excretion profiles were similar to those of the low-dose groups except for increased elimination in faeces, possibly resulting from the passage of unabsorbed material. Excretion in urine, faeces and expired air accounted for 37%, 58% and 2% respectively of the AR by 168 hours post dosing, . After 168 hours post treatment, 2% of the AR was still present in the carcass. There were no significant sex-related differences in the excretion profile of [*pyrimidinyl-4(6)*-^{14}C]benzpyrimoxan.

Table 12. Excretion rates in urine, faeces and expired air (% administered dose)

Radiolabel		[*pyrimidinyl-4(6)*-^{14}C]benzpyrimoxan			
Dose		1 mg/kg bw		100 mg/kg bw	
Sex		Males	Females	Males	Females
Urine	0–24 hours	35.0	42.7	34.1	NA
	0–72 hours	37.2	44.3	36.0	NA
	0–168 hours	38.7	45.4	36.5	NA
Faeces	0–24 hours	39.6	40.8	51.2	NA
	0–72 hours	43.3	43.1	57.3	NA
	0–168 hours	44.2	43.9	57.6	NA
Expired air	0–24 hours	4.93	4.00	1.80	NA
	0–72 hours	5.66	4.57	2.22	NA
	0–168 hours	6.28	5.01	2.37	NA
Cage washings[a]		0.09	0.32	0.06	NA
Carcasses[a]		6.89	3.29	1.83	NA

[a] Collected at 168 hours post dose; Source: Yasunaga, 2018b

NA: Not analyzed; not conducted for the 100 mg/kg bw female dose group as no sex differences were noted in absorption
ND: Not detected.

Metabolism

Excretion of the administered radioactivity via urine and faeces was almost complete in all groups within 48 hours post dosing. Therefore excreta obtained up to 48 hours post treatment were subjected to metabolite analysis. Additionally, plasma, liver and kidney samples obtained at around T_{max} (three or six hours post dosing), and plasma samples at 24 and 168 hours post dosing were also subjected to metabolite analysis. There were no significant sex- or dose-related differences in the metabolic profile of [*pyrimidinyl-4(6)*-^{14}C]benzpyrimoxan.

In urine, no unchanged parent compound was detected. Metabolite NNI-1501-acid-2-OH (DH-05 or M5; 14–19% AR) was the only major (greater than 10% AR) metabolite. The second most abundant metabolite was NNI-1501-acid-2,4-OH (DH-205 or M14), accounting for 3–6% of AR. In addition the following were also identified as minor metabolites:

- NNI-1501-acid (DH-01 or M1),
- NNI-1501-CH_2OH (DH-02 or M2),
- NNI-1501-CH_2OH-2-OH (DH-06 or M6),
- NNI-1501-CH_2OH-oxide (DH-22 or M9),
- NNI-1501-CH_2OH (DH-02 or M2) conjugates,
- NNI-1501-CH_2OH-oxide (DH-22 or M9) conjugates
- NNI-1501-acid-2,4-OH (DH-205 or M14) conjugates.

In faeces no unchanged parent compound was detected at the low dose, but a significant amount (25% AR) was present at the high dose in males (there was no high-dose female group). NNI-1501-acid-2-OH (DH-05 or M5) was the most abundant metabolite, accounting for 20–31% of AR. In addition the following were also identified as minor metabolites:

- NNI-1501-acid (DH-01 or M1),
- NNI-1501-CH_2OH (DH-02 or M2)
- NNI-1501-CH_2OH-2-OH (DH-06 or M6).

Table 13. Metabolites in urines and faeces following a single oral administration of [pyrimidinyl-4(6)-^{14}C]benzpyrimoxan to male and female rats

	Metabolites excreted [% of administered radioactivity] [a]					
	Males				Females	
	1 mg/kg bw		100 mg/kg bw		1 mg/kg bw	
Metabolites	Urine	Faeces	Urine	Faeces	Urine	Faeces
NNI-1501	ND	ND	ND	24.99	ND	ND
NNI-1501-acid (DH-01)	0.27	0.29	ND	ND	0.39	0.28
NNI-1501-CH_2OH (DH-02)	ND	1.23	0.12	3.35	0.22	1.01
NNI-1501-acid-2-OH (DH-05)	14.03	27.78	13.69	19.78	19.15	30.73
NNI-1501-CH_2OH-2-OH (DH-06)	0.53	ND	1.45	1.11	0.77	ND
NNI-1501-CH_2OH-oxide (DH-22)	2.40	ND	0.79	ND	1.54	ND
NNI-1501-acid-2,4-OH (DH-205)	3.86	ND	2.88	ND	6.01	ND
DH-02 conjugates (glucronide/sulfate)	1.23 [1.06/0.17]	NE	3.31 [2.89/0.42]	NE	1.46 [1.25/0.21]	NE
DH-22 conjugates (glucronide/sulfate)	1.11 [0.98/0.13]	NE	0.71 [0.58/0.13]	NE	0.59 [0.51/0.08]	NE
DH-205 conjugates (glucronide/sulfate)	2.89 [1.59/1.30]	NE	2.62 [1.81/0.82]	NE	2.86 [1.85/1.01]	NE
Polar metabolites	8.97 [b]	4.47	8.69 [c]	2.84	9.44 [d]	3.86
Others	0.99	ND	0.79	ND	1.12	ND
MeCN/0.1N HCl extract [e]	–	3.20	–	1.24	–	2.81
MeCN/1N HCl extract [e]	–	1.74	–	0.76	–	1.27
Unextractable	0.20	3.98	0.64	2.74	0.25	2.76
Total	36.48	42.69	35.69	56.82	43.80	42.73

ND: Not detected; Source: Yasunaga, 2018b

NE: Not examined, because radioactivity retained at TLC origin in faeces was less than 5% of dose

[a] All values are mean of four individuals; urine and faeces obtained until 48 hours post dose were analyzed

[b] No individual component exceeded 2.91% of dose in polar metabolites

[c] No individual component exceeded 2.95% of dose in polar metabolites

[d] No individual component exceeded 3.59% of dose in polar metabolites

[e] In urine, MeCN/0.1N HCl extraction and MeCN/1N HCl extraction were not examined because unextractable radioactivity was less than 5% of dose

In plasma no unchanged parent compound was detected. At three or six hours post dosing (around T_{max}), the most abundant metabolites were:

- NNI-1501-acid (DH-01 or M1; 9–27% plasma TRR),
- NNI-1501-CH_2OH (DH-02 or M2; 2–16% plasma TRR),
- NNI-1501-acid-2-OH (DH-05 or M5; 5–11% plasma TRR),
- NNI-1501-CH_2OH-oxide (DH-22 or M9; 2–13% plasma TRR)
- NNI-1501-acid-2,4-OH (DH-205 or M14; 1–11% plasma TRR).

In addition, NNI-1501-CH_2OH-2-OH (DH-06 or M6) was also identified as a minor metabolite.

Table 14. Plasma metabolites (at T_{max}) following a single oral administration of [pyrimidinyl-4(6)-^{14}C] benzpyrimoxan to male and female rats

	Radioactivity concentration in µg eq.NNI-1501/g [% TRR] [a]		
	Males		Females
Metabolites	**1 mg/kg bw**	**100 mg/kg bw**	**1 mg/kg bw**
NNI-1501	ND [ND]	ND [ND]	ND [ND]
NNI-1501-acid (DH-01)	0.035 [9.35]	5.6 [27.18]	0.108 [26.98]
NNI-1501-CH_2OH (DH-02)	0.011 [3.04]	2.7 [15.55]	0.007 [1.72]
NNI-1501-acid-2-OH (DH-05)	0.020 [5.25]	1.6 [6.83]	0.041 [10.54]
NNI-1501-CH_2OH-2-OH (DH-06)	0.002 [0.56]	0.7 [3.87]	ND [ND]
NNI-1501-CH_2OH-oxide (DH-22)	0.047 [12.85]	0.3 [1.96]	0.018 [4.96]
NNI-1501-acid-2,4-OH (DH-205)	0.042 [11.41]	0.2 [0.55]	0.034 [9.16]
Polar metabolites	0.096 [b] [26.27]	1.7 [8.62]	0.069 [c] [19.09]
Others	0.047 [d] [12.74]	2.0 [e] [13.33]	0.012 [3.01]
Unextractable	0.068 [18.53]	4.5 [22.11]	0.091 [24.54]
Total	0.367 [100.00]	19.3 [100.00]	0.379 [100.00]

ND: Not detected; Source: Yasunaga, 2018b

[a] All values are mean of four individuals; the numbers in parentheses are percentage toward total radioactive residue in plasma (% of TRR)

[b] No individual component exceeded 0.024 µg equiv.NNI-1501/g (6.62% of TRR) in polar metabolites

[c] No individual component exceeded 0.019 µg equiv.NNI-1501/g (5.19% of TRR) in polar metabolites

[d] No individual component exceeded 0.043 µg equiv.NNI-1501/g (11.66% of TRR) in others

[e] No individual component exceeded 2.0 µg equiv.NNI-1501/g (13.33% of TRR) in others

In liver, unchanged parent was detected in low-dose females (0.65% liver TRR) and high-dose males (1.32% liver TRR), but not in low-dose males. At three or six hours post dosing (around T_{max}), the most abundant metabolites were:

- NNI-1501-CH_2OH (DH-02 or M2; 30–53% liver TRR), and
- NNI-1501-acid-2-OH (DH-05 or M5; 8–23% liver TRR).

In addition the following were also identified as minor metabolites:

- NNI-1501-acid (DH-01 or M1),
- NNI-1501-CH_2OH-2-OH (DH-06 or M6),
- NNI-1501-acid-2,4-OH (DH-205 or M14).

Table 15. Liver metabolites (at T_{max}) following a single oral administration of [pyrimidinyl-4(6)-^{14}C] benzpyrimoxan to male and female rats

	Concentration of radioactivity in µg equiv. NNI-1501/g [% TRR] [a]		
	Males		Females
Metabolites	**1 mg/kg bw**	**100 mg/kg bw**	**1 mg/kg bw**
NNI-1501	ND	0.9	0.008
	[ND]	[1.32]	[0.65]
NNI-1501-acid (DH-01)	0.003	0.5	0.014
	[0.18]	[0.72]	[1.38]
NNI-1501-CH_2OH (DH-02)	0.705	26.9	0.263
	[52.85]	[50.41]	[29.80]
NNI-1501-acid-2-OH (DH-05)	0.136	4.8	0.208
	[10.19]	[8.25]	[23.44]
NNI-1501-CH_2OH-2-OH (DH-06)	0.041	5.3	0.029
	[3.10]	[9.42]	[3.26]
NNI-1501-CH_2OH-oxide (DH-22)	ND	ND	ND
	[ND]	[ND]	[ND]
NNI-1501-acid-2,4-OH (DH-205)	0.024	ND	0.047
	[1.83]	[ND]	[5.54]
Polar metabolites [b]	0.246	12.1	0.179
	[18.50]	[23.42]	[20.88]
Others	0.044	0.3	0.042
	[3.27]	[0.36]	[4.98]
Unextractable	0.134	3.4	0.084
	[10.08]	[6.11]	[10.07]
Total	1.333	54.3	0.874
	[100.00]	[100.00]	[100.00]

ND: Not detected; Source: Yasunaga, 2018b

[a] All values are mean of four individuals;
the numbers in parentheses are percentage of total radioactive residue in liver (% of TRR)

[b] Polar metabolites are partially DH-02 conjugates

In kidney, no unchanged parent compound was detected. At three or six hours post dosing (around T_{max}), the most abundant metabolites were:

- NNI-1501-CH_2OH (DH-02 or M2; 11–38% kidney TRR),
- NNI-1501-acid-2-OH (DH-05 or M5; 16–50% kidney TRR), and
- NNI-1501-CH_2OH-2-OH (DH-06 or M6; 2–12% kidney TRR).

In addition the following were also identified as minor metabolites:

- NNI-1501-acid (DH-01 or M1),
- NNI-1501-CH_2OH-oxide (DH-22 or M9), and
- NNI-1501-acid-2,4-OH (DH-205 or M14).

Table 16. Kidney metabolites (at T_{max}) following a single oral administration of [pyrimidinyl-4(6)-^{14}C] benzpyrimoxan to male and female rats

	Concentration of radioactivity in µg equiv. NNI-1501/g [% TRR] [a]		
	Males		Females
Metabolites	**1 mg/kg bw**	**100 mg/kg bw**	**1 mg/kg bw**
NNI-1501	ND	ND	ND
	[ND]	[ND]	[ND]
NNI-1501-acid (DH-01)	0.010	0.6	0.021
	[1.12]	[1.04]	[1.92]
NNI-1501-CH_2OH (DH-02)	0.324	11.1	0.116
	[37.78]	[29.35]	[10.69]
NNI-1501-acid-2-OH (DH-05)	0.140	10.4	0.536
	[16.44]	[21.03]	[50.26]
NNI-1501-CH_2OH-2-OH (DH-06)	0.029	4.7	0.016
	[3.34]	[12.26]	[1.51]
NNI-1501-CH_2OH-oxide (DH-22)	0.077	1.6	0.015
	[8.80]	[4.60]	[1.32]
NNI-1501-acid-2,4-OH (DH-205)	0.033	1.7	0.062
	[3.94]	[2.69]	[5.62]
Polar metabolites [b]	0.143	7.0	0.151
	[16.80]	[18.24]	[14.35]
Others	0.025	1.8	0.052
	[2.88]	[2.88]	[4.65]
Unextractable	0.076	3.6	0.104
	[8.90]	[7.91]	[9.67]
Total	0.858	42.6	1.072
	[100.00]	[100.00]	[100.00]

ND: Not detected; Source: Yasunaga, 2018b

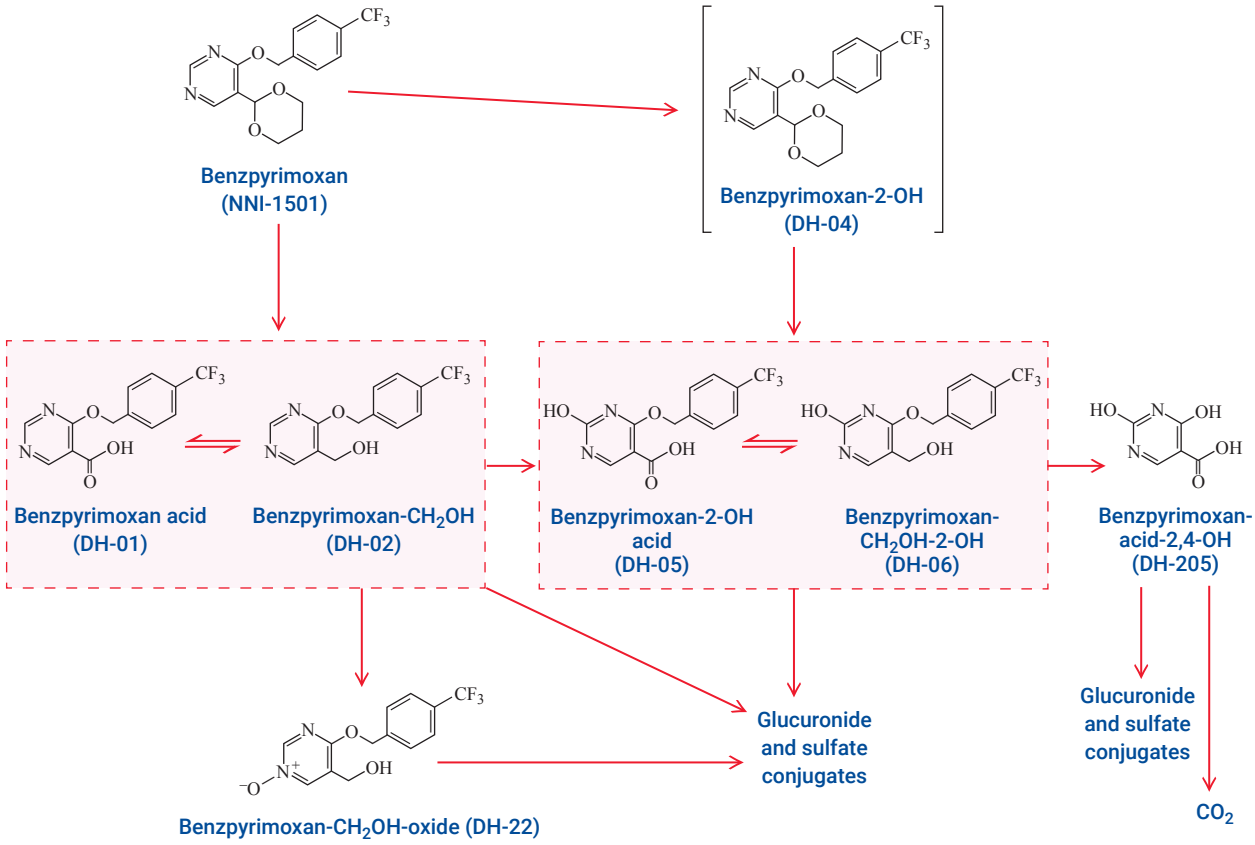
[a] All values are mean of four individuals;
the numbers in parentheses are percentage toward total radioactive residue in kidney (% of TRR)

[b] Polar metabolites are partially DH-02 conjugates, DH-05 conjugates and DH-22 conjugates.

From the results of the metabolite analysis, the following metabolic pathway was proposed. The [*pyrimidinyl-4(6)*-^{14}C]benzpyrimoxan was primarily metabolized by hydrolysis of the acetal ring to form the carboxylic acids (DH-01 or M1, and DH-05 or M5) or the hydroxymethyl derivatives (DH-02 or M2, and DH-06 or M6), followed by conjugation to glucuronide and sulfate derivatives. Hydroxylation (to generate DH-04 or M4) and *N*-oxidation (to generate DH-22 or M9) of the pyrimidine ring was also observed. In addition, metabolites formed by the loss of the phenyl ring (DH-205 or M14) and other highly polar metabolites were also produced and eventually mineralized to carbon dioxide. The proposed pathways are shown in Fig. 5.

Overall no significant differences were seen between the absorption, distribution, metabolism, excretion or toxicokinetic profiles of the phenyl-labelled benzpyrimoxan and the pyrimidinyl-labelled benzpyrimoxan.

Figure 5. Proposed metabolic pathway of [pyrimidinyl-4(6)-^{14}C] benzpyrimoxan in rats



(Redrawn from Yasunaga, 2018b)

Metabolism and excretion study with phenyl- and pyrimidinyl-labelled benzpyrimoxan in bile duct-cannulated rats

A study (Yasunaga, 2018c; OECD 417) was conducted to investigate the biliary excretion and biliary metabolites of benzpyrimoxan following a single oral administration of [*phenyl-U*-^{14}C]benzpyrimoxan or [*pyrimidinyl-4(6)*-^{14}C]benzpyrimoxan to bile duct-cannulated rats. Wistar Hannover [RccHan^{TM}:WIST] male rats (five per label) were administered 1 mg/kg bw of [*phenyl-U*-^{14}C]benzpyrimoxan or [*pyrimidinyl-4(6)*-^{14}C]benzpyrimoxan suspended in a 0.5% (w/v) aqueous solution of sodium CMC containing 0.1% (w/v) Tween 80). Excreta (bile, urine and faeces) were collected at 24, 48 and 72 hours post dosing. After the last excreta collection the animals were terminated and the GI contents, GI tract and liver removed, and the radioactivity content of these isolates was measured.

Excretion

Recovery of radioactivity by 72 hours post treatment was greater than 95% of the initially administered radioactivity for both labels, and hence acceptable. Radioactivity in the carcass was not quantified given that total recovery was greater than 95% of the AD.

In the animals treated with [*phenyl-U*-^{14}C]benzpyrimoxan the absorbed radioactivity was rapidly excreted. Radioactivity in bile, urine and faeces accounted for 46%, 36% and 16% respectively of the AR by 72 hours post dosing, . At 72 hours post treatment, residual radioactivity in the cage wash, GI contents and GI tract plus liver combined, accounted for 0.29%, 0.01% and 0.10% respectively of the AR.

In the animals treated with [*pyrimidinyl-4(6)*-^{14}C]benzpyrimoxan, the absorbed radioactivity was also rapidly excreted. Radioactivity in bile, urine and faeces accounted for 29%, 47% and 19% respectively of the AR by 72 hours post dosing. At 72 hours post treatment, residual radioactivity in the cage wash, GI contents and GI tract plus liver combined accounted for 0.72%, 0.17% and 0.13% respectively of the AR.

It is noted that with the phenyl label, excretion was greater in bile compared to urine, whilst with the pyrimidinyl label, excretion was greater in urine compared to bile. Oral absorption for benzpyrimoxan following a single oral administration at the low dose of 1 mg/kg bw was calculated to be 76.2–82.5% based on the sum of radioactivity excreted in bile and urine, and residual radioactivity in the GI tract and liver. Oral absorption at the high dose of 100 mg/kg bw could not be determined from this study as only the low dose was tested.

Table17. Excretion of radioactivity in bile duct-cannulated rats following a single oral administration

Time (hours post dose)	Cumulative excretion [% of administered radioactivity][a]			
	Bile	**Urine**	**Faeces**	**Total**
		[*phenyl-U*-^{14}C]benzpyrimoxan		
0–24	45.72 ± 7.67	34.90 ± 12.14	13.58 ± 8.08	94.19 ± 1.16
0–48	46.02 ± 7.60	36.02 ± 12.33	15.36 ± 8.04	97.40 ± 0.80
0–72	46.08 ± 7.60	36.32 ± 12.28	15.54 ± 8.11	97.95 ± 0.90
Cage wash (obtained at 72 hours post dose)				0.29 ± 0.06
Gastrointestinal content (obtained at 72 hours post dose)				0.01 ± 0.01
Sum of liver and GI tract (obtained at 72 hours post dose)				0.10 ± 0.01
Absorption (absorption extent was calculated as the sum of radioactivity in bile, urine, liver and gastrointestinal tracts)				82.51 ± 7.50
Total (recovery)				98.35 ± 0.95
		[*pyrimidinyl-4(6)*-^{14}C]benzpyrimoxan		
0–24	28.29 ± 5.92	45.38 ± 16.53	14.81 ± 12.68	88.48 ± 2.22
0–48	28.82 ± 6.02	46.80 ± 16.66	18.53 ± 11.77	94.14 ± 0.74
0–72	28.95 ± 6.04	47.61 ± 16.61	18.83 ± 11.69	94.94 ± 0.78
Cage wash (obtained at 72 hours post dose)				0.72 ± 0.27
Gastrointestinal content (obtained at 72 hours post dose)				0.17 ± 0.04
Sum of liver and GI. tracts (obtained at 72 hours post dose)				0.13 ± 0.02
Absorption (absorption extent was calculated as the sum of radioactivity in bile, urine, liver and GI tract)				76.23 ± 12.42
Total (recovery)				95.95 ± 0.58

[a] Mean and its standard deviation ($n = 5$); Source: Yasunaga, 2018c

Metabolism

The bile obtained up to 72 hours post dosing was subjected to metabolite analysis. No unchanged parent was detected. The most abundant metabolites were:

- NNI-1501-CH_2OH (DH-02 or M2) conjugates (14% AR),
- NNI-1501-acid-2-OH (DH-05 or M5; 6–12% AR), and
- NNI-1501-benzyl-cysteine (DH-403 or M17) conjugates (9% AR).

In addition the following were also identified as minor metabolites:

- NNI-1501-benzyl-alcohol (DH-102 or M11),
- NNI-1501-benzyl-cysteine (DH-403 or M17), and
- NNI-1501-CH_2OH-oxide (DH-22 or M9) conjugate.

In urine, no unchanged parent was detected. NNI-1501-acid-2-OH (DH-05 or M5; 19–20% AR) was the only major metabolite found in urine. In addition the following were also identified as minor metabolites:

- NNI-1501-acid-2, 4-OH (DH-205 or M14),
- NNI-1501-CH_2OH-2-OH (DH-06 or M6),
- NNI-1501-CH_2OH-oxide (DH-22 or M9),
- NNI-1501-benzoic acid (DH-101 or M10),
- NNI-1501-benzyl-*N*-Ac-cysteine (DH-401 or M15),
- NNI-1501-benzoyl-glycine (DH-402 or M16),
- NNI-1501-CH_2OH (DH-02 or M2) conjugates,
- NNI-1501-acid-2-OH (DH-05 or M5) conjugates,
- NNI-1501-CH_2OH-oxide (DH-22 or M9) conjugates, and
- NNI-1501-benzoic acid (DH-101 or M10) conjugates.

In faeces, unchanged parent was detected in small amount (0.2% AR) in the phenyl-labelled group. The most abundant metabolite was NNI-1501-acid-2-OH (DH-05 or M5) accounting for 11% of AR. In addition the following were also identified as minor metabolites:

- NNI-1501-CH_2OH (DH-02 or M2),
- NNI-1501-benzoic acid (DH-101 or M10), and
- NNI-1501-acid-2, 4-OH (DH-205 or M14).

The metabolite profiles observed in bile seemed to be consistent with those in faeces and urine seen in this study and in previous ADME studies, except for the presence of NNI-1501-benzyl-cysteine (DH-403 or M17), which was the precursor of NNI-1501-benzyl-N-Ac-cysteine (DH-401 or M15).

Table 18. Metabolites in bile duct-cannulated rats following a single oral administration of 1 mg/kg bw of [phenyl-U-^{14}C] benzpyrimoxan and [pyrimidinyl-4(6)-^{14}C] benzpyrimoxan

	Metabolites excreted [% of administered radioactivity][a]					
	[*phenyl-U*-^{14}C]NNI-1501			**[*pyrimidinyl-4(6)*-^{14}C]NNI-1501**		
Metabolites	**Bile**	**Urine**	**Faeces**	**Bile**	**Urine**	**Faeces**
NNI-1501	ND	ND	0.20	ND	ND	ND
NNI-1501-acid (DH-01)	ND	ND	ND	ND	ND	ND
NNI-1501-CH_2OH (DH-02)	ND	ND	ND	ND	ND	0.92
NNI-1501-2-OH (DH-04)	ND	ND	ND	ND	ND	ND
NNI-1501-acid-2-OH (DH-05)	11.61	19.30	11.26	5.50	20.49	11.08
NNI-1501-CH_2OH-2-OH (DH-06)	ND	0.51	ND	ND	0.74	ND
NNI-1501-CH_2OH-oxide (DH-22)	ND	0.58	ND	ND	1.03	ND
NNI-1501-benzoic acid (DH-101)	ND	0.41	0.49	-	-	-
NNI-1501-benzyl alcohol (DH-102)	2.10	ND	ND	-	-	-
NNI-1501-benzaldehyde (DH-103)	ND	ND	ND	-	-	-
NNI-1501-benzyl-*N*-Ac-cysteine (DH-401)	ND	2.16	ND	-	-	-
NNI-1501-benzoyl-glycine (DH-402)	ND	1.81	ND	-	-	-
NNI-1501-benzyl-cysteine (DH-403)	ND	ND	ND	-	-	-

	Metabolites excreted [% of administered radioactivity][a]					
	[*phenyl-U*-^{14}C]NNI-1501			[*pyrimidinyl-4(6)*-^{14}C]NNI-1501		
Metabolites	**Bile**	**Urine**	**Faeces**	**Bile**	**Urine**	**Faeces**
NNI-1501-acid-2,4-OH (DH-205)	-	-	-	ND	6.06	0.66
DH-02 conjugates (glucuronide/sulfate)	13.62 [4.37/9.25]	1.76 [1.48/0.28]	NE	14.08 [3.83/10.25]	4.93 [3.68/1.25]	NE
DH-05 conjugates (glucuronide/sulfate)	ND [ND/ ND]	1.20 [1.17/0.03]	NE	ND [ND/ ND]	2.86 [2.43/0.43]	NE
DH-22 conjugates (glucuronide/sulfate)	1.37 [ND/1.37]	0.40 [0.32/0.09]	NE	1.00 [ND/1.00]	1.08 [0.86/0.21]	NE
DH-101 conjugates (glucuronide/sulfate)	ND [ND/ ND]	1.72 [1.30/0.42]	NE	- -	- -	-
DH-403 conjugates (glucuronide/sulfate)	8.91 [0.87/8.05]	ND [ND/ ND]	NE	- -	- -	-
Polar metabolites[b]	5.16	3.91	1.01	4.86	6.97	1.88
Others[c]	1.88	2.41	ND	3.31	2.04	ND
Unextractable	0.07	0.15	2.58	0.20	0.96	4.29
Total	46.08	36.32	15.54	28.95	47.15	18.83

ND: Not detected; Source: Yasunaga, 2018c

NE: Not examined because radioactivity retained at TLC origin in faeces was less than 5% of dosed radioactivity

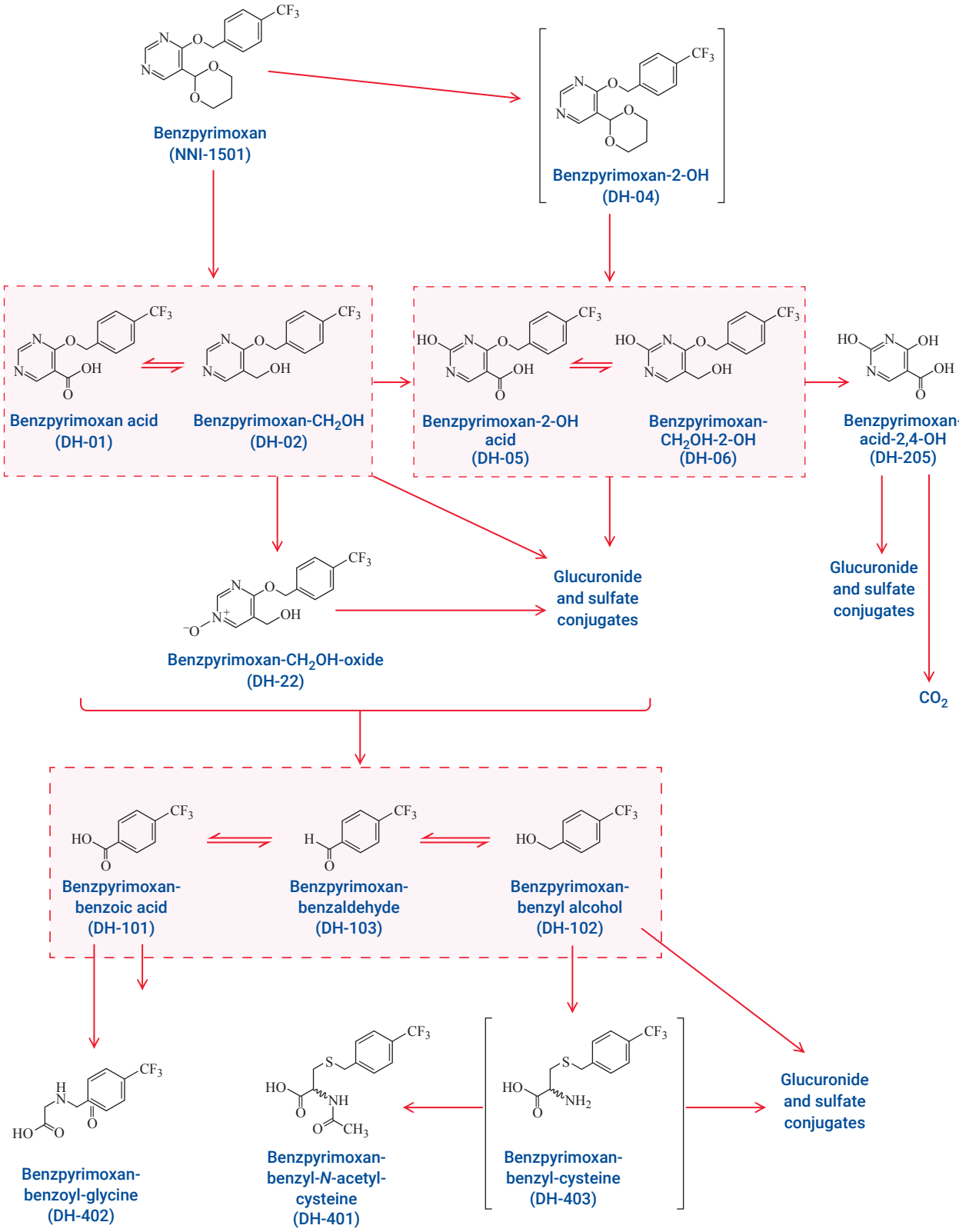
[a] All values are mean of five individuals

[b] No individual component exceeded 3.91% of dosed radioactivity in polar metabolites

[c]: No individual component exceeded 1.92% of dosed radioactivity in others

The proposed metabolic pathways from the result of the three toxicokinetic studies (Yasunaga. 2018a, b, c) are summarized in Fig. 6.

Figure 6. Proposed metabolic pathways of benzpyrimoxan in rats



(Redrawn from Yasunaga, 2018a, b, c)

(b) Dermal route

No studies were available.

1.2 Biotransformation

Please refer to the studies presented in section 1.1 above. Benzpyrimoxan was extensively metabolized, with up to nine metabolites identified in plasma, up to 15 in urine and six in bile. Unchanged parent compound was detected only in faeces, in small amounts (0.19–0.39% AR) at the low dose but at much higher levels at the high dose (24–29% AR). Benzpyrimoxan was mainly metabolized by hydrolysis of the acetal ring to form the carboxylic acid (DH-05 or M5) or the hydroxymethyl derivative (DH-06 or M6), followed by conjugation to form the glucuronide and sulfate. Hydroxylation (to generate DH-04 or M4) and *N*-oxidation (to generate DH-22 or M9) of the pyrimidine ring were also observed. In addition various metabolites involving the loss of the pyrimidine ring (DH-101 or M10 and DH-102 or M11) were also produced. These were further conjugated, leading to the glycine (DH-402 or M16), cysteine (DH-401 or M15), glucuronide and sulfate conjugates. Metabolites formed via loss of the phenyl ring (DH-205 or M14) and other highly polar metabolites were also produced and eventually mineralized to carbon dioxide. There was no significant impact of sex or dose on metabolism. DH-05 (or M5) was the only major metabolite in urine, accounting for 14–28% of AD at the low dose, and the DH-02 conjugates (or M2 conjugates) were the only major metabolites in bile, accounting for 13–14% of AD at the low dose. In plasma at the low dose, DH-01 (or M1) was a major metabolite, accounting for 79–85% of the TRR in this matrix at six hours post dosing (around T_{max}). At the high dose, DH-01 (or M1) and DH-101 (or M10) were significant metabolites, accounting for 21–32% and 23–30% respectively of the plasma TRR at nine hours post dosing (around T_{max}) (Yasunaga, 2018a, b, c).

1.3 Effects on enzymes and other biochemical parameters

The metabolite profiles of benzpyrimoxan were compared among various animal species and humans, in an in vitro (non-GLP) metabolism study using liver microsomes (Yasunaga, 2019).

Metabolite identification and quantification were performed by adding [*phenyl-U*-^{14}C]benzpyrimoxan at a final concentration of 2 µM to pooled liver microsomes of Wistar rats (male and female), ICR mice (male and female), Beagle dogs (male and female) and humans (pooled samples from males and females mixed; total of 10 and 50 donors of mixed sex, no further information provided). The mixtures were incubated at 37°C for one hour or 24 hours. Following incubation, the reaction mixture was extracted with acetone/methanol and then quantified by two-dimensional thin layer chromatography-radioluminography. Metabolites were identified by co-chromatography with reference standards.

Metabolites in liver microsomes from various animal species and humans are shown in Table 19.

Table 19. Metabolites in the liver microsomes of various animal species and humans, expressed as % of applied radioactivity

		Rat		Mouse		Dog		Human
Reaction time	**Metabolite**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male + female**
1 hour	Benzpyrimoxan	ND	ND	ND	0.3	23.5	23.4	0.8
	DH-01	6.7	3.1	7.7	10.0	21.2	23.9	23.4
	DH-02	83.9	91.8	78.4	63.6	35.7	33.9	68.1
	DH-03	ND	ND	2.1	1.2	2.6	3.5	1.5
	DH-04	ND	ND	1.1	1.8	4.6	4.4	2.5
	DH-05	2.4	1.9	1.5	2.9	1.7	1.3	0.3
	DH-06	0.3	0.3	ND	ND	ND	ND	0.3
	DH-07	ND	ND	ND	ND	ND	ND	ND
	DH-08	ND	ND	ND	ND	ND	ND	ND
	DH-22	5.4	0.7	7.6	16.6	0.5	0.5	2.5
	DH-101	0.4	ND	1.1	1.7	ND	ND	ND

Reaction time	Metabolite	Rat		Mouse		Dog		Human
		Male	Female	Male	Female	Male	Female	Male + female
24 hours	Benzpyrimoxan	ND	ND	ND	ND	1.6	1.7	0.7
	DH-01	ND	ND	2.3	3.9	38.6	36.7	16.0
	DH-02	62.4	83.9	56.3	25.2	9.1	26.4	61.1
	DH-03	ND	ND	0.7	0.7	ND	0.7	1.0
	DH-04	ND	ND	1.1	1.5	3.4	4.7	0.3
	DH-05	9.3	6.2	14.9	14.6	14.5	10.7	12.1
	DH-06	1.4	1.9	< 0.1	0.2	ND	ND	1.0
	DH-07	ND	ND	ND	ND	ND	ND	ND
	DH-08	ND	ND	ND	ND	ND	ND	ND
	DH-22	18.5	1.5	16.8	39.8	3.3	2.5	2.1
	DH-101	3.0	1.0	6.7	12.0	6.8	3.3	3.2

ND: Not detected; Source: Yasunaga , 2019

After one hour of incubation, DH-01 (M1), DH-02 (M2), and DH-22 (M9) were observed with all microsomes, DH-01 and DH-02 being the main metabolites in dog and human microsomes, while DH-22 also represented a significant metabolite with rat and mouse microsomes. After 24 hours of incubation the percentages of metabolites DH-05 (M5), DH-06 (M6), DH-22 (M9), and DH-101 (M10) increased compared to the proportions seen after one hour of incubation. Metabolite DH-04 (M4) was not observed with rat microsomes but small amounts were detected with mouse, dog and human microsomes. However, from the results on DH-05 and DH-06, it was concluded that DH-04, which is produced by hydroxylation of position 2 of the pyrimidine ring before hydrolysis of the acetal ring (to generate DH-05 and DH-06), must be generated as an intermediate metabolite in rats.

Regardless of species or sex, benzpyrimoxan was primarily metabolized by hydrolysis of the acetal ring to form the carboxylic acid (DH-05) or the hydroxymethyl derivative (DH-06), but also by hydroxylation of position 2 of the pyrimidine ring (to form DH-04), and *N*-oxidation of the pyrimidine ring (to form DH-22). Cleaved metabolites of the phenyl ring (DH-101) were also observed. No significant unique human metabolites were detected.

Based on the above results, the main metabolites detected in liver microsomes of all animal species and humans were DH-01 (M1), DH-02 (M2), DH-05 (M5), DH-22 (M9), and DH-101 (M10). There were no qualitative differences in metabolite profiles among the species. Furthermore the results from the in vitro metabolism study of benzpyrimoxan with rat liver microsomes resembled those from the phase-I metabolism seen in the in vivo ADME studies in rats. These results suggest that the metabolic pathway of benzpyrimoxan in mouse, dog and human is qualitatively comparable to the metabolism proposed from the rat ADME studies.

2. Toxicological studies

2.1 Acute toxicity

The results of acute oral, dermal and inhalation toxicity studies with benzpyrimoxan are summarized in Table 20, below.

Table 20. Studies of the acute toxicity of benzpyrimoxan

Species	Strain	Sex	Route	Purity	Result	Reference
Rat	RccHan™: Wistar	Male	Oral	93.7%	LD_{50} > 2000 mg/kg bw	Tsukushi, 2016a
Rat	Sprague Dawley Slc:SD	Male + female	Dermal	93.7%	LD_{50} > 2000 mg/kg bw	Yoshida, 2016
Rat	Sprague Dawley Crl:CD(SD)	Male + female	Inhalation	93.7%	LC_{50} > 3.9 mg/L (MMAD 3 to 3.3 µm)	van Huygevoort, 2017

LC_{50}: Median lethal concentration; LD_{50}: Median lethal dose; MMAD: Mass median aerodynamic diameter

(a) Lethal doses

Oral

In an acute oral toxicity study (acute toxic class method), benzpyrimoxan (purity 93.7%) in aqueous solution of 0.5% (w/v) sodium CMC containing 0.1% (v/v) Tween 80 was administered to three male Wistar rats via oral gavage at 2000 mg/kg bw with a dosing volume of 10 mL/kg. In the absence of mortality, an additional group of three animals was treated. Animals were observed for mortality and clinical signs over the subsequent 14 days. Clinical observations were made before fasting, pre and post dosing and 0.25, 0.5, 1, 3, and 6 hours after dosing, then once daily for 14 days. Mortality and morbidity were checked at least once a day. Individual body weights were recorded before fasting, on day 0 (the day of dosing) and on days 1, 7 and 14. The bilateral kidneys in all animals tested and the urinary bladder in two animals were removed for histopathological examination as the organs suggested treatment-related change in gross findings at necropsy.

No mortality was observed. No signs of abnormal behaviour or adverse clinical effects were noted.

Kidney enlargement, coarse surface and/or discolouration, all bilateral, were observed in all six animals. Renal tubular basophilic change and dilatation of renal tubules and collecting ducts were found in all tested animals. Three animals showed renal inflammatory cell infiltrate, two animals showed fibrosis, and two showed focal urothelial hyperplasia. Characteristic rounded crystals were seen in the renal collecting ducts of four animals. In the urinary bladder, yellow contents were found in two animals, but no histopathological findings were observed.

The acute oral LD_{50} of benzpyrimoxan in male rats was greater than 2000 mg/kg bw. Effects on the kidney were detected at necropsy and in histopathological examination (Tsukushi, 2016a).

Dermal

In an acute dermal toxicity study (OECD 402), groups of five female and five male Sprague Dawley rats were treated topically for 24 hours with a suspension of benzpyrimoxan (purity 93.7%) in 0.5% (w/v) sodium CMC containing 0.1% (v/v) Tween 80 solution under occlusive conditions at the limit dose of 2000 mg/kg bw. Observations for mortality and clinical/behavioural signs of toxicity were made before and immediately after treatment commenced, then 15, 30 and 60 minutes, three and six hours after administration, after which observations continued at least once daily for 14 days.

No mortality was observed. No signs of gross toxicity, abnormal behaviour, adverse clinical effects, or effects on body weight were noted. The acute dermal LD_{50} of benzpyrimoxan in female and male rats was greater than 2000 mg/kg bw (Yoshida, 2016).

Inhalation

In an acute inhalation toxicity study (OECD 403), groups of five male and five female Sprague Dawley rats were exposed (nose only) to a dust aerosol of benzpyrimoxan (purity 93.7%) for four hours at a mean actual concentration of 3.9 mg/L. This was the maximum attainable concentration. Animals were observed twice on the day of exposure and daily thereafter for 14 days. Body weights were recorded before exposure and on days 2, 4, 8 and 15 following exposure. At termination rats underwent a gross necropsy. The geometric standard deviation (GSD) and mass median aerodynamic diameter (MMAD) of the treatment material were determined twice during the exposure period. The first measurement showed a MMAD of 3.3 µm (GSD 1.9) with 62% of the particles below 4 µm; the second measurement showed a MMAD of 3.0 µm (GSD 1.9) with 66% of the particles below 4 µm.

There were no mortalities. Clinical signs of reaction to treatment during the exposure period were confined to shallow respiration in two males. Clinical signs noted post exposure were hunched posture in all animals and lethargy in two females. The animals had recovered from all signs by day 2. Reduced body weight gain and body weight loss were seen during the first week following exposure. All animals regained weight during the second week. No macroscopic abnormalities were observed. The acute median lethal concentration (LC_{50}) of the test article exceeded 3.9 mg/L following a four hour exposure period (van Huygevoort, 2017).

(b) Dermal irritation

The results of the skin and eye irritation and skin sensitization studies with benzpyrimoxan are summarized below in Table 21.

Table 21. Studies of irritation and skin sensitization due to benzpyrimoxan

Species	Strain	Sex	Route	Purity	Result	Reference
Rabbit	Japanese White (Kbl:JW)	Male	Skin irritation	93.7%	Slightly irritating	Munechika, 2017a
Rabbit	Japanese White (Kbl:JW)	Male	Eye irritation	93.7%	Slightly irritating	Tsukushi, 2017a
Rabbit cornea cells	Statens Serum Institut rabbit cornea (SIRC) cells	Unknown	Eye irritation	93.7%	No irritation potential	Munechika, 2017b
Guinea pig	Slc:Hartley [SPF]	Female	Skin sensitization	93.7%	Sensitizing	Takehara, 2016
Mouse	CBA/J [SPF]	Female	Skin sensitization	93.7%	Not sensitizing	Fujishima, 2016

In a dermal irritation study (OECD 404), three male Japanese white rabbits were exposed to 0.5 g benzpyrimoxan (purity 97.3%) moistened with 0.45 mL distilled water. The test substance was held in contact with the skin (exposed area: approximately 2.5 cm × 2.5 cm) for four hours under a semi-occlusive patch. Skin irritation reactions were evaluated 1, 24, 48 and 72 hours after removal of the test article.

No mortality or clinical signs were observed in any animal during the observation period of 72 hours following the removal of patches. Very slight erythema (score 1) was observed in one rabbit treated with benzpyrimoxan one hour after removal of the test substance. The erythema had disappeared within 48 hours of removal of the test substance. Neither erythema nor oedema was observed in the other two rabbits. It was concluded that benzpyrimoxan is very slightly irritating to rabbit skin (Munechika, 2017a).

(c) Ocular irritation

The potential of benzpyrimoxan to cause eye irritation was evaluated in an in vitro test followed by the standard in vivo eye irritation study in rabbits.

A non-GLP in vitro eye irritation test was performed to evaluate the eye irritation hazard potential of benzpyrimoxan based on its ability to induce cytotoxicity using the OECD "short time exposure in vitro test method" (OECD 491, July 2015).

Suspensions of benzpyrimoxan in mineral oil were prepared at concentrations of 0.05% and 5% (w/w). As a positive control a saline solution containing 0.01% (w/v) sodium lauryl sulfate (SLS) was used. A confluent monolayer of Statens Serum Institut rabbit cornea cells cultured in a 96-well microplate was exposed to benzpyrimoxan and SLS for five minutes. Medium and solvent control wells were exposed in the same manner. After exposure, cells were washed with Dulbecco's phosphate buffer. Cell viability was measured by MTT assay. MTT formazan, extracted with a 0.04 M hydrochloric acid/isopropanol mixture was quantified by measuring its optical density at 570 nm with a microplate reader. Three replicate wells per assay were used, and three independent experiments conducted.

The cell viability of medium, the solvent and positive controls satisfied the acceptance criteria of the guideline. No significant difference was found in cell viability at 0.05% or 5% benzpyrimoxan compared to the solvent control. The relative cell viability compared to the solvent control was 100% and 98% at 0.05% and 5% benzpyrimoxan, respectively. Cell viability with the SLS positive control was 50%.

Since cell viability with benzpyrimoxan at both concentrations was higher than 70%, benzpyrimoxan was judged to be non-irritant according to this in vitro assay It is noted that since a clear negative result was obtained in the study, an in vivo follow-up study was not required. However, the results of such a study were submitted in any case (Munechika, 2017b).

The eye irritation potential of benzpyrimoxan was investigated in a study (OECD 405) in male Japanese White rabbits (Kbl:JW). Test animals were treated with a topical anaesthetic (tetracaine HCl) and systemic analgesics (buprenorphine and meloxicam) to minimize any pain and distress. Benzpyrimoxan (48 mg in a volume of 0.1 mL) was instilled into the conjunctival sac of the left eye. The treated left eye and untreated right eye were not washed. Eyes were observed macroscopically and comprehensively at 1, 24, 48 and 72 hour(s) after the application. As benzpyrimoxan had shown to be a very slight irritant to the skin, initially one rabbit was treated in this study. Since no severe irritation was observed in this animal, two additional rabbits were treated to confirm the results.

No mortality or clinical signs were observed in any animal during the observation period. No corneal opacity was noted during the study. For the iris, minimal irritation response (grade 1) was observed in one treated eye at one hour after the application. For the conjunctiva, redness (grade 1) was observed in all treated eyes until 24 hours after application. In addition, discharge (grade 1 or 2) was also noted in two treated eyes at one hour after application. All treated eyes appeared normal at 48 hours after application.

It was concluded that benzpyrimoxan is slightly irritating to the eyes of rabbits (Tsukushi, 2017a).

Overall, benzpyrimoxan is only slightly irritating to the eye in rabbits.

(d) Dermal sensitization

The skin sensitization potential of benzpyrimoxan was investigated in a maximization test in Guinea pigs and a mouse local lymph node assay (LLNA).

In a maximization test (Magnusson & Kligmann; OECD 406) 10 Hartley female Guinea pigs were tested with benzpyrimoxan (purity 93.7%) in corn oil. The 10 test animals were intradermally injected with 2% benzpyrimoxan in corn oil. Subsequently a 50% test substance suspension in corn oil was applied topically. At challenge, a suspension of 50% benzpyrimoxan in olive oil was applied topically to the skin at a naïve site. Negative and positive control groups each consisting of five animals were established and treated with the vehicle alone or α-hexylcinnamaldehyde (HCA), respectively. The application site was observed for skin reactions 24 and 48 hours post injection and 24 and 48 hours after the topical applications.

No mortality was observed. As a result of the challenge treatment, skin reactions (score 1 or 2) were seen in all 10 Guinea pigs of the test group at 24 or 48 hours after removal of the challenge patch. Skin reactions (score 3) were also seen in all five animals of the positive control group. No animals from the negative control group showed a skin reaction at the challenge sites. The skin sensitization ratio was therefore 100%.

Benzpyrimoxan was judged to be a skin sensitizer under the conditions of this study (Takehara, 2016).

In a mouse local lymph node assay (LLNA; radioactive incorporation; OECD 429), 25 female CBA/J [SPF] mice were divided into five dose groups, each group consisting of five mice. these were treated topically with benzpyrimoxan at concentration levels of 2.5%, 5% or 10% (w/v), positive control substance (25% v/v HCA) or the vehicle (acetone : olive oil at 4 : 1 v/v). The highest test substance concentration that could be achieved was reported as 10%. Mice were observed daily from day 1–3 and then at day 6 (five days after administration). Ear thickness was assessed before treatment, on day 3 (24 hours after treatment) and on day 6. Body weight was assessed before treatment and on day 6.

The stimulation indices (SI) for the 2.5%, 5% and 10% w/v groups were calculated to be 0.9, 1.1 and 1.0, respectively. As these indices were less than 3 for any of the treated groups, benzpyrimoxan was judged to be a non-sensitizer. The SI of the positive control was 14.5, confirming the test's validity.

No animals showed abnormal clinical signs, findings at the application sites or increases in ear thickness during the observation period. The weight of the lymph nodes in the positive control group was higher than that of the vehicle control group and of the test substance groups. There were no effects on body weight.

Benzpyrimoxan was judged to be a non-sensitizer under the conditions of this study. It is however noted that it is unclear whether or not the concentration of test substance was maximized. It is possible that if a different vehicle (perhaps corn oil) had been chosen, a higher concentration could have been tested (Fujishima, 2016).

2.2 Short-term studies of toxicity

The short-term toxicity of benzpyrimoxan was investigated by the oral route in 28-day and 90-day studies in mice and rats and in 14-day, 28-day, 90-day and one-year studies in dogs. Additionally, in rats, a 28-day dermal study and five- and 28-day inhalation studies were available for evaluation.

(a) Oral administration

Mouse

A non-GLP but guideline (OECD 407) 28-day study in mice was conducted to make a preliminary assessment of the toxicity potential of benzpyrimoxan in this species and to aid selection of dietary concentrations for the subsequent 90-day study. Groups of five male and five female ICR [Crl:CD1] mice were five weeks old, weighing 28–32 g (males) and 23–27 g (females). These were fed benzpyrimoxan (purity 94%) in the diet for 28 days at concentrations of 0, 400, 1000, 4000 or 8000 ppm (equivalent to 0, 65, 161, 632 and 1203 mg/kg bw per day for males, 0, 73, 190, 759 and 1428 mg/kg bw per day for females). During the study the animals were examined for mortality, clinical signs of toxicity, body weight, food consumption and haematology as well as clinical chemistry parameters. At necropsy, the animals were subjected to gross pathology, organ weight measurements and histopathology.

One male receiving 8000 ppm presented clinical signs of wasting during days 5–7. Body weights at 4000 ppm in males were reduced (statistically significant) on day 2 by 8% compared to controls. By week 1 weights were still lower than for controls, but only marginally so. Body weights were also reduced (statistically significant) at 8000 ppm in males from days 2–5 (by up to 24%) up to week 2 (by 10%). By week 4 weights were still lower than for controls (by 9%) but not with statistical significance. Accordingly, body weight gains were significantly reduced (in some cases, animals lost weight) in males at 4000 and 8000 ppm, with decreases being much more substantial during the first two weeks, but still apparent at the end of the study, in particular at the top dose. There were no effects on body weights or body weight gains in females. Food consumption was reduced (by 29%; statistically significant) in top dose males during week 1.

In top dose males, there were statistically significant increases in platelet count, segmented neutrophil count and reticulocyte count, and slight(not statistically significant) decreases in haematocrit (Ht), haemoglobin (Hb) concentration and erythrocyte count, indicative of haematological effects. From the clinical chemistry examination, changes indicative of liver and kidney toxicity were noted from 4000 ppm. Statistically significant increases in plasma aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), cholinesterase (ChE), creatinine, total protein and calcium, in addition to statistically significant decreases in triglycerides and glucose were observed in top dose males. In top dose females AST and ALT were also decreased, although not with statistical significance. In males receiving 4000 ppm, ALT and ChE were also increased with statistical significance.

At necropsy, yellow contents in the urinary bladder were observed in two top-dose males and in one male receiving 4000 ppm. Discolouration was observed in the kidneys of four males and two females given 8000 ppm (bilateral in four males and unilateral in two females). In addition, a rough surface was observed on the kidneys of all males and one female given 8000 ppm (bilateral in four males and unilateral in one male and one female). Relative liver weight was increased (statistically significant) in females at 4000 ppm and above (by 24% and 17% at 4000 and 8000 ppm respectively) and in males at 1000 ppm and above (by 16%, 22% and 22% at 1000, 4000 and 8000 ppm respectively). Absolute liver weights were also increased (by 15% and more) at the same dose levels, although this change was not statistically significant. The increase in liver weight observed in males at 1000 ppm was not considered adverse by the Meeting in the absence of associated histopathology findings (see below) or changes in clinical chemistry parameters. The sponsor, however, did regard the effect in males at 1000 ppm as adverse. Absolute and relative spleen weights were increased (statistically significant) in both sexes given 8000 ppm (absolute by 45% in males and 66% in females).

At histopathological examination, changes were noted in the liver, urinary bladder, kidneys, spleen and thyroid. In the liver, centrilobular hepatocellular hypertrophy was observed in both sexes at 4000 ppm and above. Hepatocellular single cell necrosis was observed in four males and two females given 8000 ppm and two males given 4000 ppm. Additionally in males given 8000 ppm, focal necrosis, increased mitotic figures of hepatocytes, and proliferation and inflammation of the bile ducts were noted. Crystals were observed in the urinary bladders of males at 4000 ppm and above. In the kidneys, crystals were observed in the renal tubules, collecting ducts, pelvis and interstitium of three males and one female given 8000 ppm and four males given 4000 ppm. Degeneration/necrosis and basophilic changes of the renal tubules were observed in males from 4000 ppm and in top-dose females. Luminal dilatation and degeneration/necrosis of the collecting ducts were noted in males from 4000 ppm and in one top-dose female. There were also cellular casts in the collecting ducts of males at 4000 ppm and above, and luminal dilatation and increased mitotic figures of the renal tubules in top-dose males. Interstitial cellular infiltration and/or fibrosis were observed in males at 1000 ppm and above and in top-dose females. At 1000 ppm in males there was a non-significant increase (2/5 compared to 0/5 in controls) in the incidence of interstitial cellular infiltration. No such effects were observed in the kidneys of mice treated for 13 weeks (see below) up to the much higher dose of 4000 ppm, so this finding was considered equivocal. Incidence and severity of the kidney changes were greater in males than in females. In the spleen, increased extramedullary haemopoiesis of the red pulp was observed in four top-dose females. In the thyroid, minimal follicular cell hypertrophy was observed in one top-dose female. A summary of the histopathological findings from the 28-day mouse study is shown in Table 22.

Table 22. Selected histopathology findings in the 28-day mouse study

	Dose level (ppm), sex									
	0		**400**		**1000**		**4000**		**8000**	
Parameter	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Liver										
Hypertrophy, hepatocyte, centrilobular	0	0	0	0	0	0	**5****	**4***	**4***	**4***
Focal necrosis, hepatocyte	0	0	0	0	0	0	0	1	2	0
Single cell necrosis, hepatocyte	0	0	0	0	0	0	2	1	**4***	2
Mitotic figures, hepatocyte, increased	0	0	0	0	0	0	0	1	2	0
Proliferation/inflammation, bile duct	0	0	0	0	0	0	0	0	3	0

	Dose level (ppm), sex									
	0		400		1000		4000		8000	
Parameter	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Urinary bladder										
Crystals, A type	0	0	NE	NE	0	NE	0	NE	3	0
Crystals, B type	0	0	NE	NE	0	NE	3	NE	**5****	0
Kidney										
Crystals, B type	0	0	0	0	0	0	**4***	0	3	1
Dilatation, luminal (renal tubule)	0	0	0	0	0	0	0	0	**5****	1
Degeneration / necrosis (renal tubule)	0	0	0	0	0	1	2	1	**5****	2
Mitotic figures, increased (renal tubule)	0	0	0	0	0	1	0	0	3	0
Basophilic change (renal tubule)	0	0	0	1	0	1	1	1	**5****	**4***
Dilatation, luminal (collecting duct)	0	0	0	0	0	0	1	0	1	1
Degeneration/necrosis (collecting duct)	0	0	0	0	0	0	2	0	**4***	1
Casts, cellular (collecting duct)	0	0	0	0	0	0	1	0	**4***	0
Cellular infiltration (interstitium)	0	0	0	0	2	0	3	0	**5****	1
Fibrosis (interstitium)	0	0	0	0	0	0	2	0	4*	**5****
Spleen										
Extramedullary haemopoiesis, increased	0	0	NE	NE	NE	NE	NE	0	0	**4***
Thyroid gland										
Hypertrophy (follicular cell)	0	0	NE	NE	NE	NE	NE	NE	0	1

NE: Not examined; Source: Yoshimitsu, 2017

Significantly different from control by Fisher's exact probability test: * $p < 0.05$; ** $p < 0.01$

In conclusion, dietary administration of benzpyrimoxan to mice for 28 days was adverse at the top dose of 8000 ppm, causing effects on body weights, body weight gains and food consumption in males, haematological effects in males, clinical chemistry changes indicative of liver and kidney toxicity in both sexes, increased liver and spleen weights in both sexes and histopathologically observed changes to the liver (both sexes), kidney (both sexes), urinary bladder (males only) and spleen (females only). At 4000 ppm, there were still effects on body weight and body weight gain in males (although milder), changes in fewer clinical chemistry parameters in males, increased liver weights in both sexes and histopathological changes to the liver (mainly hypertrophy in both sexes, with single cell necrosis in males), kidney (males only) and urinary bladder (males only). There were no adverse effects at 1000 ppm, with the exception of an equivocal increase in renal interstitial cellular infiltration in males. The kidney effects are not considered relevant to humans (see below in 2.6 Special studies). Overall a NOAEL of 1000 ppm (equal to 161 mg/kg bw per day) was identified from this study, based on decreases in body weight and body weight gain in males, changes in clinical chemistry parameters in males, increased liver weights in both sexes and histopathology of the liver (mainly hypertrophy in both sexes, with single cell necrosis in males) at the LOAEL of 4000 ppm (equal to 632 mg/kg bw per day). It was concluded that the high dose for the subsequent 90-day study needed to be between 4000 and 8000 ppm, to induce clear toxic signs in both sexes (Yoshimitsu, 2017).

A GLP but non-guideline (although similar to OECD 408) preliminary 90-day study in mice was conducted to aid selection of dietary concentrations for the subsequent 78-week carcinogenicity study. Groups were of 12 male and 12 female ICR [Crl:CD1] mice, which were five weeks old and weighed 25–34 g (males) and 19–24 g (females). They were administered benzpyrimoxan (purity 93.7%) at 0, 400, 2000 or 4000 (males)/6000 (females) ppm (equivalent to 0, 56, 282 and 523 mg/kg bw per day in males, 0, 66, 327 and 971 mg/kg bw per day in females). During the study, clinical condition, body weights, food consumption, haematology, blood chemistry, organ weight, gross pathology and histopathology investigations were undertaken.

There were no treatment-related clinical signs of toxicity but there was one treatment-related death. One female receiving 6000 ppm died in week 5. At necropsy, marked bilateral obstructive nephropathy was noted. This finding was considered to have compromised renal function in this animal contributing to its death. Similar lesions were seen in the remaining top-dose females, therefore, the death of this animal was clearly due to treatment. Body weights were lower (by 6–8%, statistically significant) compared to controls at various time points throughout the study in top-dose males only. However, the overall body weight gain was lower (statistically significant) compared to controls in top-dose males (by 24%) and females (by 26%). In males this was mainly due to a marked reduction in body weight gain during week 1, whilst in females a moderate reduction in body weight gain was observed throughout the 13 weeks of treatment. Food consumption was reduced to a statistically significant extent in top-dose males at week 1 (by 18%) and week 4 (by 14%), and in top-dose females at week 1 (by 14%).

The haematological examination after 13 weeks of treatment revealed a statistically significant lowering of Ht, Hb and erythrocyte count in top-dose males and females. In males these changes were associated with lower reticulocyte counts and higher mean cell volumes, whilst in females there was an increase in reticulocyte count. There was also a statistically significant increase in neutrophil, lymphocyte and large unstained cell counts in both sexes from the mid-dose, and a small increase in eosinophil count in top-dose females. Platelet counts were higher than in controls in top-dose females. Selected haematology results are shown in Table 23 below.

Table 23. Selected haematological parameters after 13 weeks of treatment in 90-day mouse study (mean ± SD shown)

Parameter	Dose level (ppm)							
	Males				Females			
	0	400	2000	4000	0	400	2000	6000 [a]
Haematocrit (L/L)	0.438 ± 0.0206	0.449 ± 0.0233	0.470 ± 0.0287	**0.401** ± 0.0374**	0.474 ± 0.0176	0.474 ± 0.0163	0.456 ± 0.0229	**0.387** ± 0.0308**
Haemoglobin (g/dL)	13.1 ± 0.71	13.6 ± 0.80	13.8 ± 0.56	**11.3** ± 0.96**	13.8 ± 0.54	13.8 ± 0.54	13.5 ± 0.77	**11.2** ± 0.98**
Red blood cells (10^{12}/L)	8.38 ± 0.421	8.81 ± 0.372	8.76 ± 0.630	**7.39** ± 0.466**	8.60 ± 0.363	8.50 ± 0.437	8.42 ± 0.334	**7.11** ± 0.533**
Reticulocytes (10^{12}/L)	0.311 ± 0.0824	0.291 ± 0.0310	0.302 ± 0.0627	**0.254* ± 0.0585**	0.255 ± 0.0859	0.262 ± 0.0770	**0.393* ± 0.1044**	**0.314* ± 0.1078**
MCHC (g/dL)	30.0 ± 0.52	30.2 ± 0.54	**29.3* ± 1.18**	**28.2** ± 0.62**	29.0 ± 0.75	29.0 ± 1.21	29.6 ± 0.95	28.9 ± 0.80
MCV (fL)	52.3 ± 1.91	50.9 ± 1.46	53.7 ± 2.30	**54.2* ± 2.05**	55.2 ± 1.48	55.8 ± 2.03	54.2 ± 1.74	54.5 ± 1.49
WBC (10^9/L)	3.13 ± 1.558	2.86 ± 1.481	**6.16** ± 2.434**	**8.40** ± 2.362**	4.36 ± 1.419	4.30 ± 2.270	**7.48** ± 1.810**	**8.04** ± 1.922**
Neutrophils (10^9/L)	1.00 ± 1.039	1.17 ± 1.168	**2.20* ± 1.090**	**1.73* ± 0.663**	0.69 ± 0.477	0.58 ± 0.317	**1.10* ± 0.404**	**1.28** ± 0.300**
Lymphocytes (10^9/L)	1.91 ± 0.952	1.53 ± 0.542	**3.58** ± 1.562**	**6.41** ± 2.133**	3.36 ± 1.014	3.44 ± 1.870	**5.89** ± 1.480**	**6.25** ± 1.782**
Eosinophils (10^9/L)	0.11 ± 0.052	0.08 ± 0.038	0.17 ± 0.110	0.13 ± 0.134	0.17 ± 0.093	0.14 ± 0.53	0.18 ± 0.075	**0.25* ± 0.129**
LUC [#] (10^9/L)	0.02 ± 0.019	0.01 ± 0.007	**0.07* ± 0.046**	**0.12* ± 0.078**	0.07 ± 0.050	0.07 ± 0.054	**0.19** ± 0.077**	**0.17** ± 0.086**
Platelets (10^9/L)	1270 ± 162.0	1231 ± 146.6	1173 ± 122.4	1193 ± 214.8	967 ± 154.2	955 ± 131.1	1074 ± 154.0	**1289** ± 221.9**

MCHC: Mean corpuscular haemoglobin concentration; LUC: Large unstained cells; Source: Coleman, 2017

WBC: White blood cell (leucocyte) count; MCV: Mean corpuscular volume;

[a] Animal number was reduced from 12 to 11 due to one death; [#] Shirley's test used for male animals;

Significantly different from control by Williams' test: * $p < 0.05$; ** $p < 0.01$

At the clinical chemistry analysis, the following treatment-related effects indicative of liver and kidney toxicity were observed: increased ALP in top-dose males and increased AST and ALT activities in males at the mid-dose and above; increased urea and creatinine in top-dose males and females; higher triglyceride concentrations in top-dose males; lower sodium and chloride concentrations in top-dose females; higher calcium levels in males at the mid dose and above, and in top-dose females; and lower albumin/globulin ratio in top-dose males and females. Table 24 below summarizes selected clinical chemistry results.

Table 24. Selected clinical chemical parameters after 13 weeks of treatment in a 90-day mouse study (mean ± SD are shown)

Parameter	Dose level (ppm)							
	Males				Females			
	0	400	2000	4000	0	400	2000	6000 [a]
ALP (U/L)	36 ± 4.6	44 ± 8.5	41 ± 6.8	**79** ± 24.0**	60 ± 13.6	66 ± 15.8	54 ± 15.5	59 ± 17.1
ALT (U/L)	45 ± 48.6	45 ± 28.9	64 ± 31.8	**105** ± 46.6**	24 ± 7.2	24 ± 4.5	27 ± 10.2	29 ± 8.5
CK (U/L)	128 ± 118.9	221 ± 286.5	305 ± 176.1	146 ± 107.7	69 ± 54.2	69 ± 48.5	52 ± 21.2	**164* ± 267.6**
Urea (mmol/L)	9.25 ± 1.950	9.58 ± 1.722	**7.12* ± 0.834**	**15.71** ± 4.187**	7.15 ± 0.694	7.51 ± 1.060	6.43 ± 1.016	**14.71** ± 5.812**
Creatine (µmol/L)	8 ± 2.0	6 ± 1.5	7 ± 2.6	**19** ± 5.8**	9 ± 1.1	9 ± 1.4	10 ± 2.7	**23** ± 9.7**
Triglycerides (mmol/L)	1.15 ± 0.444	1.07 ± 0.310	1.07 ± 0.531	**1.51* ± 0.94**	1.07 ± 0.366	1.45 ± 0.479	1.19 ± 0.420	0.84 ± 0.510
Sodium (mmol/L)	152 ± 1.9	153 ± 2.3	154 ± 1.6	154 ± 1.2	152 ± 0.9	151 ± 1.6	149* ± 2.6	**149* ± 5.4**
Calcium (mmol/L)	2.26 ± 0.062	2.24 ± 0.059	**2.35** ± 0.092**	**2.48** ± 0.065**	2.35 ± 0.080	2.36 ± 0.043	2.35 ± 0.068	**2.49** ± 0.099**
Phosphate (mmol/L)	2.02 ± 0.285	2.05 ± 0.365	**2.48* ± 0.599**	1.91 ± 0.245	2.30 ± 0.501	2.17 ± 0.318	2.10 ± 0.299	2.18 ± 0.450
A/G	1.67 ± 0.196	1.73 ± 0.128	1.56 ± 0.166	**1.45** ± 0.105**	1.83 ± 0.163	1.80 ± 0.135	1.80 ± 0.120	**1.61** ± 0.160**

[a] Animal number was reduced from 12 to 11 due to one death Source: Coleman, 2017

ALP: Alkaline phosphatase; ALT: Alanine transaminase; CK: Creatine kinase; A/G: Albumin : globulin ratio

Significantly different from control using Fisher's exact test: * $p < 0.05$; ** $p < 0.01$

At necropsy there was a statistically significant reduction in absolute and relative kidney weight in top-dose males (by 35–38%) and females (by 23–26%). Absolute and relative liver weights were increased (statistically significant) in both sexes from the mid dose (by 17% and 22% for males and 9% and 21% for females). In addition, absolute and relative spleen weights were increased, with statistical significance, in females at the mid dose (by 31% and 41%) and at the top dose in males (by 34%). A statistically significant increase in relative adrenal weights was seen from the lowest dose (by 28%, 28% and 49%) in males, although no clear dose–response relationship was apparent. Absolute adrenal weights were also significantly increased at the mid dose and above in males. However, similar increases were not seen in the preceding 28-day study, where males were administered up to the higher dose of 8000 ppm. There were no effects in females and no associated histopathology. In addition, no effects were seen on adrenal weights at doses of up to 2000 ppm in the 78-week study (see below). On this basis, although relationship to treatment cannot be excluded, these organ changes were not considered adverse. All other organ weight changes were minor (for example a 5% decrease in brain weights in top-dose males), lacked a dose–response relationship (for example thymus weights in males) or were secondary to the observed reductions in body weight (For example brain and testis weights). Gross

pathology examination revealed small size and depressions in the kidney at the top dose in both sexes and enlargement of the spleen at the mid dose and above in both sexes.

Table 25. Changes in organ weights in 90-day mouse study (mean ± standard deviation shown)

Parameter		Dose level (ppm)							
		Males				Females			
		0	400	2000	4000	0	400	2000	6000 [a]
Adrenals	A	0.0052 ± 0.0013	0.0063 ± 0.0014	**0.0065* ± 0.0013**	**0.0076** ± 0.0015**	0.0100 ± 0.0020	0.0090 ± 0.0028	0.0102 ± 0.0029	0.0104 ± 0.0022
	R	0.0134 ± 0.0038	**0.0172* ± 0.0042**	**0.0168* ± 0.0028**	**0.0204** ± 0.0036**	0.0347 ± 0.0082	0.0312 ± 0.0117	0.0344 ± 0.0111	0.0370 ± 0.0071
Brain	A	0.4892 ± 0.0209	0.4806 ± 0.0259	0.4757 ± 0.0200	**0.4631** ± 0.0246**	0.4794 ± 0.0234	0.4741 ± 0.0154	0.4691 ± 0.0291	0.4748 ± 0.0207
	R	1.25 ± 0.11	1.30 ± 0.08	1.24 ± 0.08	1.24 ± 0.09	1.66 ± 0.17	1.62 ± 0–14	1.58 ± 0.11	1.69 ± 0.15
Kidney	A	0.5347 ± 0.0856	0.5152 ± 0.0615	0.5433 ± 0.0842	**0.3288** ± 0.0587**	0.3429 ± 0.0246	0.3516 ± 0.0322	0.3450 ± 0.0414	**0.2576** ± 0.0400**
	R	1.35 ± 0.15	1.39 ± 0.16	1.41 ± 0.16	**0.88** ± 0.12**	1.18 ± 0.09	1.20 ± 0.10	1.16 ± 0.10	**0.91** ± 0.13**
Liver	A	1.8664 ± 0.3040	1.7537 ± 0.2248	2.1299* ± 0.2243	**2.1544** ± 0.1834**	1.4302 ± 0.2150	1.5314 ± 0.2291	1.5917 ± 0.1693	**1.6643** ± 0.1433**
	R	4.72 ± 0.53	4.71 ± 0.35	**5.54** ± 0.44**	**5.77** ± 0.40**	4.89 ± 0.45	5.19 ± 0.43	**5.33** ± 0.34**	**5.91** ± 0.33**
Spleen	A	0.1140 ± 0.0451	0.0812 ± 0.0186	0.1146 ± 0.0338	**0.1441* ± 0.0261**	0.1060 ± 0.0207	0.1045 ± 0.0152	**0.1412** ± 0.0396**	**0.1457** ± 0.0246**
	R	0.288 ± 0.103	0.218 ± 0.045	0.296 ± 0.077	**0.385** ± 0.059**	0.367 ± 0.086	0.355 ± 0.041	**0.477** ± 0.153**	**0.517** ± 0.077**
Testes	A	0.2353 ± 0.0293	0.2403 ± 0.0289	0.2477 ± 0.0285	0.2535 ± 0.0270	-	-	-	-
	R	0.600 ± 0.088	0.649 ± 0.076	0.646 ± 0.076	**0.681* ± 0.083**	-	-	-	-
Thymus	A	0.0340 ± 0.0070	0.0286 ± 0.0078	**0.0246* ± 0.0057**	**0.0305* ± 0.0066**	0.0341 ± 0.0074	0.0367 ± 0.0093	0.0377 ± 0.0063	0.0372 ± 0.0090
	R	0.0869 ± 0.0207	0.0769 ± 0.0197	0.0644 ± 0.0164	0.0817 ± 0.0182	0.117 ± 0.027	0.124 ± 0.027	0.127 ± 0.024	0.131 ± 0.027

A: Absolute weight change (g); R: Relative weight change; organ weight/body weight; Source: Coleman, 2017

[a] Animal number was reduced from 12 to 11 due to one death

Significantly different from control using Fisher's exact test: * $p < 0.05$; ** $p < 0.01$

Treatment-related histopathological findings were observed in the kidneys, urinary bladder, liver and spleen. In the kidneys, slight to marked bilateral obstructive nephropathy was seen in all top-dose males and females. It was characterized by tubular dilatation, tubular basophilia, fibrosis, tubular inflammatory cell infiltrate, tubular eosinophilic material and/or crystals and glomerular atrophy. Slight unilateral obstructive nephropathy was also seen in two mid-dose females and crystals were noted in the pelvis of some top-dose animals of both sexes. In the urinary bladder, crystals were present in one top-dose male and one top-dose female. In the liver, centrilobular hypertrophy was noted at the mid dose and above in both sexes. In addition, there was bile duct proliferation/inflammation at the top dose in both sexes. In the spleen, there was an increased incidence of extramedullary haemopoiesis in females at the mid dose and above.

Table 26. Treatment-related histopathological findings in 90-day mouse study (number of mice in each group of 12 showing signs is shown)

Parameter			Dose level (ppm)							
			Males				Females			
			0	400	2000	4000	0	400	2000	6000 [a]
Kidneys	Nephropathy, (obstructive)	slight	0	0	0	1	0	0	2	1
		moderate	0	0	0	5	0	0	0	1
		marked	0	0	0	6	0	0	0	9
		total	0	0	0	12	0	0	2	11
	Crystals (pelvic)	present	0	0	0	4	0	0	0	3
		total	0	0	0	4	0	0	0	3
Urinary bladder	Crystals	present	0	0	0	1	0	0	0	1
		total	0	0	0	1	0	0	0	1
Liver	Hypertrophy, (centrilobular)	minimal	0	0	2	7	0	0	1	4
		slight	0	0	0	3	0	0	0	1
		total	0	0	2	10	0	0	1	5
	Proliferation/ inflammation, (bile ducts)	minimal	0	0	0	4	0	0	0	1
		slight	0	0	0	3	0	0	0	0
		moderate	0	0	0	1	0	0	0	0
		total	0	0	0	8	0	0	0	1
Spleen	Extramedullary haemopoiesis	minimal	3	1	3	4	3	4	4	5
		slight	1	0	1	0	0	0	2	2
		total	4	1	4	4	3	4	6	7

[a] Animal number was reduced from 12 to 11 due to one death

Source: Coleman, 2017

In conclusion, dietary administration of benzpyrimoxan to mice for 90 days was adverse at the top dose of 4000/6000 ppm, causing one death in females, effects on body weight in males, reductions in body weight gain and food consumption in both sexes, haematological effects in both sexes, clinical chemistry changes indicative of liver and kidney toxicity in both sexes, changes in kidney, liver and spleen weights in both sexes and histopathological changes in liver (both sexes), kidney (both sexes), urinary bladder (both sexes) and spleen (females only). At the mid dose of 2000 ppm, there were still apparent changes in haematology and clinical chemistry parameters in both sexes, increased liver weight in both sexes and increased spleen weight in females, and histopathological changes in liver (hypertrophy in both sexes), kidney (females only) and spleen (females only). There were no adverse effects at 400 ppm. The kidney effects were considered not relevant to humans (see section 2.6 Special studies). Overall, a NOAEL of 400 ppm (equal to 56 mg/kg bw per day) was identified from this study based on liver and spleen histopathological changes associated with changes in organ weights and/or haematological and clinical chemistry findings at the LOAEL of 2000 ppm (equal to 282 mg/kg bw per day). It was concluded that the high dose for the subsequent carcinogenicity study needed to be 2000 ppm in males and below 2000 ppm in females mainly because of the effects seen in the kidney in this sex at 2000 ppm (Coleman, 2017).

Rat

A non-GLP and non-guideline (although similar to OECD 407) preliminary 28-day study in rats was conducted to aid selection of dietary concentrations for the subsequent 90-day study. Groups of five male and five female Wistar Hannover rats used rats that were six weeks old and weighed 154–171 g (males) and 129–145 g (females). They were administered benzpyrimoxan (purity 99.1%) over 28 days via the diet at concentrations of 0, 300, 1000 or 5000 ppm (equivalent to 0, 25.5, 86.7 and 317.4 mg/kg bw per day for males, 0, 26.1, 89.1 and 324.5 mg/kg bw per day for females). During the administration period,

mortality and clinical signs of toxicity were checked once a day. Body weight and food consumption were measured twice during the first week and then once a week. Before commencement of treatment and at four weeks, urinalysis and ophthalmology were performed. After four weeks of treatment haematology and blood chemistry assessments were made and animals were subjected to necropsy, organ weight measurements and histopathological examination.

No deaths occurred, but one top-dose female showed emaciation and hypothermia from day 6 to day 8 of treatment. Body weights were lower (statistically significant) compared to controls throughout the study in top-dose males (by 11–35%) and females (by 10–27%). In addition, significantly lower terminal body weights were seen in mid-dose males (by 8%). Similarly, body weight gains were significantly reduced in males at the mid dose and above (by 16% and 66% respectively) and in top-dose females (by 80%). Lower food consumption and food efficiency were also observed at the top dose in both sexes.

At the haematology examination, significant decreases in Hb concentration, Ht and mean corpuscular volume were noted in top-dose females. Significant increases in counts of leukocytes, lymphocytes (males only), stab neutrophils, segmented neutrophils, and platelets were observed in top-dose males and females. Haemoglobin was also lower in the mid-dose females. These findings indicated general haematological effects with signs of anaemia. At the urinalysis, a statistically significant decrease in pH was recorded in top-dose males and a statistically significant increase in leukocytes and epithelial cells of the urinary sediment was seen in top-dose females. In addition, crystals were present in the sediment from rats at the mid-dose and above in both sexes. Clinical chemistry investigations, revealed significant changes indicative of liver and kidney toxicity in both sexes mainly at the top dose. These included increases in total serum (or plasma) cholesterol, glucose (females only), urea nitrogen, creatinine, uric acid (females only), cholinesterase (males only), inorganic phosphorus, chloride, calcium and potassium, and decreases in protein (females only), albumin and the albumin : globulin ratio. Total cholesterol was also significantly increased in mid-dose females.

At necropsy, a coarse surface, discolouration and enlargement were observed in the kidneys of top-dose males and females. With these animals there was also deposition of a yellow material in the renal cortex and medulla. This yellow content was also observed in the urinary bladders of top-dose males. Numerous organs (brain, heart, epididymides, prostate, seminal vesicles, testes, ovaries, uterus, pituitary, thymus and thyroid) showed significant weight changes at the top dose. However, in the absence of associated histopathology or clinical pathology, these were most likely secondary to the decrements in body weights observed at this dose. Organ weight changes considered toxicologically significant were noted in both sexes at the top dose; significant changes were to the adrenal (relative weight increased by 5–25%), kidney (absolute weight increased by 78–88%), liver (relative weight increased by 26–33%) and spleen (relative weight increased by 13–36%).

Table 27. Selected organ weights in 28-day rat study (mean ± standard deviation shown)

		Dose level (ppm)							
		Males				Females			
Parameter		**0**	**300**	**1000**	**5000**	**0**	**300**	**1000**	**5000**
Mean final body weight (g)		318 ± 14	315 ± 19	**291* ± 12**	**207** ± 15**	198 ± 8	202 ± 16	198 ± 12	**141** ± 8**
Adrenals	absolute (g)	0.076 ± 0.007	0.079 ± 0.006	0.078 ± 0.012	**0.062* ± 0.009**	0.082 ± 0.006	0.095 ± 0.016	0.076 ± 0.017	**0.060# ± 0.003**
	relative (%)	0.024 ± 0.003	0.025 ± 0.001	0.027 ± 0.003	**0.030* ± 0.004**	0.041 ± 0.004	0.047 ± 0.005	0.039 ± 0.001	0.043 ± 0.004
Brain	absolute (g)	1.983 ± 0.055	2.043 ± 0.085	1.992 ± 0.090	1.933 ± 0.033	1.901 ± 0.053	1.863 ± 0.094	1.862 ± 0.055	**1.758# ± 0.014**
	relative (%)	0.623 ± 0.016	0.650 ± 0.054	0.686 ± 0.044	**0.940** ± 0.082**	0.960 ± 0.032	0.925 ± 0.059	0.945 ± 0.059	**1.249** ± 0.064**
Epididymides	absolute (g)	0.971 ± 0.088	0.994 ± 0.091	0.940 ± 0.077	**0.838* ± 0.054**	-	-	-	-
	relative (%)	0.305 ± 0.023	0.317 ± 0.044	0.323 ± 0.022	**0.405# ± 0.008**	-	-	-	-

		Dose level (ppm)							
		Males				Females			
Parameter		**0**	**300**	**1000**	**5000**	**0**	**300**	**1000**	**5000**
Heart	absolute (g)	0.934 ± 0.026	0.914 ± 0.089	0.912 ± 0.101	**0.680** ± 0.083**	0.641 ± 00.86	0.668 ± 0.079	0.648 ± 0.045	**0.497* ± 0.053**
	relative (%)	0.293 ± 0.006	0.290 ± 0.014	0.314 ± 0.032	**0.328# ± 0.023**	0.323 ± 0.039	0.330 ± 0.020	0.328 ± 0.004	0.352 ± 0.027
Kidneys	absolute (g)	2.288 ± 0.202	2.111 ± 0.068	1.973 ± 0.192	**4.300# ± 0.659**	1.464 ± 0.154	1.478 ± 0.215	1.412 ± 0.046	**2.609# ± 0.468**
	relative (%)	0.718 ± 0.044	0.671 ± 0.039	0.678 ± 0.054	**2.069# ± 0.180**	0.737 ± 0.049	0.729 ± 0.048	0.716 ± 0.040	**1.854# ± 0.330**
Liver	absolute (g)	9.41 ± 0.44	9.02 ± 0.63	8.84 ± 0.63	**7.72** ± 0.82**	5.62 ± 0.40	5.68 ± 0.68	5.85 ± 0.57	5.31 ± 0.27
	relative (%)	2.96 ± 0.10	2.86 ± 0.05	3.03 ± 0.20	**3.73** ± 0.20**	2.83 ± 0.11	2.81 ± 0.13	2.96 ± 0.22	**3.77** ± 0.25**
Ovaries	absolute (g)	-	-	-	-	0.103 ± 0.017	0.092 ± 0.017	0.093 ± 0.010	**0.053** ± 0.007**
	relative (%)	-	-	-	-	0.052 ± 0.007	0.045 ± 0.006	0.048 ± 0.007	**0.038* ± 0.007**
Pituitary	absolute (g)	0.011 ± 0.001	0.011 ± 0.001	0.010 ± 0.001	**0.007# ± 0.000**	0.014 ± 0.002	0.014 ± 0.002	0.013 ± 0.001	**0.007** ± 0.001**
	relative (%)	0.0035 ± 0.0003	0.0033 ± 0.0002	0.0034 ± 0.0004	0.0035 ± 0.0003	0.0070 ± 0.0007	0.0068 ± 0.0005	0.0067 ± 0.0008	**0.0053** ± 0.0006**
Prostate	absolute (g)	0.347 ± 0.059	0.363 ± 0.036	0.312 ± 0.081	**0.224* ± 0.084**	-	-	-	-
Seminal vesicles	absolute (g)	1.040 ± 0.207	0.897 ± 0.176	0.857 ± 0.218	**0.426** ± 0.132**	-	-	-	-
	relative (%)	0.327 ± 0.063	0.287 ± 0.066	0.294 ± 0.071	**0.206* ± 0.060**	-	-	-	-
Spleen	absolute (g)	0.702 ± 0.055	0.632 ± 0.051	0.598 ± 0.100	0.637 ± 0.093	0.535 ± 0.068	0.460 ± 0.043	**0.435* ± 0.043**	**0.431** ± 0.027**
	relative (%)	0.221 ± 0.021	0.201 ± 0.018	0.206 ± 0.039	**0.308** ± 0.033**	0.271 ± 0.039	0.228 ± 0.012	**0.221* ± 0.014**	0.307 ± 0.033
Testes	relative (%)	1.052 ± 0.112	1.061 ± 0.056	1.162 ± 0.055	**1.535** ± 0.080**	-	-	-	-
Thymus	absolute (g)	0.585 ± 0.037	0.583 ± 0.098	0.557 ± 0.072	**0.282# ± 0.018**	0.503 ± 0.048	0.453 ± 0.102	0.472 ± 0.042	**0.253** ± 0.061**
	relative (%)	0.184 ± 0.013	0.184 ± 0.022	0.192 ± 0.030	**0.136# ± 0.005**	0.254 ± 0.029	0.223 ± 0.039	0.238 ± 0.014	0.179 ± 0.038
Thyroids	relative (%)	0.0077 ± 0.0010	0.0075 ± 0.0008	0.0080 ± 0.0013	**0.0105** ± 0.0010**	0.0097 ± 0.0008	0.0103 ± 0.0011	0.0107 ± 0.0014	0.0110 ± 0.0021
Uterus	absolute (g)	-	-	-	-	0.377 ± 0.092	0.370 ± 0.025	0.463 ± 0.171	**0.122# ± 0.014**
	relative (%)	-	-	-	-	0.191 ± 0.048	0.184 ± 0.024	0.238 ± 0.101	**0.087# ± 0.009**

Significantly different from the control using Dunnett's test: * $p < 0.05$; ** $p < 0.01$; Source: Nagai, 2018

Significantly different from the control using Steel's test: # $p < 0.05$;

Treatment-related histopathological findings were observed in the kidneys, urinary bladder, liver, adrenals and bone marrow. In the kidney, various findings were noted in both sexes at the top dose. These included obstructive nephropathy characterized by unusual crystals (round, brown, laminal structure, and/or occasionally coated with eosinophilic material) in the pelvis and lumen of the renal tubules/collecting ducts, and basophilic changes of the renal tubules accompanied by luminal dilatation, degeneration and/or necrosis with regenerative changes of the renal tubules/collecting ducts, and

interstitial inflammation. In the urinary bladder the same unusual crystals were noted in top-dose males and females. Two mid-dose males also showed hyperplasia of the urothelium, however given the lack of a dose–response relationship this finding was not considered treatment-related. In the liver there was centrilobular hepatocellular hypertrophy in males at the mid-dose and above and in top-dose females. In the absence of organ weight changes and/or associated findings in clinical chemistry parameters in mid-dose males, the hypertrophy in this group was considered non-adverse. In the adrenals, hypertrophy of the zona glomerulosa was seen at the top dose in both sexes. In the bone marrow (of sternum and femur), there was atrophy in one male and four females at the top dose.

Table 28. Selected histopathological findings from 28-day rat study; (number of affected animals [number examined] is shown)

		Dose level (ppm)							
		Males				Females			
Parameter		**0**	**300**	**1000**	**5000**	**0**	**300**	**1000**	**5000**
Adrenals	Hypertrophy, zona glomerulosa	0 [5]	0 [0]	0 [5]	**5** [5]**	0 [5]	0 [0]	0 [5]	**5** [5]**
Bone marrow (sternum and femur),	atrophy	0 [5]	0 [0]	0 [0]	1 [5]	0 [5]	0 [0]	0 [0]	**4* [5]**
Kidneys	Crystal(s), luminal, renal tubule	0 [5]	0 [5]	0 [5]	**5** [5]**	0 [5]	0 [5]	0 [5]	**5** [5]**
	Dilatation, luminal, renal tubule	0 [5]	0 [5]	0 [5]	**5** [5]**	0 [5]	0 [5]	0 [5]	**5** [5]**
	Degeneration/nuclear pycnosis/necrosis, renal tubule	0 [5]	0 [5]	0 [5]	**5** [5]**	0 [5]	0 [5]	0 [5]	**4* [5]**
	Basophilic change, renal tubule	0 [5]	0 [5]	0 [5]	**5** [5]**	0 [5]	0 [5]	0 [5]	**5** [5]**
	Cellular cast, renal tubule	0 [5]	0 [5]	0 [5]	**5** [5]**	0 [5]	0 [5]	0 [5]	**5** [5]**
	Crystal(s), luminal, collecting duct	0 [5]	0 [5]	0 [5]	**5** [5]**	0 [5]	0 [5]	0 [5]	**5** [5]**
	Crystal(s), pelvis	0 [5]	0 [5]	0 [5]	3 [5]	0 [5]	0 [5]	0 [5]	3 [5]
	Crystal(s), urothelium	0 [5]	0 [5]	1 [5]	0 [5]	0 [5]	0 [5]	0 [5]	0 [5]
	Dilatation, luminal, collecting duct	0 [5]	0 [5]	0 [5]	**5** [5]**	0 [5]	0 [5]	0 [5]	**5** [5]**
	Hyperplasia, collecting duct	0 [5]	0 [5]	0 [5]	**5** [5]**	0 [5]	0 [5]	0 [5]	**4* [5]**
	Cellular cast, collecting duct	0 [5]	0 [5]	0 [5]	3 [5]	0 [5]	0 [5]	0 [5]	**4* [5]**
	Mononuclear cell infiltration, interstitial	0 [5]	0 [5]	0 [5]	**5** [5]**	0 [5]	0 [5]	0 [5]	**4* [5]**
	Fibrosis, interstitial	0 [5]	0 [5]	0 [5]	**5** [5]**	0 [5]	0 [5]	0 [5]	**5** [5]**
	Hyperplasia, urothelium	0 [5]	0 [5]	0 [5]	0 [5]	0 [5]	0 [5]	0 [5]	**4* [5]**
Liver	Hypertrophy, hepatocyte, centrilobular	0 [5]	0 [5]	2 [5]	**5** [5]**	0 [5]	0 [5]	0 [5]	**5** [5]**
Urinary bladder	Crystal(s)	0 [5]	0 [5]	0 [5]	**4* [5]**	0 [5]	0 [5]	0 [5]	1 [5]
	Urothelium hyperplasia	0 [5]	0 [5]	2 [5]	0 [5]	0 [5]	0 [5]	0 [5]	0 [5]

Significantly different from the control using Fisher's Exact test: * $p < 0.05$; ** $p < 0.01$; Source: Nagai, 2018

In conclusion, dietary administration of benzpyrimoxan to rats for 28 days was adverse at the top dose of 5000 ppm in both sexes, causing reductions in body weight, body weight gain, food consumption and food efficiency, haematological effects indicative of anaemia, clinical chemistry and causing urinalysis changes indicative of liver and kidney, changes in kidney, liver, adrenal and spleen weight and histopathological changes to kidney, liver, urinary bladder, adrenals and bone marrow. At the mid dose of 1000 ppm, there were still adverse effects on body weight and body weight gain in males, decreased Hb and increased total cholesterol in females, and the presence of crystals in the urinary sediment of both sexes. There were no adverse effects at 300 ppm. The kidney and urinary bladder effects were considered not relevant to humans (see section 2.6 Special studies). Overall, a NOAEL of 300 ppm (equal to 25.5 mg/kg bw per day) was identified from this study based on effects on body weight and body weight gain and haematological and clinical chemistry findings at the LOAEL of 1000 ppm (equal to 86.7 mg/kg bw per day) (Nagai, 2018).

In a 90-day study (OECD 408), benzpyrimoxan (purity 94%) was administered in the diet for 90 days (13 weeks) to groups of 10 male and 10 female Wistar Hannover rats, five weeks old, and weighing 156–177 g (males) or 117–137 g (females). Dietary concentrations were 0, 100, 300, 1000 or 3000 ppm (equivalent to 0, 6.26, 18.7, 64.2 and 194 mg/kg bw per day for males, 0, 7.41, 22.2, 78.1 and 227 mg/kg bw per day for females) . All animals were observed daily for mortality and general clinical signs of toxicity, and their body weights and food consumption recorded weekly during the treatment period. Detailed clinical observations were performed once prior to the initiation of treatment and once a week during the treatment period. Functional observation was carried out at 11 weeks of treatment. Ophthalmological examination was conducted prior to the initiation of treatment on all animals and at 13 weeks of treatment on all animals in the control and 3000 ppm groups. Urinalysis was conducted at 13 weeks of treatment. At the end of treatment all surviving animals were subjected to examination of haematology and blood biochemistry, necropsy and organ weight analysis were conducted. Histopathological examination was performed on the prescribed organs of all animals from the 0 and 3000 ppm groups and also on the liver, kidney and urinary bladder of all animals in the 100, 300 and 1000 ppm groups.

There were no significant changes in clinical or functional observation in any dose group. At the top dose, body weights were significantly lower than in controls from week 1 in males (by 6–19% at week 13) and from week 3 in females (by 8–11% at week 13). Similarly, body weight gains were reduced over the course of the study in top dose males (by 29%) and females (by 23%). Food consumption and food efficiency were also decreased at the top dose in both sexes, although more markedly in males.

At the haematology examination various changes, including changes indicative of anaemia, were noted at the top dose. There were in both sexes statistically significant increases in platelet count and decreases in Ht, Hb and prothrombin time. In addition, differential neutrophil counts were increased in males and mean corpuscular volume and mean corpuscular Hb were decreased in females. Reticulocyte counts were unchanged. Clinical chemistry investigations revealed significant changes in parameters indicative of liver and kidney toxicity, mainly at the top dose. Statistically significant increases in plasma γ-glutamyl transpeptidase (GGTP), total cholesterol and chloride were noted in both sexes. Males also showed statistically significant increases in ALP, AST, creatinine, urea nitrogen and calcium, and a statistically significant decrease in glucose. At 1000 ppm, statistically significant increases in AST in males and GGTP and total cholesterol in females were noted. At urinalysis, a statistically significant increase in urine volume and the colourless appearance of urine was noted in top-dose males and females. In addition crystals were present in the urinary sediment of all top-dose males and females. The urine of top-dose males also showed a statistically significant decrease in specific gravity, ketone levels and pH. Urinary sediment crystals were also present in four females at 1000 ppm.

At necropsy, changes were noted at the top dose. Males and females showed a statistically significant increase in the incidence of spots on the surface of the kidney. Males showed statistically significant increases in the incidences of dark-coloured liver and coarse surface to the kidney. In females, a statistically significant increase in the incidence of depressed areas of the kidney was also noted. Numerous organs (brain, heart, epididymides, testes, adrenals and thymus) showed significant weight changes at the top dose. However, in the absence of associated histopathology or clinical pathology, these were most likely secondary to the decrements in body weights observed at this dose. Organ weight

changes considered toxicologically significant were noted in both sexes at the top dose in the kidney (relative weight increased by 18–53%), liver (relative weight increased by 16–26%) and spleen (relative weight increased by 21–33%). Relative liver weights were also significantly increased at 1000 ppm in males (by 9%) and females (by 12%). Detail of selected organ weights is shown in Table 29.

Table 29. Selected organ weights in 90-day rat study (mean ± standard deviation shown)

Parameter		Dose level (ppm)									
		Males					Females				
		0	**100**	**300**	**1000**	**3000**	**0**	**100**	**300**	**1000**	**3000**
Mean final body weight (g)		428 ± 26	418 ± 32	414 ± 24	420 ± 33	347** ± 34	234 ± 23	233 ± 10	239 ± 17	231 ± 15	208** ± 18
Brain (mg)	A	2004 ± 70	2007 ± 51	1997 ± 58	2027 ± 92	1973 ± 54	1844 ± 46	1834 ± 57	1823 ± 62	1817 ± 68	1806 ± 58
	R	0.47 ± 0.04	0.48 ± 0.03	0.49 ± 0.03	0.48 ± 0.04	0.57** ± 0.05	0.79 ± 0.07	0.79 ± 0.04	0.77 ± 0.06	0.79 ± 0.05	0.87* ± 0.06
Heart (mg)	A	1061 ± 63	1008 ± 86	1018 ± 72	1065 ± 83	955* ± 88	684 ± 61	683 ± 23	702 ± 57	715 ± 58	678 ± 70
	R	0.25 ± 0.01	0.24 ± 0.02	0.25 ± 0.02	0.26 ± 0.01	0.28** ± 0.02	0.29 ± 0.02	0.29 ± 0.01	0.29 ± 0.01	0.31 ± 0.02	0.33* ± 0.03
Thymus (mg)	A	419 ± 71	412 ± 89	392 ± 63	404 ± 74	311** ± 74	346 ± 64	339 ± 57	384 ± 51	296 ± 48	306 ± 47
	R	0.099 ± 0.022	0.099 ± 0.021	0.095 ± 0.012	0.097 ± 0.020	0.090 ± 0.021	0.149 ± 0.028	0.146 ± 0.025	0.162 ± 0.028	0.129 ± 0.026	0.148 ± 0.024
Liver (g)	A	10.14 ± 0.90	10.01 ± 1.01	10.07 ± 0.81	10.82 ± 1.05	9.46 ± 0.62	5.66 ± 0.39	5.65 ± 0.32	5.96 ± 0.64	6.28* ± 0.62	6.38* ± 0.67
	R	2.37 ± 0.10	2.39 ± 0.14	2.43 ± 0.10	2.58** ± 0.18	2.74** ± 0.16	2.43 ± 0.13	2.43 ± 0.14	2.49 ± 0.15	2.72** ± 0.22	3.07** ± 0.18
Kidneys (mg)	A	2428 ± 148	2387 ± 238	2389 ± 118	2436 ± 179	2978 ± 538	1520 ± 159	1519 ± 59	1525 ± 153	1536 ± 105	1595 ± 155
	R	0.57 ± 0.02	0.57 ± 0.05	0.58 ± 0.03	0.58 ± 0.04	0.87** ± 0.19	0.65 ± 0.04	0.65 ± 0.03	0.64 ± 0.04	0.66 ± 0.04	0.77** ± 0.07
Spleen (mg)	A	654 ± 67	609 ± 81	607 ± 61	676 ± 91	699 ± 70	451 ± 54	476 ± 86	481 ± 86	469 ± 46	475 ± 53
	R	0.15 ± 0.02	0.15 ± 0.02	0.15 ± 0.01	0.16 ± 0.02	0.20** ± 0.02	0.19 ± 0.02	0.20 ± 0.03	0.20 ± 0.03	0.20 ± 0.02	0.23** ± 0.03
Adrenals (mg)	A	72.4 ± 9.7	67.3 ± 8.7	66.8 ± 6.3	71.6 ± 6.3	75.9 ± 8.9	74.1 ± 8.6	73.8 ± 9.9	74.5 ± 9.5	75.3 ± 11.4	67.7 ± 11.3
	R	0.017 ± 0.003	0.016 ± 0.003	0.016 ± 0.002	0.017 ± 0.002	0.022** ± 0.004	0.032 ± 0.003	0.032 ± 0.004	0.031 ± 0.004	0.033 ± 0.004	0.033 ± 0.005
Testes (mg)	A	3668 ± 294	3514 ± 191	3393* ± 289	3512 ± 168	3484 ± 226	-	-	-	-	-
	R	0.86 ± 0.11	0.84 ± 0.07	0.82 ± 0.07	0.84 ± 0.07	1.01** ± 0.12	-	-	-	-	-
Epididymides (mg)	A	1285 ± 94	1244 ± 56	1251 ± 65	1226 ± 106	1191* ± 68	-	-	-	-	-
	R	0.30 ± 0.03	0.30 ± 0.03	0.30 ± 0.02	0.29 ± 0.02	0.34** ± 0.04	-	-	-	-	-

A: Absolute weight; R: Relative weight (organ weight/body weight); Source: Ohtsuka, 2018a

* Significantly different from the control using Dunnett's (or Dunnett-type) test: $p < 0.05$;

** Significantly different from the control using Dunnett's (or Dunnett-type) test: $p < 0.01$

Treatment-related histopathological findings were seen in the kidneys, urinary bladder and liver. In the kidney, obstructive nephropathy was reported for all males and eight of 10 females at the top dose. This condition was characterized by lesions in the cortex, which extended into the medulla and up to the tip of the papilla. The lesions were associated with the presence of crystals in the lumen of the renal tubules and/or collecting ducts. From these findings, it was inferred that the renal lesions were produced due to obstruction of the renal tubules/ducts by the crystals, most likely derived from the test substance or its metabolites. Crystals were also present in the pelvis in both sexes from 1000 ppm. In the liver, centrilobular hepatocyte hypertrophy was noted at the top dose. In the urinary bladder, urothelial hyperplasia was observed in one male and two females, at the top dose. Two top-dose females showed signs of cystitis, and luminal crystals were observed in two top-dose males. Noteworthy histopathological findings are detailed in Table 30.

Table 30. Selected histopathological findings from 90-day rat study (Number affected in each dose group of 10 rats)

Parameter	Dose level (ppm)									
	Males					Females				
	0	**100**	**300**	**1000**	**3000**	**0**	**100**	**300**	**1000**	**3000**
Liver										
Fatty change, hepatocyte, centrilobular	0	0	0	1	0	0	0	0	0	0
Hypertrophy, hepatocyte, centrilobular	0	0	0	0	**6****	0	0	0	0	**5***
Kidneys										
Basophilic change, renal tubule	1	1	2	4	0	0	0	0	0	**0**
Calcification, fornix	1	0	0	0	0	2	0	0	1	1
Crystal(s), luminal, collecting duct	0	0	0	0	0	0	0	0	1	1
Crystal(s), pelvis	0	0	0	4*	**8****	0	0	0	4*	**7****
Dilatation, pelvis	2	0	0	0	0	2	0	0	0	0
Nephropathy, obstructive	0	0	0	0	**10****	0	0	0	0	**8****
Pyelitis/pyelonephritis	0	0	0	0	0	0	0	0	0	2
Hyperplasia, urothelium	1	0	0	1	3	1	0	0	0	4
Nephroblastematosis	0	0	0	0	0	0	0	1	0	0
Urinary bladder										
Crystal(s), luminal	0	0	0	0	2	0	0	0	0	0
Cystitis	0	0	0	0	0	0	0	0	0	2
Hyperplasia, urothelium	0	0	0	0	1	0	0	0	0	2

* Significantly different from the control using Fisher's exact probability test: $p < 0.05$; Source: Ohtsuka, 2018a

** Significantly different from the control using Fisher's exact probability test: $p < 0.01$

In conclusion, dietary administration of benzpyrimoxan to rats for 90 days was adverse at the top dose of 3000 ppm, causing reductions in body weight, body weight gain, food consumption and food efficiency in both sexes, haematological effects indicative of anaemia, clinical chemistry and urinalysis changes indicative of liver and kidney toxicity in both sexes, changes in kidney, liver, and spleen weight in both sexes, and histopathological changes in kidney, liver and urinary bladder in both sexes. At 1000 ppm, there were adverse effects on some clinical chemistry parameters (increased plasma GGTP and total cholesterol in females and increased AST in males) and urinalysis parameters (presence of crystals in the urinary sediment of females), increased liver weight in both sexes and kidney histopathology findings (presence of crystals in the pelvis) in females. There were no adverse effects at 300 or 100 ppm. The kidney and urinary bladder effects are considered not relevant to humans (see section 2.6 Special studies). Overall, a NOAEL of 300 ppm (equal to 18.7 mg/kg bw per day) was identified from this study based on clinical-chemistry findings and increased liver weight at the LOAEL of 1000 ppm (64.2/78.1 mg/kg bw per day in males/females) (Ohtsuka, 2018a).

Dog

Preliminary studies
In a preliminary 14-day study in dogs, benzpyrimoxan (purity 99.1%) was administered daily in the diet for seven or 14 days to 12-month-old beagle dogs (one male weighing 12.5 kg and one female weighing 10.1 kg) at dose levels of 0, 1000, 3000, 10 000 and 30 000 ppm (equivalent to 0, 24, 71, 236 and 295 mg/kg bw per day for males, 0, 30, 88, 210 and 335 mg/kg bw per day for females) . Animals were checked daily for general condition and mortality, and individual food consumption measured. Body weights were recorded on days 1, 3, 7, 10 and/or 14. Urinalysis was performed on all animals prior to initiation of treatment, in week 1 (3000, 10 000 and 30 000 ppm) and week 2 (1000 ppm). Before initiation of treatment and after the end of administration, all top dose animals were subjected to haematology, blood chemistry, necropsy and organ weight measurements.

During the administration period no mortalities occurred and there were no effects on urinalysis or haematology parameters. At 10 000 ppm, vomiting was noted in the male, with slight body weight loss and decreased food consumption in the female. At 30 000 ppm, gradual body weight loss and decreased food consumption were noted in males and females. In addition, the male showed high ALP values and both sexes produced high values for total cholesterol and phospholipid. At necropsy, liver enlargement and increased absolute and relative liver weights were noted at the top dose in both sexes. Based on these results, the study director concluded that the top dose for the subsequent 28-day study should be set below 30 000 ppm (Ishii, 2014a).

In a 28-day study (OECD 409), benzpyrimoxan (purity 94.1%) was administered to six-month-old beagle dogs (two males weighing 6.8–8.0 kg and two females weighing 7.3–7.9 kg per group) in the diet at concentrations of 0, 1000, 5000 and 20 000 ppm (equivalent to 0, 35, 180 and 297 mg/kg bw per day for males, 0, 36, 152 and 465 mg/kg bw per day for females. The required standard examinations were performed.

No mortalities occurred. Clinical signs of toxicity (vomiting, no defaecation, emaciation) were observed at the top dose during the first three days of treatment. Body weights were reduced by 22–24% and food consumption was decreased at the top dose. Haematology showed that reticulocyte percentage and reticulocyte counts were reduced (by 31–65%) in both sexes at the top dose. Clinical chemistry investigations revealed increased levels of AST, ALT, ALP, total cholesterol, triglycerides and phospholipids at the top dose. Increased levels of total cholesterol, triglycerides and phospholipids were also noted at 5000 ppm. Liver weight was increased at 5000 (relative weight by 31–45%) and 20 000 ppm (relative weight by 37–70%). Thymus weight was reduced (relative weight by 62–63%) at the top dose. In the liver, single cell necrosis of hepatocytes (minimal) was observed in one female at 20 000 ppm, and hypertrophy of centrilobular hepatocytes (minimal or mild) in both males at 5000 ppm and in both males and one female at 20 000 ppm. In addition, hypocellularity of the bone marrow (femur and sternum) and atrophy of the thymus were noted at the top dose.

In conclusion, dietary administration of benzpyrimoxan to dogs for 28 days caused significant toxicity at the top dose of 20 000 ppm (297 mg/kg bw per day), including clinical signs of toxicity, reductions in body weight and food consumption, decreases in reticulocytes, clinical chemistry changes indicative of liver toxicity, increased liver weight with associated histopathology, decreased thymus weight with associated atrophy and hypocellularity of the bone marrow. At 5000 ppm, there were adverse effects mainly in the liver, including increased weight, hypertrophy of centrilobular hepatocytes and changes in some clinical chemistry parameters. There were no adverse effects at 1000 ppm. Based on these findings, a NOAEL of 1000 ppm (equal to 35 mg/kg bw per day) was identified from this study based on liver toxicity at the LOAEL of 5000 ppm (equal to 152 mg/kg bw per day) (Ishii, 2014b).

Study 1
In a 90-day study (OECD 409), benzpyrimoxan (purity 93.7%) was administered in the diet to six-month-old beagle dogs (four males weighing 6.3–9.2 kg and four females weighing 6.3–8.3 kg per group) at concentrations of 0, 500, 2500 or 10 000 ppm, (equivalent to 0, 17, 79 and 302 mg/kg bw per day for males, 0, 16, 81 and 246 mg/kg bw per day for females). The required standard examinations were performed.

No mortalities occurred during the study. Emaciation was noted in one top-dose female and vomiting was observed in both sexes at the mid dose and above during the first three days of treatment. Terminal body weights were substantially reduced at the top dose in males (by 18%; not statistically significant) and in females (by 30%; statistically significant). In addition, top-dose animals lost weight over the duration of the study rather than gaining weight as seen with controls. Food consumption was decreased (by up to 24–41%; statistically significant) over the duration of the study at the top dose.

From the haematology examination, reduced levels of erythrocyte count, Hb, Ht, reticulocyte percentage and reticulocyte count were noted in top-dose females, with increased levels of leukocyte and neutrophil counts in top-dose males. In addition, platelet count was increased at the top dose in both sexes. Some parameters were affected also at the mid dose, however in the absence of a dose–response relationship and with values being similar to those for samples taken pretreatment, the changes were considered to be incidental. At the clinical chemistry examination, changes indicative of liver toxicity (increased levels of ALP, total cholesterol, triglyceride and phospholipid) were noted in both sexes at the mid dose and above. In addition, increased levels of ALT, AST and GGTP and lower levels of glucose and creatinine were seen at the top dose.

Table 31. Selected clinical chemistry parameters in 90-day dog study (mean ± standard deviation)

		Dose level (ppm)							
		Males				Females			
Parameter	**Week**	**0**	**500**	**2500**	**10 000**	**0**	**500**	**2500**	**10 000**
Glucose [mg/dL]	−1	109 ± 6	107 ± 4	104 ± 8	104 ± 4	105 ± 6	98 ± 5	109 ± 7	101 ± 6
	5	104 ± 2	104 ± 7	102 ± 8	96 ± 2	102 ± 8	99 ± 4	98 ± 4	**88* ± 7**
	8	104 ± 5	106 ± 4	100 ± 5	**95* ± 3**	99 ± 2	102 ± 4	96 ± 3	**87* ± 8**
	13	103 ± 4	104 ± 2	100 ± 6	**90** ± 7**	98 ± 6	98 ± 4	95 ± 3	**86* ± 6**
Sodium [mmol/L]	−1	149 ± 1	149 ± 1	150 ± 1	149 ± 0	149 ± 2	149 ± 1	150 ± 1	150 ± 1
	5	148 ± 1	**150* ± 1**	**150* ± 1**	**150* ± 1**	150 ± 1	150 ± 1	150 ± 1	150 ± 1
	8	149 ± 1	149 ± 2	150 ± 1	149 ± 1	150 ± 1	150 ± 1	150 ± 1	148 ± 1
	13	150 ± 1	149 ± 1	149 ± 1	**148* ± 1**	149 ± 2	149 ± 1	150 ± 1	149 ± 1
Potassium [mmol/L]	−1	4.4 ± 0.1	4.5 ± 0.2	4.1 ± 0.4	4.2 ± 0.1	4.3 ± 0.4	4.3 ± 0.1	4.1 ± 0.3	4.1 ± 0.1
	5	4.5 ± 0.1	4.5 ± 0.3	**4.1* ± 0.2**	4.3 ± 0.2	4.3 ± 0.3	4.3 ± 0.1	4.1 ± 0.1	4.2 ± 0.2
	8	4.3 ± 0.2	4.2 ± 0.2	4.1 ± 0.2	4.1 ± 0.1	4.1 ± 0.3	4.0 ± 0.2	3.9 ± 0.1	4.1 ± 0.2
	13	4.3 ± 0.2	4.3 ± 0.3	3.9 ± 0.2	4.0 ± 0.2	4.0 ± 0.3	4.1 ± 0.2	3.8 ± 0.1	3.9 ± 0.3
Calcium [mg/dL]	−1	11.1 ± 0.1	11.0 ± 0.2	11.2 ± 0.3	11.0 ± 0.2	10.8 ± 0.3	10.8 ± 0.1	10.9 ± 0.2	10.9 ± 0.1
	5	11.1 ± 0.1	10.9 ± 0.1	11.3 ± 0.3	11.1 ± 0.3	10.6 ± 0.4	10.8 ± 0.2	10.8 ± 0.4	11.0 ± 0.3
	8	10.6 ± 0.2	10.4 ± 0.1	**10.9* ± 0.1**	10.7 ± 0.2	10.3 ± 0.3	10.5 ± 0.2	10.5 ± 0.3	10.5 ± 0.3
	13	10.8 ± 0.1	10.6 ± 0.2	11.0 ± 0.2	10.5 ± 0.3	10.4 ± 0.3	10.6 ± 0.1	10.5 ± 0.5	10.6 ± 0.3
Albumin [g/dL]	–1	3.2 ± 0.1	3.1 ± 0.4	3.3 ± 0.3	3.1 ± 0.1	3.1 ± 0.1	3.1 ± 0.2	3.1 ± 0.1	3.3 ± 0.2
	5	3.2 ± 0.1	3.1 ± 0.3	3.4 ± 0.2	3.2 ± 0.1	3.1 ± 0.1	3.3 ± 0.2	3.2 ± 0.2	**3.5* ± 0.3**
	8	3.1 ± 0.0	3.0 ± 0.3	**3.3* ± 0.2**	3.2 ± 0.2	3.1 ± 0.1	3.2 ± 0.1	3.2 ± 0.1	3.3 ± 0.3
	13	3.2 ± 0.1	3.0 ± 0.2	3.4 ± 0.2	3.0 ± 0.2	3.1 ± 0.1	3.3 ± 0.1	3.2 ± 0.1	3.2 ± 0.3
Aspartate trans-aminase [IU/L]	−1	26 ± 3	25 ± 2	26 ± 2	27 ± 5	28 ± 2	31 ± 4	28 ± 2	33 ± 5
	5	27 ± 3	27 ± 2	25 ± 4	23 ± 2	30 ± 3	31 ± 6	27 ± 3	**20* ± 2**
	8	29 ± 5	28 ± 3	24 ± 5	23 ± 2	31 ± 3	34 ± 8	27 ± 3	24 ± 3
	13	30 ± 2	27 ± 2	25 ± 4	25 ± 6	32 ± 5	30 ± 7	27 ± 4	33 ± 17

		Dose level (ppm)							
		Males				Females			
Parameter	**Week**	**0**	**500**	**2500**	**10 000**	**0**	**500**	**2500**	**10 000**
GGTP [IU/L]	-1	4 ± 0	4 ± 1	4 ± 1	4 ± 1	3 ± 1	3 ± 1	3 ± 1	4 ± 1
	5	4 ± 1	3 ± 0	3 ± 1	4 ± 1	3 ± 1	3 ± 0	3 ± 1	3 ± 1
	8	3 ± 1	3 ± 0	3 ± 1	3 ± 1	2 ± 0	3 ± 1	3 ± 1	3* ± 0
	13	3 ± 1	3 ± 1	3 ± 1	4 ± 1	3 ± 0	3 ± 1	3 ± 1	5* ± 2
Total cholesterol [mg/dL]	−1	131 ± 10	133 ± 30	127 ± 22	136 ± 26	121 ± 10	107 ± 16	122 ± 19	132 ± 16
	5	128 ± 6	141 ± 50	187 ± 53	159 ± 46	111 ± 13	119 ± 17	**166* ± 24**	**208** ± 48**
	8	126 ± 5	136 ± 55	190 ± 46	161 ± 43	105 ± 4	120 ± 19	168 ± 41	214 ± 59
	13	120 ± 6	137 ± 56	191 ± 46	149 ± 44	108 ± 8	130 ± 38	172 ± 57	**229* ± 72**
Platelets [10^3/µL]	-1	280 ± 22	283 ± 48	274 ± 41	282 ± 37	265 ± 8	248 ± 32	269 ± 37	297 ± 34
	5	272 ± 20	289 ± 75	375 ± 52	331 ± 56	244 ± 26	265 ± 34	**349** ± 36**	**400** ± 61**
	8	267 ± 11	279 ± 88	364 ± 44	334 ± 61	232 ± 17	264 ± 37	**343* ± 60**	**391** ± 81**
	13	262 ± 8	275 ± 88	369 ± 43	315 ± 68	242 ± 22	277 ± 58	340 ± 84	**403* ± 101**
Blood urea nitrogen [mg/dL]	−1	12 ± 3	11 ± 2	11 ± 1	12 ± 2	11 ± 1	12 ± 1	11 ± 2	12 ± 1
	5	12 ± 2	11 ± 2	12 ± 1	15 ± 4	12 ± 1	12 ± 1	13 ± 1	11 ± 2
	8	14 ± 2	11 ± 1	13 ± 1	14 ± 3	15 ± 3	14 ± 1	15 ± 2	**12* ± 1**
	13	13 ± 3	12 ± 1	12 ± 2	14 ± 4	14 ± 2	13 ± 2	14 ± 1	12 ± 2
CRNN [mg/dL]	−1	0.51 ± 0.03	0.51 ± 0.02	0.48 ± 0.04	0.50 ± 0.05	0.56 ± 0.05	0.56 ± 0.09	0.56 ± 0.04	0.52 ± 0.03
	5	0.52 ± 0.03	0.55 ± 0.04	0.50 ± 0.07	0.54 ± 0.07	0.58 ± 0.03	0.61 ± 0.09	0.58 ± 0.04	0.53 ± 0.04
	8	0.57 ± 0.07	0.61 ± 0.03	0.54 ± 0.06	0.56 ± 0.07	0.64 ± 0.05	0.65 ± 0.08	0.63 ± 0.06	**0.51* ± 0.04**
	13	0.56 ± 0.05	0.62 ± 0.05	0.54 ± 0.06	0.55 ± 0.05	0.65 ± 0.05	0.66 ± 0.08	0.63 ± 0.08	**0.50* ± 0.07**
Chloride [mmol/L]	−1	112 ± 2	111 ± 0	111 ± 2	111 ± 2	112 ± 1	112 ± 1	113 ± 1	112 ± 1
	5	111 ± 1	112 ± 1	110 ± 1	111 ± 2	114 ± 1	113 ± 2	113 ± 1	112 ± 1
	8	111 ± 1	111 ± 1	111 ± 2	111 ± 1	113 ± 1	113 ± 1	114 ± 2	111 ± 1
	13	113 ± 1	113 ± 1	111 ± 2	112 ± 1	115 ± 1	114 ± 1	115 ± 1	**113* ± 1**
Inorganic phosphorus [mg/dL]	−1	6.2 ± 0.5	6.4 ± 0.4	6.3 ± 0.3	6.5 ± 0.4	6.1 ± 0.9	6.2 ± 0.5	6.6 ± 0.8	6.6 ± 0.2
	5	5.5 ± 0.3	5.6 ± 0.4	5.4 ± 0.4	5.4 ± 0.6	5.5 ± 0.5	4.9 ± 0.3	5.2 ± 0.4	**4.8* ± 0.2**
	8	5.1 ± 0.4	5.0 ± 0.3	5.0 ± 0.2	5.4 ± 0.7	4.9 ± 0.6	4.4 ± 0.3	4.9 ± 0.4	4.7 ± 0.4
	13	4.8 ± 0.4	4.7 ± 0.3	4.5 ± 0.5	4.9 ± 0.7	4.4 ± 0.8	4.4 ± 0.3	4.3 ± 0.4	4.2 ± 0.2

CRNN: Not defined in study report

* Significantly different from the control using Dunnett's test, 2-sided test: $p \leq 0.05$; Source: Ohtsuka, 2018a

** Significantly different from the control using Dunnett's test, 2-sided test: $p \leq 0.01$

At necropsy, increased liver weights were noted at 2500 ppm and 10 000 ppm in both sexes (relative weight by 49% and 77% in males, and by 63% and 122% in females, respectively) and reduced thymus weight was also seen at the top dose. Other changes in organ weights (uterus, thyroid, heart, adrenal, brain and kidney) were mainly observed at the top dose and were considered of no toxicological significance as they were most likely a secondary consequence of the reduced body weights reported

at this dose or they were not associated with histopathology or clinical pathology findings. At histopathology, changes were found in the liver, thymus and bone marrow. In the liver, hypertrophy of centrilobular hepatocytes was noted in males from the mid dose and in females at the top dose, with increased pigmentation of hepatocytes observed in both sexes at the top dose. In the thymus, atrophy was noted in two top-dose females. In the bone marrow, hypocellularity was noted at the mid dose and above, and gelatinous change seen at the top dose.

In conclusion, dietary administration of benzpyrimoxan to dogs for 90 days caused significant toxicity at the top dose of 10 000 ppm (equal to 246 mg/kg bw per day), including clinical signs of toxicity, reductions in body weight and food consumption, haematological changes indicative of anaemia, clinical chemistry changes indicative of liver toxicity, increased liver weight with associated histopathology, decreased thymus weight with associated atrophy and hypocellularity of the bone marrow. At the mid dose of 2500 ppm, there were adverse effects mainly in the liver in both sexes, including increased weight, hypertrophy of centrilobular hepatocytes and changes in some clinical chemistry parameters (increased ALP, total cholesterol, triglycerides and phospholipids) and hypocellularity of femoral bone marrow in females. There were no adverse effects at 500 ppm. Based on these findings, a NOAEL of 500 ppm (equal to 16 mg/kg bw per day) was identified from this study based on liver toxicity and bone marrow histopathology at the LOAEL of 2500 ppm (equal to 79 mg/kg bw per day) (Ishii, 2016).

Study 2

In a one-year study (OECD 452), benzpyrimoxan (purity 93.7%) was administered in the diet to six-month-old beagle dogs (four males weighing 6.8–9.1 kg and four females weighing 6.8–8.3 kg per group) at concentrations of 0, 100, 500 or 2500 ppm (equivalent to 0, 2.9, 14.6 and 71 mg/kg bw per day for males, 0, 2.7, 14.3 and 67 mg/kg bw per day for females. During the treatment period dogs were examined for clinical signs of toxicity, body weight and food consumption recorded, and ophthalmological, haematological, clinical chemistry and urinalysis examinations performed. After treatment animals were necropsied; organ weights were determined and gross pathological as well as histopathological examinations performed. One male animal of the mid-dose group (500 ppm) was excluded from the terminal investigations as it was diagnosed with demodicosis in week 44.

No mortalities occurred and there were no treatment-related effects on clinical signs of toxicity, body weight, food consumption, ophthalmology or urinalysis. Haematology revealed an increase in platelet counts in both sexes at the top dose. Clinical chemistry investigations revealed changes indicative of liver toxicity (increased ALP, ALT, triglyceride, total cholesterol and phospholipids) at the top dose. Alkaline phosphatase in females and triglycerides in males were also increased to a statistically significant extent at the mid dose of 500 ppm. Clinical chemistry findings in the mid-dose groups were minor and highly variable, hence they were not considered to be adverse and/or substance-related. Selected results for the one-year dog study are shown in Table 32.

Table 32. Selected clinical chemistry parameters in the one-year dog study (mean ± standard deviation)

Parameter	Week	Dose level (ppm)							
		Males				Females			
		0	**100**	**500**	**2500**	**0**	**100**	**500**	**2500**
Alkaline phosphatase (IU/L)	13	230 ± 46	237 ± 104	233 ± 24	**383* ± 26**	180 ± 16	219 ± 41	358** ± 94	314* ± 23
	26	146 ± 13	140 ± 37	202 ± 14	**346** ± 61**	125 ± 11	154 ± 53	266* ± 119	243 ± 43
	52	139 ± 36	129 ± 37	207 ± 20	**323** ± 62**	124 ± 30	119 ± 49	265 ± 155	295 ± 93
Alanine transaminase (IU/L)	13	36 ± 5	36 ± 4	36 ± 12	35 ± 12	38 ± 4	35 ± 3	32 ± 9	34 ± 6
	26	41 ± 10	40 ± 9	37 ± 7	43 ± 19	35 ± 6	41 ± 4	74 ± 81	36 ± 4
	52	41 ± 10	39 ± 9	33 ± 7	43 ± 16	44 ± 21	39 ± 5	38 ± 14	42 ± 10

Parameter	Week	Dose level (ppm)							
		Males				Females			
		0	100	500	2500	0	100	500	2500
Total cholesterol (mg/dL)	13	118 ± 26	133 ± 8	157 ± 21	**189* ± 43**	136 ± 26	133 ± 24	159 ± 31	196 ± 43
	26	112 ± 26	131 ± 6	163 ± 22	**190* ± 52**	153 ± 31	128 ± 20	163 ± 27	169 ± 15
	52	118 ± 24	134 ± 20	170 ± 30	**191** ± 37**	171 ± 75	149 ± 13	179 ± 76	221 ± 91
Triglycerides (mg/dL)	13	20 ± 8	23 ± 5	25 ± 7	45 ± 29	26 ± 3	31 ± 16	30 ± 6	26 ± 10
	26	18 ± 6	20 ± 4	23 ± 3	**34** ± 5**	21 ± 6	22 ± 15	25 ± 5	22 ± 8
	52	13 ± 6	20 ± 3	27* ± 5	**35** ± 6**	28 ± 13	19 ± 9	23 ± 7	28 ± 10
Platelets [10^3/µL]	13	253 ± 36	277 ± 9	317 ± 29	**370** ± 50**	280 ± 52	275 ± 38	327 ± 53	**384* ± 61**
	26	245 ± 40	279 ± 14	322 ± 36	**375* ± 86**	308 ± 53	268 ± 43	324 ± 44	349 ± 29
	52	258 ± 46	292 ± 20	344 ± 49	**392** ± 68**	344 ± 114	321 ± 30	348 ± 92	430 ± 113

* Significantly different from the control using Dunnett's test (or Dunnett-type test): $p < 0.05$; Source: Ishii, 2018

** Significantly different from the control using Dunnett's test (or Dunnett-type test): $p < 0.01$

At necropsy, liver weights were increased in both sexes at mid (500 ppm) and high (2500 ppm), relative weights by 17% and 49% in males, 31% and 41% in females, respectively. Other organ weight changes (thyroid, pituitary, adrenal, kidney and testis) were regarded to be of no toxicological significance due to lack of dose–response relationship or lack of associated histopathology or clinical pathology findings. Dark discolouration of the liver was noted from at mid dose and above and liver enlargement at the top dose. Histopathology examination revealed changes in the liver. Minimal hypertrophy of the centrilobular hepatocytes was noted in two top-dose males and pigmentation of hepatocytes was observed in both sexes at the mid dose (3/3 males and 2/4 females at 500 ppm) and high dose (4/4 males and 3/4 females at 2500 ppm). The pigments involved produced positive reactions with the Schmorl method and periodic acid-Schiff (PAS) reaction, indicating the presence of lipofuscin. The incidence and severity of pigmentation increased with dose. In the absence of a consistent pattern of clinical chemistry changes at the mid dose, the hepatocyte pigmentation and increased liver weight observed at this dose, were considered not to be adverse as they did not result in functional clinical pathology findings. Selected histopathological findings in the liver are detailed in Table 33.

***Table 33. Histopathological liver findings in the one-year dog study (Number of animals affected* [*severity*])**

Parameter		Dose level (ppm)							
		Males				Females			
		0	100	500	2500	0	100	500	2500
Number of animals examined		4	4	3	4	4	4	4	4
Liver	Pigmentation, hepatocytic	-	-	3 2 [2] 1 [3]	4 1 [2] 3 [3]	-	-	2 1 [1] 1 [2]	3 1 [2] 2 [3]
	Hypertrophy, hepatocytic, centrilobular	-	-	-	2 [1]	-	-	-	-

[Severity of the lesion]: 1 minimal, 2 mild, 3 moderate Source: Ishii, 2018

* Significantly different from the control using Fisher's exact probability test: $p < 0.05$;

** Significantly different from the control using Fisher's exact probability test: $p < 0.01$

In conclusion, dietary administration of benzpyrimoxan to dogs for one year caused moderate toxicity at the top dose of 2500 ppm (equal to 67 mg/kg bw per day), characterized by increased platelet counts, clinical chemistry changes indicative of liver toxicity and increased liver weight with associated histopathology (hypertrophy and pigmentation). There were no adverse effects at the mid dose of 500 ppm. Based on these findings, a NOAEL of 500 ppm (equal to 14.3 mg/kg bw per day) was identified based on increased platelet count in both sexes and liver toxicity (relative weight increased by 41–49 in both sexes), hepatocyte brown pigmentation (lipofuscin) in both sexes, hypertrophy in males, and a statistically significant increase in levels of total cholesterol, triglycerides, ALP and phospholipids (in males) at the LOAEL of 2500 ppm (equal to 67 mg/kg bw per day) (Ishii, 2018).

(b) Dermal application

A GLP and guideline (OECD 410) dermal 28-day study in rats was carried out. Benzpyrimoxan (purity 93.7%) in vehicle consisting of 0.5% (w/v) sodium CMC solution containing 0.2% (w/v) Tween 80) was applied to the skin of the dorsal area of Sprague Dawley rats (10 animals/sex per group) for 28 days for 6–7 hours per day, five times or more per week) under semi-occlusive conditions at doses of 40, 200 and 1000 mg/kg bw. The required standard examinations were performed.

No effect on mortality or clinical signs of toxicity were observed. The only treatment-related effects were seen in top-dose males. In this group, terminal body weight was reduced by 8% and body weight gain decreased by 28%. Haematology revealed changes related to red blood cells, however these were minor (around 5% reduced from control values) and were not accompanied by any histopathological changes in the haematopoietic system including the bone marrow and spleen. On this basis they were not considered of toxicological significance. No other effects were noted.

In conclusion, dermal application of benzpyrimoxan to rats for 28 days had no effect on females up to the limit dose of 1000 mg/kg bw per day, but caused decreases in body weight in top-dose males. On the basis on these results, a NOAEL of 200 mg/kg bw per day was identified from this study (Serizawa, 2017).

(c) Exposure by inhalation

In a five-day inhalation range-finding study, groups of three male and three female Sprague Dawley rats were exposed nose-only to an aerosol of benzpyrimoxan (purity 93.7%) powder for six hours per day at concentrations of 0, 0.39, 1.0 or 3.9 mg/L. The MMADs were 3.17, 2.89 and 1.96 µm at the low, mid and top concentrations, respectively. Clinical signs of toxicity, body weight, body weight gain and food consumption were investigated during the study. Animals were killed on day 6 and gross necropsy, organ weights measurements and histopathological examinations were performed.

No mortality occurred during the course of the study. Partially and completely closed eyes and ungroomed fur were seen at all test concentrations. A body weight loss of 1% was noted in males on day 6 at the top concentration. Reduction in food consumption over the five-day dosing period was noted in males at the mid and high concentrations. At necropsy, mottled discolouration and/or enlargement of the kidneys were seen mainly in males at the top concentration. Increased kidney weights were noted in males at the mid concentration and above and in females at low, mid and high concentrations.

Histopathological findings were observed in the kidney, urinary bladder, nasal cavity, pharynx and lung. In the kidney, obstructive nephropathy was observed in males and females at the lowest concentration and above. Crystals were also noted in the lumen of the urinary bladder of one male at 3.9 mg/L and in the renal pelvis of one male at 1 mg/L. In the nasal cavity, mucous cell hypertrophy/hyperplasia was observed in males and females at the lowest concentration and above. The pharynx of some animals showed a minimal degree of mucous cell hypertrophy/hyperplasia at mid and high concentrations. In the lung, alveolar infiltration by mixed inflammatory cells was seen in males at the lowest concentration and above, and in two females at 1 mg/L.

Based on the findings, a concentration of 0.39 mg/L was considered by the sponsor as an appropriate top concentration for the subsequent 28-day study (Cow, 2018a).

In a 28-day inhalation study (OECD 412), groups of five male and five female Sprague Dawley rats were exposed nose-only to an aerosol of benzpyrimoxan (purity 93.7%) powder for six hours per day, five days per week at concentrations of 0, 0.039, 0.1 or 0.39 mg/L. The MMADs were 2.75–2.92, 2.65–2.90 and 2.67–2.94 µm at the low, mid and top concentrations, respectively. Clinical observations, body weight, body weight gain, food consumption, clinical pathology parameters (haematology, coagulation, clinical chemistry and urinalysis), gross necropsy findings, organ weights, and histopathological examinations were performed.

Body weight gain was noted as lower in top concentration males (46.4% lower compared to controls; statistically significant). This was associated with a reduction in food consumption (up to 14%). There were no effects of treatment on clinical pathology parameters or organ weights. Histopathological examination revealed findings in the nasal cavity, pharynx and kidney. In the nasal cavity and pharynx, minimal hypertrophy/hyperplasia of epithelial mucous cells was observed at all concentrations. These findings were regarded as adaptive as no degeneration occurred. In the kidney, obstructive nephropathy (various findings related to crystal deposition in the tubules) was noted at 0.390 mg/L in males.

In conclusion, administration of benzpyrimoxan to rats by inhalation for 28 days caused moderate adverse effects at the top concentration of 0.39 mg/L in males only, indicating a greater sensitivity of this sex compared to females. Effects consisted of decreased body weight gain, reduced food consumption and kidney obstructive nephropathy. The kidney effects were considered not relevant to humans (see section 2.6 Special studies). Overall, a no-observed-adverse-effect concentration (NOAEC) of 0.1 mg/L was identified from this study based on decreases in body weight gain and food consumption in males at 0.39mg/L. The NOAEC value of 0.1mg/L is equal to 29 mg/kg bw per day, assuming 100% inhalation absorption and a respiratory rate for the rat of 0.8 L/minute per kg bw, which is a NOAEL similar to the oral NOAEL from the 28-day rat study (Cow, 2018b).

Table 34 summarizes the available short-term studies.

Table 34. Summary of the short term studies available for benzpyrimoxan

Species/strain No. of animals Guidelines	Duration Route Purity	Dose levels (mg/kg bw/day) [ppm in diet]	NOAEL[a] (mg/kg bw/day)	LOAEL[a] (mg/kg bw/day)	Effects at LOAEL[a]	Reference
Mouse						
ICR [Crl:CD1] 5/sex/group OECD 407 GLP	4 weeks Dietary 94.0%	**M**: 65, 161, 632 and 1203 **F**: 73, 190, 759 and 1428 [400, 1000, 4000 and 8000 ppm]	**M**:161 **F**: 190 [1000 ppm]	**M**: 632 **F**: 759 [4000 ppm]	Decreases in body weight and body weight gain in males; Changes in clinical chemistry parameters in males; Increased liver weights in both sexes; Histopathology of the liver	Yoshimitsu 2017
Crl:CD1(ICR) 12/sex/group GLP Similar to OECD 408	13 weeks Dietary 93.7%	**M**: 56, 282 and 523 **F**: 66, 327 and 971 [400, 2000, 4000/6000 ppm]	**M**: 56 **F**: 66 [400 ppm]	**M**: 282 **F**: 327 [2000 ppm]	Liver and spleen weights and associated histopathology; haematology and clinical-chemistry changes	Coleman, 2017

Species/strain No. of animals Guidelines	Duration Route Purity	Dose levels (mg/kg bw/day) [ppm in diet]	NOAEL[a] (mg/kg bw/day)	LOAEL[a] (mg/kg bw/day)	Effects at LOAEL[a]	Reference
Rat						
Wistar Hannover (RccHan™: WIST) 5/sex/group GLP Similar to OECD 407	4 weeks Dietary 99.1%	**M**: 25.5, 86.7 and 317.4 **F**: 26.1, 89.1 and 324.5 [300, 1000, 5000 ppm]	**M**: 25.5 **F**: 26.1 [300 ppm]	**M**: 86.7 **F**: 89.1 [1000 ppm]	Decreased body weights; Body weight gains; Clinical pathology findings;	Nagai, 2018
Wistar Hannover rats 10/sex/group GLP OECD 408	13 weeks Dietary 94%	**M**: 6.26, 18.7, 64.2 and 194 **F**: 7.41, 22.2, 78.1 and 227 [100, 300, 1000, 3000 ppm]	**M**: 18.7 **F**: 22.2 [300 ppm]	**M**: 64.2 **F**: 78.1 [1000 ppm]	Increased liver weight; Clinical chemistry;	Ohtsuka, 2018a
Sprague Dawley Crl:CD(SD) 10/sex/group GLP OECD 407	4 weeks Percutaneous 93.7%	40, 200 and 1000	**M**: 200 **F**: 1000	**M**: 1000 **F**: > 1000	Decreased body weight (M)	Serizawa, 2017
Sprague Dawley Crl:CD(SD) 5/sex/group GLP OECD 412	4 weeks Inhalation (6 hours/day 5 days/week) 93.7%	0.039, 0.1 and 0.39 mg/L	**M**: 0.1 mg/L **F**: 0.39 mg/L	**M**: 0.39 mg/L **F**: > 0.39 mg/L	Reduced body weight gain (M); Reduced food consumption (M)	Cow, 2018b
Dog						
HRA beagle 1/sex/group Non GLP Non-guideline	14 days Dietary 99.1%	**M**: 23, 71, 236 and 295 **F**: 29, 88 210 and 335 [1000, 3000, 10 000, 30 000 ppm]	**M**: 71 **F**: 88 [3000 ppm]	**M**: 236 **F**: 210 [10 000 ppm]	Vomiting (M); Slight body weight loss and decreased food consumption (F)	Ishii, 2014a
Beagle 2/sex/group GLP OECD 409	4 weeks Dietary 94.0%	**M**: 35, 180, 297 **F**: 36, 152,465 [1000, 5000, 20 000 ppm]	**M**: 35 **F**: 36 [1000 ppm]	**M**: 180 **F**: 152 [5000 ppm]	Liver effects (increased weight, hypertrophy and clinical-chemistry)	Ishii, 2014b
HRA beagle 4/sex/group GLP OECD 409	13 weeks Dietary 93.7%	**M**: 17, 79 and 302 **F**: 16, 81 and 246 [500, 2500, 10 000 ppm]	**M**: 17 **F**: 16 [500 ppm]	**M**: 79 **F**: 81 [2500 ppm]	Liver effects (increased weight, hypertrophy and clinical chemistry); Hypocellularity of bone marrow	Ishii, 2016
HRA Beagle dogs 4/sex/group GLP OECD 452	52 weeks Dietary 93.7%	2.9/2.7, 14.6/14.3 71/67 [100, 500, 2500 ppm]	**M**: 14.6 **F**: 14.3 [500 ppm]	**M**: 71 **F**: 67 [2500 ppm]	Liver effects (increased wt, hepatocyte pigmentation, clicincal chemistry)	Ishii, 2018

[a] In the case of inhalation studies NOAEC and LOAEC

2.3 Long-term studies of toxicity and carcinogenicity

The chronic toxicity and carcinogenic potential of benzpyrimoxan was investigated in a 78-week mouse carcinogenicity study and in a combined chronic toxicity/carcinogenicity study in rats.

Mouse

In a study of carcinogenicity, benzpyrimoxan (purity 93.7%) was administered in feed for 78 weeks to CD-1 mice between five and six weeks old. Each dose group consisted of 51 males weighing 24–34 g or 51 females weighing 18–27 g. Dose levels were 0, 80, 400 or 2000 (male)/1500 (female) ppm (equivalent to 0, 7.7, 40 and 195 mg/kg bw per day for males, 0, 8.9, 44 and 163 mg/kg per day for females).

During the study, mortality, clinical condition, palpation of masses, assessment of body weight, food consumption, blood parameters, organ weights, and gross pathology was investigated as well as the histopathology of neoplastic and non-neoplastic findings.

Treatment had no effect on mortality, with survival remaining relatively high (75–86%) across all groups at the end of the study. No clinical signs of toxicity or differences in palpable swellings across groups were seen. The overall body weight gain (week 0–78) of males receiving 2000 ppm was approximately 11% lower than for controls, but there was no such effect in females.

There were no consistent or substantial effects of treatment on haematological parameters at weeks 52 or 78. At necropsy there were no treatment-related macroscopic findings. Liver weights were increased after 78 weeks in males given 2000 ppm (relative weights by 25%) and in females given 1500 ppm (relative weights by 15%). The sponsor considered the hepatic changes as adaptive, rather than adverse. This conclusion was reached although clinical chemistry investigations were not performed in this study, but in a 13-week study in mice described above, similar histopathological changes in the liver were accompanied by changes in serum AST and ALP levels. Hence, it was not possible to dismiss the toxicological significance of the observations in the liver in this 78-week study. All other intergroup differences from controls were minor, lacked dose–effect relationship or were attributed to differences in terminal body weight. The ovary weight of females receiving 1500 ppm was higher than in controls, but this was not statistically significant and the difference was considered to be just the result of high values for a few individual animals.

There was no statistically significant difference in the incidence or trend of any tumour type in treated animals compared with the control groups. Non-neoplastic findings were noted in the kidney, urinary bladder, gall bladder and liver. Rounded brown crystals were seen in the bladder of the top-dose males and in the renal pelvis of one single top-dose male. There was an increased incidence of calculi in the gall bladder in males given 400 or 2000 ppm. The difference in the incidence at 400 ppm was not statistically significant compared to controls and was only slightly higher than in controls. Therefore, this findings at the mid dose of 400 ppm was not considered substance-related. There was also an increased incidence and severity of centrilobular/diffuse hypertrophy of the liver in treated males and females at the lowest dose and above. Only the effect seen at the top dose in the presence of an increase in liver weight was considered adverse.

Table 35. Selected histopathological non-neoplastic findings in 78-week mouse carcinogenicity study; shown as individuals affected [animals examined]

		Dose level (ppm)							
		Males				Females			
Parameter		**0**	**80**	**400**	**2000**	**0**	**80**	**400**	**1500**
Kidneys; crystal(s), pelvis	Present	0 [51]	0 [51]	0 [51]	1 [51]	0 [51]	0 [51]	0 [51]	0 [51]
	Total	0 [51]	0 [51]	0 [51]	1 [51]	0 [51]	0 [51]	0 [51]	0 [51]
Urinary bladder; crystals	Minimal	0 [50]	0 [50]	0 [50]	4 [51]	0 [50]	0 [46]	0 [48]	0 [47]
	Slight	0 [50]	0 [50]	0 [50]	7 [51]	0 [50]	0 [46]	0 [48]	0 [47]
	Moderate	0 [50]	0 [50]	0 [50]	3 [51]	0 [50]	0 [46]	0 [48]	0 [47]
	Total	0 [50]	0 [50]	0 [50]	14 [51]	0 [50]	0 [46]	0 [48]	0 [47]

		Dose level (ppm)							
		Males				Females			
Parameter		**0**	**80**	**400**	**2000**	**0**	**80**	**400**	**1500**
Gall bladder; calculi	Present	7 [46]	8 [44]	11 [42]	**18* [49]**	5 [48]	0 [10]	0 [8]	4 [47]
	Total	7 [46]	8 [44]	11 [42]	**18* [49]**	5 [48]	0 [10]	0 [8]	4 [47]
Gall bladder; hyperplasia	Minimal	2 [46]	0 [44]	4 [42]	3 [49]	1 [48]	0 [10]	0 [8]	2 [47]
	Slight	3 [46]	0 [44]	1 [42]	2 [49]	0 [48]	0 [10]	0 [8]	0 [47]
	Moderate	0 [46]	0 [44]	0 [42]	2 [49]	0 [48]	0 [10]	0 [8]	0 [47]
	Total	5 [46]	0 [44]	5 [42]	7 [49]	1 [48]	0 [10]	0 [8]	2 [47]
Liver; hypertrophy, centrilobular/diffuse	Minimal	6 [51]	9 [51]	7 [51]	19 [51]	2 [51]	6 [51]	5 [51]	5 [51]
	Slight	3 [51]	5 [51]	7 [51]	8 [51]	0 [51]	2 [51]	3 [51]	5 [51]
	Total	9 [51]	14 [51]	14 [51]	27 [51]	2 [51]	8 [51]	8 [51]	10 [51]

* statistically significant; Source: Coleman, 2018

In conclusion, when administered in the diet to mice for 78 weeks benzpyrimoxan was not carcinogenic in either sex up to the top dose of 1500/2000 ppm (equivalent to 195 and 163 mg/kg bw per day in males and females respectively). Therefore, the NOAEL for carcinogenicity was 1500 ppm (equal 163 mg/kg bw per day), the highest dose tested. The top dose caused only moderate toxicity, characterized by decreased body weight gain in males, increased liver weight with associated hypertrophy in both sexes, crystals in the kidney and urinary bladder and gall bladder calculi in males only. The kidney and urinary bladder effects are considered not relevant to humans (see section 2,6 Special studies). It is questionable whether the maximum tolerated dose (MTD) was reached in females as only increased liver weight with associated hypertrophy was noted at the highest dose tested. Whilst the top dose in females might not have been adequate for hazard identification and classification from a risk perspective, the highest dose tested was still many orders of magnitude above the expected levels of human exposure. There were no adverse effects at 400 ppm. Therefore, a NOAEL for chronic toxicity of 400 ppm (equal to 40 mg/kg bw per day) was identified based on decreased body weight gain (11%) and gall bladder calculi in males, and liver effects in both sexes at the LOAEL of 1500 ppm (equal to 163 mg/kg bw per day) (Coleman, 2018).

Rat

In a chronic toxicity/carcinogenicity study, benzpyrimoxan (purity 93.7%) was administered in the diet for one year (chronic toxicity group) or two years (carcinogenicity group) to five-week-old Wistar rats weighing 146–177 g (males) and 125–153 g (females). Dose levels were 0, 60, 300 or 1500 ppm (in the chronic phase equivalent to 0, 2.3, 12 and 59 mg/kg bw per day for males, 0, 2.9, 15 and 78 mg/kg bw per day for females; in the carcinogenicity phase it was equal to 0, 2.7, 14 and 69 mg/kg bw per day for males, 0, 3.6, 17.5 and 90 mg/kg bw per day for females). Each dose group consisted of 21 or 12 males and 21 or 12 females in the chronic toxicity groups, and 51 males and 51 females in the carcinogenicity groups.

Table 36. Animal assignment in the two-year rat study

Test group		**Dose Level (ppm)**	**Males**	**Females**
Chronic toxicity	Control	0	21	21
	Low	60	12	12
	Intermediate	300	12	12
	High	1500	21	21
Carcinogenicity	Control	0	51	51
	Low	60	51	51
	Intermediate	300	51	51
	High	1500	51	51

All animals were observed for mortality and general clinical signs of toxicity, and their body weight and food consumption recorded periodically. Functional observation was carried out on 10 animals of each sex per group at 49 weeks of treatment in the chronic toxicity group. Ophthalmological examination was conducted on all animals before initiation of treatment and on all surviving animals in the control and high-dose groups at 52 weeks of treatment in the chronic toxicity group. Urinalysis (at 13, 25 and 51 weeks of treatment), haematology and blood biochemistry analysis (after 14, 26 and 52 weeks of treatment) were performed on 10 animals of each sex per group, three times during the treatment period in the chronic toxicity group. In the carcinogenicity group, haematology (total leukocyte count and differential leukocyte count) was performed on all surviving animals after 104 weeks of treatment. All animals were subjected to necropsy. Organ from all surviving animals were weighed after 52 weeks of treatment in the chronic toxicity group, and 10 animals of each sex per dose group after 104 weeks of treatment in the carcinogenicity group. In the chronic toxicity and carcinogenicity groups, histopathological examination was carried out for all animals on the liver, kidney, urinary bladder, and on all gross lesions.

Treatment had no effect on mortality, with survival rates remaining relatively high (71–82%) at the end of the study across all groups. There was no clear dose-related pattern to any effect on clinical signs of toxicity or functional observations. Body weights were reduced (by 6–15%) and body weight gains reduced (by 8–24%) to a statistically significant extent in males and females at the top dose. This effect was more pronounced in females than males, especially in the carcinogenicity phase. Food consumption was reduced (by 8%; statistically significant) in top-dose females throughout the study.

Haematology revealed top-dose males and females to have a significant decrease in Ht (chronic toxicity phase). Top-dose females also showed significant decreases in Hb and mean corpuscular Hb (chronic toxicity phase). Reticulocyte numbers did not change. From clinical chemistry investigations it was apparent that blood urea nitrogen (BUN) was significantly increased in top-dose males at 16 and 52 weeks. There were no changes in the results of liver function tests. At urinalysis, top-dose males and females showed an increase in round crystals in the urinary sediment, consistently at weeks 13, 25 and 51, and top-dose males showed a significantly and consistently lower pH.

At necropsy, top-dose males and females displayed depressed areas on the kidney. Top-dose males showed calculi in the urinary bladder, and top-dose females had spot(s) on the surface of the liver (carcinogenicity group). Relative liver weights were significantly increased in top-dose males and females in the chronic and carcinogenicity phases (by 9–10% in males and 15–31% in females) and similarly relative kidney weights (by 6–17% in males and 12–27% in females). Other organ weights (brain, heart and ovaries) showed some changes at the top dose in the chronic phase, however, given the lack of effect in the carcinogenicity phase, these were considered unrelated to treatment.

There were no treatment-related neoplastic findings. Non-neoplastic findings were noted in the liver and kidney at the top dose only. In the liver, fatty change and hypertrophy of centrilobular hepatocytes were seen in males in the chronic phase, whilst foci of cellular alteration were seen in males and females in the carcinogenicity phase. In the kidney, pelvic crystals and obstructive nephropathy were seen in males and females in the chronic and carcinogenicity phases. Details of non-neoplastic findings in the chronic phase are shown in Table 37, and in the carcinogenicity phase in Table 38.

Table 37. Selected histopathological non-neoplastic findings of the chronic phase; shown as individuals affected [animals examined]

Parameter		Dose level (ppm)							
		Males				Females			
		0	60	300	1500	0	60	300	1500
Liver	Fatty change; hepatocyte, centrilobular	1 [21]	0 [11]	1 [11]	**10** [20]**	0 [21]	0 [12]	0 [12]	0 [21]
	Hypertrophy; hepatocyte, centrilobular	0 [21]	0 [11]	0 [11]	**7** [20]**	0 [21]	0 [12]	0 [12]	4 [21]
Kidneys	Crystal(s); pelvis	0 [21]	0 [11]	0 [11]	**4** [20]*	0 [21]	0 [12]	0 [12]	4 [21]
	Nephropathy; obstructive	0 [21]	0 [11]	0 [11]	**8** [20]**	0 [21]	0 [12]	0 [12]	**6** [21]*

* Significantly different from the control using Fisher's exact probability test: $p < 0.05$; Source: Ohtsuka, 2018b

** Significantly different from the control using Fisher's exact probability test: $p < 0.01$

Table 38. Selected histopathological non-neoplastic findings of the carcinogenicity phase; shown as individuals affected [animals examined]

Parameter		Dose level [ppm]							
		Males				Females			
		0	60	300	1500	0	60	300	1500
Terminated after 104 weeks of treatment									
Liver	Spongiosis hepatis	4 [36]	**0** [41]*	5 [43]	8 [42]	0 [37]	0 [34]	0 [38]	0 [35]
	Foci of cellular alteration, basophilic cell type	18 [36]	25 [41]	24 [43]	28 [42]	23 [37]	19 [34]	22 [38]	**29** [35]*
	Foci of cellular alteration, eosinophilic cell type	15 [36]	19 [41]	25 [43]	**32** [42]**	10 [37]	6 [34]	7 [38]	**19** [35]*
Kidneys	Crystal(s), pelvis	0 [36]	0 [41]	0 [43]	**11** [42]**	0 [37]	0 [34]	0 [38]	**9** [35]**
	Nephropathy, obstructive	0 [36]	0 [41]	0 [43]	**19** [42]**	0 [37]	0 [34]	0 [38]	**8** [35]**
Terminated in extremis or found dead									
Lung	Accumulation, macrophage alveolar	6 [15]	1 [10]	1 [8]	**0** [9]*	3 [14]	1 [17]	1 [13]	1 [16]
Liver	Hyperplasia, bile duct	3 [15]	2 [10]	1 [8]	2 [9]	8 [14]	6 [17]	4 [13]	**3** [16]*
Kidneys	Calcification, fornix	4 [15]	1 [10]	0 [8]	2 [9]	10 [14]	12 [17]	8 [13]	**4** [16]*
	Nephropathy, obstructive	0 [15]	0 [10]	0 [8]	**4** [9]*	0 [14]	0 [17]	0 [13]	**6** [16]*
Ovary	Hyperplasia, cystic/papillary	-	-	-	-	4 [14]	1 [17]	2 [13]	**0** [16]*
	Hyperplasia, Sertoli cell	-	-	-	-	7 [14]	6 [17]	1* [13]	4 [16]
	Hyperplasia, sex cord stromal	-	-	-	-	5 [14]	3 [17]	3 [13]	**0** [16]*

Parameter		Dose level [ppm]							
		Males				Females			
		0	60	300	1500	0	60	300	1500
Adrenals	Hypertrophy, cortical cell, focal	4 [15]	1 [10]	0 [8]	0 [9]	6 [14]	4 [17]	1* [13]	3 [16]
	All animals examined								
Liver	Foci of cellular alteration, basophilic cell type	20 [51]	26 [51]	26 [51]	**30* [51]**	26 [51]	22 [51]	27 [51]	33 [27]
	Foci of cellular alteration, eosinophilic cell type	18 [51]	19 [51]	27 [51]	**37** [51]**	11 [51]	7 [51]	8 [51]	**22* [51]**
Kidneys	Crystal(s), pelvis	0 [51]	0 [51]	0 [51]	**12** [51]**	0 [51]	0 [51]	0 [51]	**13** [51]**
	Nephropathy, obstructive	0 [51]	0 [51]	0 [51]	**23** [51]**	0 [51]	0 [51]	0 [51]	**14** [51]**
Adrenals	Peliosis	1 [51]	0 [10#]	0 [9#]	1 [51]	33 [51]	9 [17#]	7 [15#]	**22* [51]**

Source: Ohtsuka, 2018b

[a] Examined on the animals that showed macroscopic lesions at terminal sacrifice and on all the animals sacrificed in extremis or found dead during the study. Not subjected to statistical analysis.

* Significantly different from the control using Fisher's exact probability test: $p < 0.05$;

** Significantly different from the control using Fisher's exact probability test: $p < 0.01$

In conclusion, dietary administration of benzpyrimoxan to rats for two years was not carcinogenic in either sex up to the top dose of 1500 ppm (equivalent to 69 mg/kg bw per day for males, 90 mg/kg bw per day for females). The top dose caused significant toxicity, characterized by decreased body weights and body weight gains in both sexes, reduced food consumption in females, changes in some haematological, clinical chemistry and urinalysis parameters in both sexes, calculi/crystals in the urinary bladder of males and females, and increased liver and kidney weights associated with histopathological changes in both sexes. There were no treatment-related effects at 300 or 60 ppm. The kidney and urinary bladder effects were considered not relevant to humans (see section 2.6 Special studies). A systemic toxicity NOAEL of 300 ppm (equal to 14 mg/kg bw per day) was identified based on decreased body weights and body weight gains in both sexes, reduced food consumption in females, changes in some haematological and clinical chemistry parameters in both sexes, and increased liver weights with associated histopathological changes in both sexes at 1500 ppm (equal to 59 mg/kg bw per day) in the chronic phase. The NOAEL for carcinogenicity was 1500 ppm (equal to 69 mg/kg bw per day), the highest dose tested (Ohtsuka, 2018b).

Table 39 below summarizes the available carcinogenicity studies.

Table 39. Summary of the long term studies available for benzpyrimoxan

Species/strain No. animals Guideline	Duration Route Purity	Dose levels (mg/kg bw/day)	NOAEL (mg/kg bw/day)	LOAEL (mg/kg bw/day)	Effects at LOAEL	Reference
Mouse						
ICR [Crl:CD1] 51/sex/group GLP OECD 451	78 weeks Dietary 93.7%	**M**: 7.7, 40 and 195 **F**: 8.9, 44 and 169 [80, 400, 1500/2000 ppm]	***Chronic*** **M**: 40 **F**: 44 [400 ppm]	***Chronic*** **M**: 195 **F**: 169 [1500 ppm]	***Chronic*** Decreased body weight gain (11%) (M) Gall bladder calculi (M) Increased liver weight and associated hypertrophy	Coleman, 2018
			Carcinogenicity **M**: 195 **F**: 169 [1500 ppm]	***Carcinogenicity*** **M**: > 195 **F**: > 169 [1500 ppm]	***Carcinogenicity*** No tumour findings	
Rat						
Wistar Hannover (RccHan™: WIST) 12–21/sex/group (chronic phase) 51/sex/group (carc. phase) GLP OECD 453	104 weeks Dietary 93.7%	**M**: 2.7, 14 and 69 **F**: 3.6, 17.5 and 90 [60, 300 and 1500 ppm]	***Chronic*** **M**: 14 **F**: 17.5 [300 ppm]	***Chronic*** **M**: 69 **F**: 90 [1500 ppm]	***Chronic*** Decreased body weight, body weight gain; Decreased food consumption; Changes in clinical chemistry and haematology; Increased liver weights with associated histopathology	Ohtsuka, 2018b
			Carcinogenicity **M**: 69 **F**: 90 [1500 ppm]	***Carcinogenicity*** **M**: > 69 **F**: > 90 [1500 ppm]	***Carcinogenicity*** No tumour findings	

M: Male; F: Female

2.4 Genotoxicity

The genotoxic potential of benzpyrimoxan was investigated in a standard range of three in vitro tests and in an in vivo mouse micronucleus study. Table 40 below provides a summary of the results. Benzpyrimoxan was not genotoxic in vitro or in vivo in valid guideline studies.

Table 40. Summary of the genotoxicity studies performed with benzpyrimoxan

End-point	Test organism(s)/system(s)	Concentrations	Purity	Result	Reference
In vitro					
Reverse mutation	TA98, TA100, TA1535, TA1537, WP2 *uvrA*	0, 313, 625, 1250, 2500 and 5000 µg/plate	94.0%	Negative ± S9	Oguma, 2014
Chromosome aberrations	Chinese hamster lung (CHL/IU) cells	8.64–700 mg/mL	93.7%	Negative ± S9	Tsukushi, 2017b
Gene mutation	L5178Y tk+/− (3.7.2C) mouse lymphoma cells	0.49–62.5 mg/mL	93.7%	Negative ± S9	Verspeek-Rip, 2017
In vivo					
Micronucleus test	Slc/ICR mice	0, 500, 1000 and 2000 mg/kg bw	93.7%	Negative	Tsukushi, 2015

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*

(a) In vitro studies

An Ames test (OECD 471) was conducted with benzpyrimoxan (purity 94.0%) in *Salmonella typhimurium* strains TA100, TA1535, TA98 and TA1537, and *Escherichia coli* strain WP2 *uvrA* with and without metabolic activation (S9 mix) by the pre-incubation method. Dimethyl sulfoxide (DMSO) was used as the vehicle for the test article. Duplicate plates were used in the range-finding test and triplicate plates in the two main tests.

A range-finding test was conducted with concentrations between 19.5 and 5000 µg/plate. From the result of the range-finding test, since no growth inhibition was observed in any strain with or without metabolic activation, the main test was conducted at 313, 625, 1250, 2500 and 5000 µg/plate for all strains with and without metabolic activation. The main test was repeated with the same concentrations.

Precipitation of the test item was observed at 313 µg/plate and above with and without metabolic activation in all strains. No cytotoxicity (growth inhibition) was observed in any strain with or without metabolic activation.

Treated groups in both tests, for all strains, showed no relevant (two-fold or more) increase, nor concentration-dependent increase in revertant colonies compared to the negative controls, whether with or without metabolic activation. The positive control substances gave the expected responses.

Overall, benzpyrimoxan was negative in a GLP and guideline Ames study up to the limit concentration for this test (Oguma, 2014).

In an in vitro mammalian chromosomal aberration study in cultured Chinese hamster lung (CHL/IU) cells (OECD 473), benzpyrimoxan (purity 93.7%) dissolved in DMSO was incubated with or without metabolic activation (S9 mix) for six hours (short time exposure). Following exposure, the cells were washed and cultivated for another 17 hours to cover the length of 1.5 cell cycles. In a second experiment, continuous treatment for 23 or 46.5 hours (corresponding to 1.5 and 3.0 cell cycle lengths) was employed without metabolic activation. Following exposure the cells were harvested and prepared for chromosome analysis. Range-finding tests were performed for both treatment durations to determine the concentrations of the test substance to use in the main experiments. The concentrations giving a 40–50% reduction in the relative increase in cell count (RICC) were selected as the highest concentrations for the main tests.

In the short-time exposure (six hours), four concentrations ranging from 25.9 to 700 µg/mL were employed with S9, and four concentrations ranging from 21.9 to 175 µg/mL were employed without S9. In the continuous 23-hour experiment, four concentrations ranging from 11.3 to 90 $\mu g/mL$ were employed, and in the 43.5-hour experiment, three concentrations ranging from 17.5 to 70 $\mu g/mL$ were

used. Duplicate plates for each concentration were run and 300 metaphase cells scored for structural chromosome aberrations. The number of polyploid cells was also recorded.

The number of cells with chromosome aberrations and the number of polyploid cells in the negative controls were within the laboratory historical negative control ranges. Significant increases in chromosome aberrations were observed in the positive controls. These results also fell within the laboratory historical positive control ranges, confirming the validity of the assay. Benzpyrimoxan did not induce any increase in chromosome aberrations (excluding gaps) in the short exposure tests either with or without S9, or the continuous 23-hour or 46.5-hour exposure test without S9. The number of polyploid cells increased in a concentration-related manner in the short exposure test with and without S9, becoming statistically significant at the top concentration of 700 µg/mL with S9 (4.5% compared with 0.3% in controls) and at the mid-high concentration of 87.5 µg/mL without S9 (2.9% compared with 0.3% in controls). However, there was no increase in the number of polyploid cells under the continuous exposure conditions, where interference of the test substance with cell cycle progression would have the opportunity to lead to a much greater accumulation of polyploid cells compared to shorter treatments.

Precipitation was observed at the two highest concentrations in all experiments with or without S9, except in the 46.5-hour treatment where precipitation occurred only at the top concentration. Cytotoxicity (40–50% reduction in RICC) was observed at the top concentration under all test conditions. Although the increase in polyploidy was significant, it was only seen at the two highest concentrations and only after the short treatment. In the long treatment experiment, where the likelihood of such an effect is much greater than for short-term exposure, there was no increase in polyploidy. Therefore, the finding was considered a chance observation (Tsukushi, 2017b).

Table 41. Chromosomal aberrations and polyploid cells

Assay No. 1 (+ S9)			**Concentration (µg/mL)**					
			Control[a]	**25.9**	**77.8**	**233**[b]	**700**[b]	**CP: 10.0**
Number and type of aberration	Chromatid type	ctb	1	0	0	1	0	33
		cte	0	1	0	2	1	144
	Chromosome type	csb	0	0	0	0	0	5
		cse	0	0	0	0	0	11
	Others	frg	0	0	0	0	0	0
		mul	0	0	0	0	0	4
Cells with aberrations	+ gap	N	1	4	2	6	4	145
		%	0.3	1.3	0.7	2.0	1.3	**48.3*****
	– gap	N	1	1	0	3	1	129
		%	0.3	0.3	0.0	1.0	0.3	**43.0*****
Polyploid cells [%]			**0.3**###	1.0	2.0	1.6	**4.5*****	0.3
Assay No. 2 (− S9)			**Concentration (µg/mL)**					
			Control[a]	**21.9**	**43.8**	**87.5**[b]	**175**[b]	**MMC: 0.100**
Number and type of aberration	Chromatid type	ctb	1	0	0	0	0	73
		cte	0	0	0	0	0	130
	Chromosome type	csb	0	0	0	0	0	12
		cse	0	0	0	0	0	9
	Others	frg	0	0	0	0	0	0
		mul	0	0	0	0	0	1
Cells with aberrations	+ gap	N	5	0	5	2	1	132
		%	1.7	0.0	1.7	0.7	0.3	**44.0*****
	– gap	N	1	0	0	0	0	125
		%	0.3	0.0	0.0	0.0	0.0	**41.7*****
Polyploid cells [%]			**0.3**##	0.0	0.7	**2.9***	1.6	0.7

Assay No. 3 (1.5 cell cycle; − S9)			Concentration (μg/mL)					
			Control[a]	11.3	22.5	45.0[b]	90.0[b]	MMC: 0.0700
Number and type of aberration	Chromatid type	ctb	0	0	0	0	0	66
		cte	0	1	0	0	0	93
	Chromosome type	csb	0	0	0	1	0	10
		cse	0	0	0	0	0	13
	Others	frg	0	0	0	0	0	0
		mul	0	0	0	0	0	2
Cells with aberrations	+ gap	N	1	2	1	1	0	128
		%	0.3	0.7	0.3	0.3	0.0	**42.7*****
	− gap	N	0	1	0	1	0	112
		%	0.0	0.3	0.0	0.3	0.0	**37.3*****
Polyploid cells [%]			1.6	0.3	0.7	1.6	1.3	0.3

Assay No. 4 (3.0 cell cycle; − S9)			Concentration (μg/mL)					
			Control[a]	17.5	35.0	70.0[b]	-	MMC: 0.0700
Number and type of aberration	Chromatid type	ctb	0	0	0	0	-	48
		cte	0	0	0	1	-	113
	Chromosome type	csb	0	0	0	0	-	17
		cse	0	0	0	0	-	11
	Others	frg	0	0	0	0	-	0
		mul	0	0	0	0	-	7
Cells with aberrations	+ gap	N	2	2	1	2	-	128
		%	0.7	0.7	0.3	0.7	-	**42.7*****
	− gap	N	0	0	0	1	-	111
		%	0.0	0.0	0.0	0.3	-	**37.0*****
Polyploid cells [%]			1.0	0.0	0.7	0.7	-	2.6

[a] Control dimethyl sulfoxide only; [b] Precipitation of the test substance; Source: Tsukushi, 2017b

CP: Cyclophosphamide; MMC: Mitomycin C;

ctb: Chromatid break; csb: Chromosome break; cte: Chromatid exchange; cse: Chromosome exchange; frg: Fragmentation; mul: Multiple aberration; + gap: Including gaps; − gap: Excluding gaps;

Significantly different from the control using Fisher's exact test: * $p < 0.05$, *** $p < 0.001$;

Significantly different from the control using Cochran–Armitage trend test: $p < 0.01$;

Significantly different from the control using Cochran–Armitage trend test: $p < 0.001$

In a gene mutation assay at the thymidine kinase (*TK*) locus in L5178Y mouse lymphoma cells (OECD 490), benzpyrimoxan (purity 93.7%) dissolved in DMSO was tested in the absence of metabolic activation (S9 mix) for three and 24 hours, and in the presence of S9 mix for three hours.

Range-finding tests were performed for both treatment durations to determine the concentrations of the test substance to use in the main experiments. The highest concentration was determined by the solubility of the test item in the culture medium since the test item was not particularly cytotoxic. In the first experiment benzpyrimoxan was tested at eight different concentrations up to 62.5 μg/mL in the absence and presence of S9 mix for three hours. No cytotoxicity was observed at the top concentration, but precipitation occurred. In the second experiment benzpyrimoxan was also tested at eight different concentrations up to 62.5 μg/mL in the absence of S9 mix for 24 hours. No significant cytotoxicity (relative total growth less than 10%) was observed at the top concentration, but precipitation occurred.

The mutation frequency found in the solvent control cultures was within the acceptability criteria of this assay in both experiments. The positive control chemicals produced significant increases in the mutation frequency, which were within the 95% control limits of the distribution of the laboratory historical positive control database. It was therefore concluded that the test conditions were valid. In the absence of S9 mix, using treatment periods of three and 24 hours, benzpyrimoxan did not induce a significant increase in the mutation frequency. The same result was obtained in the presence of S9 mix with a three-hour treatment period. None of the tested concentrations under any of the test conditions reached a mutation frequency (MF) of $MF_{(controls)}$ + GEF (global evaluation factor) of 126×10^{-6} (that is, the mean of negative/solvent MF distribution plus one standard deviation, as defined by the guideline).

In conclusion, in this valid guideline mouse lymphoma assay, benzpyrimoxan was not mutagenic with or without metabolic activation up to concentrations that caused precipitation (Verspeek-Rip, 2017).

(b) In vivo studies

In a bone marrow micronucleus study in mice (OECD 474), benzpyrimoxan (purity 93.7%) suspended in 0.5% (w/v) sodium CMC solution with 0.1 % (v/v) Tween 80, was given by oral gavage to groups of male Slc/ICR mice (five animals per group) at dose levels of 0 (vehicle negative control), 500, 1000 and 2000 mg/kg bw (the last being the limit dose for this type of study), once daily on two consecutive days. A positive control group was administered a single intraperitoneal injection of mitomycin C at 3 mg/kg bw.

Bone marrow smears were obtained from all animals at 24 hours after the final dosing. For each animal 4000 immature erythrocytes were examined to count any micronucleated immature erythrocytes (MNIEs). The ratio of immature erythrocytes to total (immature + mature) erythrocytes (IE : TE) was also determined in 500 erythrocytes per animal.

All doses were well tolerated and no clinical signs of toxicity were observed. At 1000 and 2000 mg/kg bw a decreasing trend in body weight gain (between days 1 and 3) compared to controls (by 3 and 6% at 1000 and 2000 mg/kg bw respectively) was noted. At necropsy, mice treated with 1000 and 2000 mg/kg bw benzpyrimoxan showed a higher incidence of white spots on the kidneys, discolouration and enlargement of the kidneys and yellow material in the urinary bladder. The incidence of white spots on the kidney was also increased at 500 mg/kg bw. These findings indicate that the test substance was systemically available. The IE : TE ratio and the frequency of MNIEs in the concurrent negative control group were within the laboratory historical negative control range. The frequency of MNIEs in the positive control group was significantly increased compared to the concurrent negative control and was within the laboratory historical positive control range. These results demonstrated the validity of the study.

In treated animals, the frequency of MNIEs was neither statistically significantly different from controls nor dose-related. The IE : TE ratio was marginally reduced at 1000 mg/kg bw, indicating slight toxicity to the bone marrow.

In conclusion, in a GLP and guideline bone marrow micronucleus study in mice, benzpyrimoxan did not show clastogenic or aneugenic effects up to a dose (the limit dose), and displayed systemic toxicity and slight toxicity to the bone marrow (Tsukushi, 2015).

2.5 Reproductive and developmental toxicity

The reproductive and developmental toxicity of benzpyrimoxan was investigated in a two-generation study in rats and in developmental toxicity studies in rats and rabbits, with associated preliminary, range-finding studies.

(a) Multigeneration studies

Rat

In a two-generation reproduction toxicity study (OECD 416), groups of 24 male and 24 female Crl:CD(SD) rats were given diets containing benzpyrimoxan for two successive generations at concentrations of 0, 60, 300 or 2000 ppm (equivalent to 0, 2.5, 12 and 85 mg/kg bw per day for males, 0, 4.7, 24 and 156 mg/kg bw per day for females, taking the lowest substance intakes at each generation and life stage) .

Parental toxicity

At the top dose, mean body weights of F1 parental males were significantly lower (by 8%) than in controls during the premating period. At this dose, mean body weights of F0 and F1 parental females decreased (by 5%) during the breeding period, that is from gestation day (GD) 7 to lactation day (LD) 14. Also statistically significant differences were noted on LDs 0, 4, 7, and 14 for F0 and F1 parental females as well as on GD 14 for F0 females or GD 7 for F1 females (by up to 9%). In addition, mean body weights of F1 parental females were significantly lower (by 8%) than controls during the premating period. Similarly, body weight gains in these groups were significantly reduced. Food consumption was reduced (by 7%) in top-dose F0 females during gestation.

At the pathological examination, an increased incidence of depressed areas in the kidneys was seen in top-dose males of the F0 and F1 generation. Increased relative (to body weight) weights of the liver were noted in both sexes (by 13–18% in males and 20–21% in females) and of the thyroid in males (by 15–25%) at the top dose. These increases were consistent across generations. Relative liver weights were also increased in F0 females at 60 and 300 ppm and in F1 males at 300 ppm, however, in the absence of associated histopathology these increases in the mid- and low-dose groups were considered not to be adverse. Other organ weight changes (ovaries, spleen, brain and pituitary) were considered unrelated to treatment as no dose–response relationship was evident or there was no consistency across generations.

Histopathological examination revealed findings at the top dose only in the liver, kidney and thyroid. In the liver, centrilobular hepatocyte hypertrophy was noted in both sexes of the F0 and F1 generations. In addition, focal hepatocyte degeneration/necrosis was seen in F1 females only. In the kidney, crystal(s) in the renal pelvis were observed in F0 and F1 males. In addition, obstructive nephropathy was noted in both sexes of both generations. The kidney effects are not considered relevant to humans (see section 2.6 Special studies). In the thyroid, follicular cell hypertrophy was observed in both sexes of the F0 and F1 generations. Histopathological findings are detailed in Table 42.

***Table 42. Selected histopathological findings in parental animals; rats affected* [*number examined*]**

		Dose level (ppm)							
		Males				**Females**			
Parameter		**0**	**60**	**300**	**2000**	**0**	**60**	**300**	**2000**
		F0 generation							
Liver	Hypertrophy, hepatocyte, centrilobular	0 [24]	0 [23]	0 [23]	**5* [22]**	0 [24]	0 [23]	0 [24]	**4* [22]**
Kidneys	Crystal(s), pelvis	0 [24]	**0** [23]	0 [23]	**10** [22]**	0 [24]	0 [23]	0 [24]	0 [22]
	Nephropathy, obstructive	0 [24]	0 [23]	0 [23]	**12** [22]**	0 [24]	**0** [23]	0 [24]	2 [22]
Thyroid	Hypertrophy, follicular cell	**0** [24]	**0** [23]	**0** [23]	**8** [22]**	1 [24]	0 [23]	1 [24]	**6* [22]**
		F1 generation							
Liver	Degeneration/necrosis, hepatocyte, focal	0 [23]	0 [24]	0 [24]	0 [22]	0 [22]	0 [23]	0 [23]	**5* [20]**
	Hypertrophy, hepatocyte, centrilobular	0 [23]	0 [24]	0 [24]	**5* [22]**	0 [22]	0 [23]	0 [23]	3 [20]
Kidneys	Crystal(s), pelvis	0 [23]	0 [24]	0 [24]	**6** [22]**	0 [22]	0 [23]	0 [23]	1 [20]
	Nephropathy, obstructive	0 [23]	0 [24]	0 [24]	**13** [22]**	0 [22]	0 [23]	0 [23]	3 [20]
Thyroids	Hypertrophy, follicular cell	1 [23]	1 [24]	0 [24]	**7* [22]**	0 [22]	0 [23]	0 [23]	3 [20]

* Significantly different from control using Dunnett's test (or Dunnett-type test): $p < 0.05$; Source: Hojo, 2018

** Significantly different from control using Dunnett's test (or Dunnett-type test): $p < 0.01$;

Reproductive toxicity

There were no effects of treatment on sexual development, estrous cycle, mating and fertility indices, duration of gestation, number of implantation sites, sperm parameters, number of ovarian primordial follicles, sex ratio or reproductive organs. The gestation index was affected (number of females with live pups/number of pregnant females) being slightly lower in the F1 generation at the top dose due to one female showing poor behaviour in nesting and nursing, as a consequence of the maternal toxicity observed at this dose (reduced body weight, body weight gain, and toxicity in liver, kidney and thyroid). A higher postimplantation loss was also noted at the top dose in both generations.

Offspring toxicity

At the top dose, viability on day 0 was reduced in both generations. Survival rates were also reduced on LDs 4 and 14.

Table 43. Group mean percent litter incidences of clinical findings in rat two-generation study; (percentage ± standard deviation shown)

		Dose level (ppm)			
Time-point		**0**	**60**	**300**	**2000**
		F1 pups			
Lactation day 0	Found dead	0.2 ± 1.2	0.6 ± 2.1	0.0 ± 0.0	**6.0** ± 11.6**
		F2 pups			
		2.3 ± 9.5	0.9 ± 2.4	0.6 ± 2.2	**15.1** ± 24.7**

* Significantly different from control using Dunnett's test (or Dunnett-type test): $p < 0.05$; Source: Hojo, 2018

** Significantly different from control using Dunnett's test (or Dunnett-type test): $p < 0.01$;

Table 44. Selected viability indices of the F1 and F2 pups in rat two-generation study; (mean index ± standard deviation shown)

	Dose level (ppm)			
Time-point	**0**	**60**	**300**	**2000**
	F1 pups			
Lactation day 0	99.8 ± 1.2	99.4 ± 2.1	100.0 ± 0.0	**94.0** ± 11.6**
	F2 pups			
Lactation day 0	97.7 ± 9.5	99.1 ± 2.4	99.4 ± 2.2	**84.9** ± 24.7**

Source: Hojo, 2018

* Significantly different from control using Dunnett's test (or Dunnett-type test): $p < 0.05$;

** Significantly different from control using Dunnett's test (or Dunnett-type test): $p < 0.01$;

Pup body weights were reduced at the top dose in both generations.

Table 45. Mean body weights of the F1 and F2 pups in rat two-generation study; (g ± SD)

	Dose level (ppm)							
	Males				**Females**			
Time-point	**0**	**60**	**300**	**2000**	**0**	**60**	**300**	**2000**
Lactation day	**F1 pups**							
7	20.4 ± 1.4	19.4 ± 2.0	19.8 ± 2.0	**18.9* ± 1.6**	19.7 ± 1.2	18.5* ± 1.7	18.9 ± 2.1	**18.4* ± 1.6**
14	41.8 ± 2.0	40.7 ± 3.2	41.1 ± 2.9	**38.7** ± 2.1**	40.6 ± 2.3	39.3 ± 3.0	39.6 ± 3.3	**37.8** ± 2.2**
21	68.6 ± 3.4	67.6 ± 4.7	67.5 ± 4.2	**64.1** ± 33**	66.0 ± 3.7	64.6 ± 3.9	64.3 ± 4.9	**62.0** ± 3.8**

	Dose level (ppm)							
	Males				Females			
Time-point	0	60	300	2000	0	60	300	2000
	F2 pups							
7	20.4 ± 2.1	19.2 ± 2.6	20.7 ± 2.5	**17.3** ± 2.5**	19.1 ± 2.2	18.2 ± 2.6	20.0 ± 2.4	17.4 ± 2.7
14	42.3 ± 3.1	41.4 ± 2.9	43.9 ± 4.1	**37.1** ± 7.0**	40.6 ± 2.7	39.9 ± 2.9	42.4 ± 4.3	**36.9** ± 6.8**
21	68.1 ± 4.5	66.2 ± 3.9	70.6 ± 6.3	**61.4** ± 10.0*	65.0 ± 3.5	63.1 ± 3.8	67.4 ± 6.4	**59.8** ± 10.0*

* Significantly different from control using Dunnett's test (or Dunnett-type test): $p < 0.05$; Source: Hojo, 2018

** Significantly different from control using Dunnett's test (or Dunnett-type test): $p < 0.01$;

In conclusion, in this guideline rat two-generation study, reproductive performance was marginally affected at the top dose, with a slight reduction in the gestation index of the F1 generation and increased post-implantation loss in both generations. On this basis, a NOAEL of 300 ppm (equal to 12 mg/kg bw per day) was identified for reproductive toxicity. Parental toxicity was also apparent at the top dose of 2000 ppm (equal to 85 mg/kg bw per day) and was characterized by effects on body weight, body weight gain, food consumption and toxicity to the liver and thyroid. A NOAEL of 300 ppm (equal to 12mg/kg bw per day) was therefore also identified for parental toxicity. Offspring toxicity, characterized by reduced pup body weights and reduced viability on LDs 0, 4 and 14, was present at the top dose of 2000 ppm. Therefore, a NOAEL for offspring toxicity also of 300 ppm (equal to 12 mg/kg bw per day) was identified from this study (Hojo, 2018).

(b) Developmental toxicity

Rat

In a preliminary (non-GLP, non-guideline) prenatal developmental toxicity study, benzpyrimoxan (purity 93.7%) was administered by gavage in an aqueous solution of 0.5 % (w/v) CMC containing Tween 80 at 0.1 % (v/v) to four groups of 12 pregnant female Crl:CD(SD) rats from GDs 6 to 19 at 0, 100, 300 or 1000 mg/kg bw per day. On GD 20 all surviving females were euthanized, and their fetuses removed and examined.

Maternal toxicity; preliminary study

Three top-dose dams showed severe signs of toxicity and died on GDs 8, 12 and 19. One animal at the mid dose also showed clinical signs of toxicity and was euthanized on GD 13. Body weights were significantly reduced (by up to 13%) at the top dose, with decreases in body weight gain of 34%. Food consumption was reduced (by up to 55%) during GDs 9–12 at the top dose and by 45% on GD 9 of at the mid dose.

At necropsy, treatment-related changes were observed in the uterus, liver and kidney. Macroscopic observation revealed a blood clot in the uterus of two top-dose animals. White spots and/or areas of discolouration were seen in the kidney at the mid dose and above. Relative liver weight was increased at the mid and high doses (by 14% and 25% respectively) and relative kidney weight was increased at the top dose by 18%. Mean placental weights were increased significantly (by 23%) at the mid dose and above.

Developmental toxicity; preliminary study

In the examination of ovary and uterus, there were no treatment-related effects in the number of corpora lutea, the number of implantations, embryo-fetal deaths, or on sex ratio. At the top dose, fetal body weight in females was statistically lower (by 7%) than in controls.

In the fetuses, external and visceral examinations revealed no treatment-related changes in any treated groups. The skeletal examination revealed no skeletal malformations in any treated groups. However, some skeletal variations (dumbbell ossification of thoracic centrum, lumbar rib and discontinuous rib cartilage) were increased mainly at the mid dose and above, although the incidence of lumbar rib was also higher at the low dose.

Based on the results of this preliminary study, the sponsor concluded that the high dose for the main study needed to be between 200 and 300 mg/kg bw per day (Matsumoto, 2017).

In the subsequent main developmental toxicity study (OECD 414), benzpyrimoxan (purity 93.7%) in an aqueous solution of 0.5% (w/v) sodium CMC including 0.2% (v/v) Tween 80 was administered by gavage to groups of 24 pregnant female Crl:CD(SD) rats, from GDs 6 to 19 at doses of 0, 10, 50, and 250 mg/kg bw per day. Live fetuses were removed from the uteri at necropsy of maternal rats on GD 20 and examined for external, visceral, and skeletal alterations.

Maternal toxicity; main study

Maternal body weights were reduced (by 5%) at the top dose especially on GDs 9 to 15, with body weight gains decreased by up to 43% and food consumption significantly lower (by up to 20%) than in controls. At necropsy, one top-dose dam showed enlargement and pale discolouration of the kidney. The kidney findings are considered not to be relevant to humans (see section 2.6 Special studies). Placental weight was significantly higher at the top dose (by 12%) compared to controls.

Developmental toxicity; main study

There were no effects of treatment on the numbers of corpora lutea, implantations, pre-implantation loss, implantation index, post-implantation loss, number of dead and live fetuses, sex ratio, or fetal weights in any treated group. The external, visceral and skeletal examinations of fetuses revealed no anomalies or variations related to the treatment. Some changes were noted, but in the absence of a dose–response relationship they were considered unrelated to treatment. One top-dose fetus showed cleft palate and another showed a defect of the ventricular septum, but given the extremely low numbers, and that they were within the ranges of the laboratory historical control data, these statistically non-significant findings were considered incidental.

In conclusion, no developmental toxicity was noted in this guideline rat developmental toxicity study up to a dose of 250 mg/kg bw per day which caused significant maternal toxicity, including effects on body weight, body weight gain, food consumption and placental weight. Therefore, the NOAEL for embryo/fetal toxicity was 250 mg/kg bw per day, the highest dose tested, and the NOAEL for maternal toxicity was 50 mg/kg bw per day based on reduced food consumption and significantly higher placental weight at a LOAEL of 250 mg/kg bw per day, (Fujii, 2017a).

Rabbit

In a preliminary (non-GLP, non-guideline) prenatal developmental toxicity study, benzpyrimoxan (purity 94.0%) was administered by gavage in 0.5% (w/v) CMC solution containing 0.2% (v/v) Tween 80 to groups of eight pregnant New Zealand White rabbits from GD 6 to 27 at doses of 0, 10, 30 or 60 mg/kg bw per day. On GD 28 all surviving females were euthanized, and their fetuses removed and examined.

Maternal toxicity

Treatment-related adverse effects on maternal rabbits in the mid- (30 mg/kg bw per day) and high- (60 mg/kg bw per day) dose groups were observed. These included decreases in body weight by 4% (not statistically significant) and 12% respectively, body weight gain by 30% (not statistically significant) and 62% respectively, food consumption by 27% (not statistically significant at most time points) and up to 90% respectively, and faecal output in one animal at the mid dose and almost all animals at the high dose. One top-dose female aborted. At necropsy, discolouration of the liver and the appearance of a white region thereon were noted at the top dose, and gravid uterine weight was reduced at 60 mg/kg bw per day.

Developmental toxicity

There were no treatment-related differences between the controls and the treated groups in the number of corpora lutea or implantations, number and litter percent of live fetuses, number of early and late resorptions, dead fetuses, or percent of pre- and post-implantation losses. Fetal body weights were decreased (by 28–30%) at 60 mg/kg bw per day. In examining for external, visceral and skeletal anomalies, some changes were noted, but in the absence of a dose–response relationship they were considered incidental. However, some variations were observed at the top dose, including increased incidences of discoloured spleen, asymmetry of sternebrae and discontinuous rib cartilage, and decreased ossification of sacral and caudal vertebrae, metacarpus and metatarsus.

Based on the results of this preliminary study, the sponsor concluded that the high dose for the main study needed to be approximately 30 mg/kg bw per day (Inagaki, 2017).

In the subsequent main developmental toxicity study (OECD 414), benzpyrimoxan (purity 93.7%) was administered by gavage in 0.5% (*w/v*) CMC solution containing 0.2% (v/v) Tween 80 to groups of 25 pregnant Kbl:New Zealand White rabbits, from GD 6 to 27 at doses of 0, 3, 10 or 30 mg/kg bw per day. Fetuses were removed on GD 28 and examined for external, visceral, and skeletal alterations.

Maternal toxicity

Maternal toxicity was observed at the top dose. Two animals aborted on GDs 21 and 27. Body weight (reduced by 5%), body weight gain (lower by up to 50%), and food consumption (down by up to 44%) were decreased throughout the administration period. Gravid uterine weight was also lower than in controls.

Developmental toxicity

There were no treatment-related differences between the controls and the treated groups in the number of corpora lutea, implantations, number and litter percent of live fetuses, early and late resorptions or dead fetuses, nor in the percentage of pre- and post-implantation losses. Fetal body weights were decreased (by 15%) at the top dose. Some changes were noted during the external, visceral and skeletal examinations of fetuses, but in the absence of a dose–response relationship and lacking statistical significance, they were considered incidental. Overall, there were no treatment-related effects on any external, visceral or skeletal parameters.

In conclusion, minimal evidence of developmental toxicity (reduced fetal weight) was noted in this guideline rabbit developmental toxicity study, and only at the top dose of 30 mg/kg bw per day, at which level significant maternal toxicity occurred (effects on body weight, body weight gain, food consumption, gravid uterine weight and abortions). No effects occurred at 10 mg/kg bw per day. Therefore, the NOAEL for embryo/fetal and for maternal toxicity were both 10 mg/kg bw per day based on various effects (reduction of maternal body weight gain and food consumption and reduction in fetal body weight, respectively) based on a LOAEL of 30 mg/kg bw per day. It is noted that the effects on fetal weights were most likely a secondary, unspecific consequence of the maternal toxicity of the substance (Fujii, 2017b).

Table 46 below summarizes the available reproductive toxicity studies.

Table 46. Summary of reproductive and developmental toxicity studies with benzpyrimoxan

Species Strain No. animals	Duration Route Purity	Dose levels	NOAEL [dietary level]	LOAEL [dietary level]	Effects at LOAEL	Reference
Two-generation study						
Rat Crl:CD(SD) 24/sex/group	2 generations Dietary 93.7%	0, 60, 300, and 2000 ppm Equal to: 0, 2.5, 12 and 85 mg/kg bw/day for M; 0, 4.7, 24 and 156 mg/kg bw/day for F	*Parental toxicity* 12 mg/kg bw per day [300 ppm]	*Parental toxicity* 85 kg bw per day [2000 ppm]	*Parental toxicity* Reduced body weight, body weight gain, food consumption; Toxicity of liver and thyroid	Hojo, 2018
			Reproductive toxicity 12 mg/kg bw per day [300 ppm]	*Reproductive toxicity* 85 kg bw per day [2000 ppm]	*Reproductive toxicity* Lowered gestation index in F1; Increased post-implanation loss (F1/F2 pups).	
			Offspring 12 mg/kg bw per day [300 ppm]	*Offspring* 85 kg bw per day [2000 ppm]	*Offspring* Low viability index at days 0, 4 and 14; Reduced body weight;	
Developmental toxicity studies						
Rat Crl:CD(SD) 24 F/group	GDs 6–19 Gavage 93.7%	0, 10, 50 and 250 mg/kg bw/day	*Maternal toxicity* 50 mg/kg bw per day	*Maternal toxicity* 250 mg/kg bw per day	*Maternal toxicity* Reduced body weight, body weight gain, food consumption; Increased placental weight	Fujii, 2017a
			Developmental toxicity 250 mg/kg bw per day	*Developmental toxicity* >250 mg/kg bw per day	*Developmental toxicity* No effects	
Rabbit SPF Kbl:NZW 25 F/group	GDs 6–27 Gavage 93.7%	0, 3, 10 and 30 mg/kg bw/day	*Maternal toxicity* 10 mg/kg bw per day	*Maternal toxicity* 30 mg/kg bw per day	*Maternal toxicity* Reduced body weight, body weight gain, food consumption, gravid uterine weight; Two abortions	Fujii, 2017b
			Developmental toxicity 10 mg/kg bw per day	*Developmental toxicity* 30 mg/kg bw per day	*Developmental toxicity* Lowered fetal body weight	

M: Male; F: Female

2.6 Special studies

(a) Neurotoxicity

The acute neurotoxicity of benzpyrimoxan was investigated in a preliminary study and a main study.

In a preliminary but guideline (OECD 424) acute neurotoxicity study, benzpyrimoxan (purity 93.7%) in an aqueous solution of 0.5% sodium CMC and 0.2% Tween 80 was administered to three male and three female Crl:CD(SD) rats per group as a single gavage dose of 0, 500, 1000 or 2000 mg/kg bw.

Following administration, clinical condition, detailed clinical observations, functional observations, and body weight measurements were performed at intervals for seven days, and then animals were necropsied and all organs and tissues subject to a macroscopic examination. The kidneys (a target organ for toxicity of benzpyrimoxan in rats) were also analysed microscopically.

No changes were noted in condition or clinical observations at any dose, however the motor activity measurements performed one hour after administration were depressed from 10 to 20 minutes after dosing in males at all doses, and the total motor activity measurements for 60 minutes were also low in males at the mid dose and above. Since depressed motor activity was still present in top-dose males at three and seven days after administration, it could not be excluded that the observed change was treatment-related. The histopathological examination revealed renal findings including basophilic change and dilatation of the renal tubule in both males and females from the mid dose upwards.

Based on these findings, the sponsor concluded that 2000 mg/kg bw was an appropriate top dose for the main acute neurotoxicity study (Namiki, 2016).

In the subsequent main acute neurotoxicity study (OECD 424), benzpyrimoxan (purity 93.7%) in an aqueous solution of 0.5% sodium CMC and 0.2% Tween 80 was administered to 10 male and 10 female Crl:CD(SD) rats per group as a single gavage dose of 0, 500, 1000 or 2000 mg/kg bw. clinical condition, detailed clinical observations, functional observations, and body weight measurements were performed during the observation period at intervals up to day 14, then the animals were necropsied. All organs and tissues were subject to a macroscopic examination. However, tissue specimens of the nervous system were prepared from all animals in the control and high-dose groups, and examined histopathologically.

No treatment-related effects were noted at any dose in clinical condition, detailed clinical observations or functional observations, including sensory and motor reactivity, motor activity and grip strength. There were no macroscopic or microscopic abnormalities at any dose. Body weight was lower (statistically significant) on day 1 after administration in males in the mid- and high-dose groups (by 1% and 2% respectively) and in top-dose females (by 6%). Given the transient nature of these effects and their modest size, the body weight decreases were not considered adverse.

In conclusion, benzpyrimoxan is not acutely neurotoxic in rats and a NOAEL of 2000 mg/kg bw was identified from this study for acute neurotoxicity. The NOAEL for general toxicity was also 2000 mg/kg bw, the highest dose tested (Namiki, 2017).

The table below summarizes the available acute neurotoxicity studies.

***Table 47. Summary of the acute neurotoxicity studies performed with* benzpyrimoxan**

Species/strain Number of animals	Route Purity	Dose levels (mg/kg bw)	NOAEL (mg/kg bw)	LOAEL (mg/kg bw)	Effects at LOAEL	Reference
Rats Crl:CD(SD) 10/sex/group	Gavage 93.7%	0, 500, 1000 and 2000	*General toxicity* 2000	*General toxicity* > 2000	*General toxicity* No adverse effects	Namiki, 2017
	Main study		*Neurotoxicity* 2000	*Neurotoxicity* > 2000	*Neurotoxicity* No effects	

(b) Immunotoxicity

No specific studies were available covering immunotoxicity. In the repeat-dose toxicity studies detailed above, haematological effects with associated histopathology of spleen, thymus and bone marrow were observed across all three species tested. However, these effects were indicative of anaemia rather than a potential effect of benzpyrimoxan on the immune system. In these studies no effects were seen on organs of the immune system, such as the thymus. Overall, it was concluded that benzpyrimoxan is not immunotoxic.

(c) Mechanistic studies

Analysis of urine crystal components in rats

In the short-term and long-term toxicity studies in rats and mice with benzpyrimoxan, toxic effects were observed primarily in the urinary system (kidneys and urinary bladder) as the consequence of the formation of crystals which were detected in the kidneys, urinary bladder and urine.

To elucidate the structural composition of the crystals, groups of Wistar Hannover rats (five males per group) were administered by gavage [*phenyl-U*-^{14}C]benzpyrimoxan or [*pyrimidinyl-4(6)*-^{14}C]benzpyrimoxan in 0.5% CMC solution containing 0.1% Tween 80. The rats were dosed daily for three days at 300 mg/kg bw per day, a dose at which crystallization and nephrotoxicity were observed in the 28-day rat study. Urine samples were collected for 72 hours after the first dosing (that is, up to 24 hours after the last dose). Crystals were separated from the urine, radioactivity in the crystals was measured and the crystals were analysed for metabolites. In addition, urine samples obtained after each dosing up to 24 hours after the last dosing and kidney samples were collected and analysed for metabolites.

The radioactive component of the urine crystals amounted to 61–68% of the urine crystals' weight. The main component of the urine crystals was an unidentified substance which accounted for 95–97% the TRR. This component was also detected in the kidney (22–26% TRR) and urine (1–2% AR). Mass spectrometric analysis revealed that the unidentified component in urine crystals was a complex of a dimer of metabolite NNI-1501-acid-2-OH (DH-05 or M5) with Mg^{2+} ions. The same complex was also identified in the kidney and urine samples. Based on these findings it was concluded that at toxic dose levels, metabolite DH-05 (M5) during the process of urinary excretion is concentrated and crystallizes in the kidney, eventually leading to nephrotoxicity (Yasunaga, 2018d).

Species differences in the toxic effects of benzpyrimoxan on the kidney

The formation of crystals and obstructive nephropathy were observed with benzpyrimoxan in rats and mice, but not in dogs. It is therefore important to understand the basis of these species differences and whether humans are more similar to rodents or dogs in their toxic response to benzpyrimoxan.

In the in vitro interspecies comparative metabolism study (Yasunaga, 2019; see biochemical section above), metabolite DH-05 (or M5), the main component of the crystals, was formed in all species including humans. Therefore, the observed species differences in nephrotoxicity between rodents and dogs cannot be due to differences in the formation of metabolite DH-05. It has been proposed by the sponsor that the crystals are formed in rodents but not in dogs because rodents concentrate their urine to a greater degree than do dogs. It has further been proposed that humans have similar urine composition and concentration to that seen in dogs (Cohen, 2018), different from rats and mice, suggesting that the findings in the urinary system seen in rats and mice with benzpyrimoxan would not be relevant to humans. Unlike some compounds producing crystals in the urinary tract, benzpyrimoxan was not carcinogenic to rats or mice.

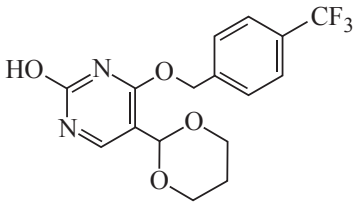
The proposed mode of action and lack of human relevance of these findings appear plausible. In addition, there will be a clear threshold for the formation of urinary tract crystals. Regardless of this lack of human relevance, since the urinary system findings were not the most sensitive effects (on their own) of benzpyrimoxan in rats or mice, the NOAELs and LOAELs identified from the rat and mouse studies are still relevant to the establishment of the toxicological reference values and the human risk assessment.

(d) Studies on metabolites

Test data have been submitted by the sponsor on metabolite DH-04 (or M4), namely an Ames test, an in vitro micronucleus test, an acute oral rat toxicity study and a 90-day rat study. the sponsor also provided an Ames test on metabolite DH-102 (or M11). Genotoxicity quantitative structure–activity relationship (QSAR) analyses were also submitted for metabolites DH-03 (or M3), DH-06 (or M6), DH-07 (or M7), DH-102 (or M11) and DH-04 (or M4), and these were compared to those obtained for the parent substance.

Studies on metabolite DH-04 (or M4)

Figure 7. Chemical structure of metabolite DH-04 (or M4)



Ames test

An Ames test (OECD 471) was conducted with metabolite DH-04 (or M4; purity 99.7%) in *Salmonella typhimurium* strain TA100, TA1535, TA98 and TA1537, and *Escherichia coli* strain WP2 *uvrA* with and without metabolic activation (S9 mix) by the pre-incubation method. The vehicle for the test article was DMSO. Triplicate plates were used in the range-finding test and in the two main tests.

The range-finding test was conducted with concentrations between 1.22 and 5000 µg/plate. No growth inhibition was observed in any strain with or without metabolic activation, but precipitation was evident at 313 µg/plate and above. From the result of the range-finding test the main tests were conducted at concentrations ranging from 3.86 to 313 µg/plate for all strains with or without S9.

In the range-finding test, no clear increase was found in the number of revertant colonies as compared with that in the negative control for any bacterial strains, either with or without S9 mix. In the main two tests no cytotoxicity (growth inhibition) was observed in any strains with or without metabolic activation, but precipitation was evident at the top concentration in all strains with and without S9 mix. Again, no clear increase was found in the number of revertant colonies in any bacterial strains as compared with that in the negative control, either with or without S9 mix. The positive control substances gave the expected responses.

Overall, metabolite DH-4 (or M4) was negative in a GLP and guideline Ames study up to the limit of solubility of the substance (Inagaki, 2016).

In vitro micronucleus test

Metabolite DH-04 (or M4) (purity 99.85%) dissolved in DMSO was tested in an in vitro micronucleus assay (OECD 487) using duplicate human lymphocyte cultures prepared from the pooled blood of two male donors in the absence and presence of metabolic activation (S9). A cytotoxicity range-finding experiment was conducted at concentrations ranging from 0.9 to 250 µg/mL with an exposure of three hours with and without S9 mix and additionally with an exposure of 24 hours without S9 mix. Due to the absence of visible precipitation at the end of the treatment period at 250 µg/mL with and without S9 mix, the main experiment was performed at concentrations ranging from 10 to 500 µg/mL with and without metabolic activation. Cytotoxicity was measured in 500 cells per culture by calculating the relative replication index (RI). Micronuclei were analysed at four concentrations (60, 100, 150 and 300 µg/mL following three-hour exposure with and without S9 mix; and 40, 80, 150 and 250 µg/mL following 24-hour exposure without S9 mix, the highest value being the lowest concentration that had resulted in precipitation.

Appropriate negative (vehicle) and positive control cultures were included in the test system under each treatment condition. Binucleate cells, 2000 per concentration, were analysed for micronuclei. All acceptance criteria were considered met and the study was considered valid. All positive control compounds induced statistically significant increases in the proportion of cells with micronuclei.

Treatment of cells with DH-04 (or M4) for three hours in the absence and presence of S9 mix and for 24 hours in the absence of S9 mix resulted in frequencies of micronucleated cells that were similar to and not significantly higher (at the $p \leq 0.05$ level) than those observed in the concurrent vehicle controls, at all concentrations analysed under all treatment conditions. The micronucleated cell frequencies were within the normal ranges at all concentrations analysed and there were no statistically significant linear trends under any treatment condition. No significant cytotoxicity was observed up to the highest concentration analysed for micronuclei, but precipitation was evident under all test conditions at the two highest concentrations. Details of the micronucleus tests on DH-04 are shown in Table 48.

Table 48. Summary of micronucleus test results with DH-04 or M4

Treatment	Concentration (µg/mL)	Cytotoxicity[a] (%)	Mean MN cell frequency (%)	Historical control range (%)[#]	Statistical significance
3 + 21 hours Without S9 mix	Vehicle[a]	-	0.15	0.10–0.70	-
	60.00	8	0.35		NS
	100.0	22	0.20		NS
	150.0	26	0.15		NS
	300.0	30	0.25		NS
	MMC[b], 0.30	32	2.60		$p \leq 0.001$
3 + 21 hours With S9 mix	Vehicle[a]	-	0.35	0.10–0.80	-
	60.00	6	0.45		NS
	100.0	31	0.55		NS
	150.0	29	0.70		NS
	300.0	31	0.50		NS
	CPA[b], 7.00	53	2.10		$p \leq 0.001$
24 + 24 hours Without S9 mix	Vehicle[a]	-	0.10	0.10–0.70	-
	40.00	4	0.30		NS
	80.00	31	0.20		NS
	150.0	29	0.15		NS
	250.0	36	0.20		NS
	VIN[b], 0.04	48	3.95		$p \leq 0.001$

[a] Based on replication index (RI); [b] Vehicle control was DMSO; [c] Positive control Source: Lloyd, 2020

MMC: Mitomycin; CPA: Cyclophosphamide; VIN: Vinblastine sufate

[#] 95th percentile of the observed range; MN: Micronucleated; NS: Not significant

In conclusion, DH-04 (or M4) did not induce micronuclei in cultured human peripheral blood lymphocytes in a GLP and guideline study when tested up to precipitating concentrations in the absence or presence of metabolic activation (Lloyd, 2020).

Genotoxicity QSAR analysis

Since a complete in vitro genotoxicity dataset had been submitted for DH-04 (or M4), the submitted QSAR analysis (using DEREK, OECD QSAR Toolbox, Vega and ToxTree) of this metabolite was not considered further.

Acute oral toxicity study of DH-04

The metabolite DH-04 (or M4) was evaluated for its acute oral toxicity potential in male Wistar rats. The "acute toxic class method" (OECD 423) was employed. The test substance (purity 99.7%) was suspended in an aqueous solution of 0.5% (w/v) sodium CMC containing 0.1% (v/v) Tween 80 and administered to two groups of three animals by oral gavage at a dose of 2000 mg/kg bw. Any mortality or clinical signs of toxicity in the subsequent 14 days were recorded.

No animals died or showed any abnormal clinical signs over the 14 days of observation after dosing. Body weight in all animals tested increased from the day of dosing (day 0) to day 14. The necropsy performed after 14 days of treatment revealed the presence of enlarged kidneys with coarse surface and discolouration. The kidneys showed treatment-related histopathological findings such as the presence of crystals, tubular basophilic change, inflammatory cell infiltrate, fibrosis, focal urothelial hyperplasia and dilatation of the renal tubules and collecting ducts.

Overall, an oral LD_{50} of greater than 2000 mg/kg bw was identified in the rat for DH-04 (or M4) (Tsukushi, 2016b).

Ninety-day dietary study of DH-04 in the rat

In a subchronic toxicity study (OECD 408), metabolite DH-04 (or M4) (purity 99.85%) was given in the diet for 13 weeks to groups of Wistar rats (10/sex per group) at 0, 100, 300, 1000 or 3000 ppm (equivalent to 0, 6.4, 19.6, 65.2 and 168 mg/kg bw per day for males, 0, 7.5, 22.8, 78.4 and 181 mg/kg bw per day for females) . Functional observation was carried out at 11 weeks of treatment. Ophthalmology was conducted prior to the initiation of treatment and at 13 weeks of treatment. Urinalysis was conducted at 13 weeks of treatment. At termination of treatment, all surviving animals were subjected to haematology, blood biochemistry, necropsy and organ weight measurement. Animals killed in extremis or found dead were also subjected to necropsy. Histopathological examination was performed on the liver, kidneys, urinary bladder, heart, bone marrow (sternum and femur), thymus, aorta, glandular stomach and adrenals in both sexes, and the testes and epididymides in males, and on all gross lesions from all animals. The other systemic organs and tissues from the animals terminated in extremis or found dead during the treatment period, and those from all animals terminated at the end of the study in the 0 ppm and 3000 ppm groups, were also subjected to a histopathology examination.

During the study period, six males and four females treated at 3000 ppm and one female treated at 1000 ppm were terminated in extremis or found dead within one week of treatment. Although there were no deaths in males treated at 1000 ppm, they showed severe treatment-related effects as did males and females treated at 3000 ppm and females treated at 1000 ppm. This showed that the dose of 1000 ppm was already in excess of the MTD. Decedent animals showed poor general condition such as decrease of spontaneous motor activity, bradypnea, tremor, and piloerection. Detailed results are shown below in Tables 49 and 50.

Table 49. Final mortality rates in a 90-day study of DH-04 toxicity in rats

Dose Level (ppm)	Males	Females
0	0/10	0/10
100	0/10	0/10
300	0/10	0/10
1000	0/10	1/10
3000	**6**/10**	**4*/10**

Significantly different from the control using Fisher's exact probability test: * $p < 0.05$; ** $p < 0.01$; Source: Katoh, 2018

Table 50. Clinical observations in a 90-day study of DH-04 toxicity in rats

		Dose level (ppm)									
		Males					Females				
Clinical sign		**0**	**100**	**300**	**1000**	**3000**	**0**	**100**	**300**	**1000**	**3000**
Appearance:	emaciation	0	0	0	0	**3**	0	0	0	0	**2**
Behaviour:	decreased spontaneous motor activity	0	0	0	0	**4***	0	0	0	1	**3**
Respiration:	bradypnea	0	0	0	0	**4***	0	0	0	1	**3**
Consciousness/nervous system:	tremor	0	0	0	0	0	0	0	0	1	**2**
Skin:	pale-coloured skin	0	0	0	0	**5***	0	0	0	0	**3**

		Dose level (ppm)									
		Males					Females				
Clinical sign		**0**	**100**	**300**	**1000**	**3000**	**0**	**100**	**300**	**1000**	**3000**
Fur:	Loss of fur	0	1	0	0	0	1	0	0	0	**4**
	Piloerection	0	0	0	2	**8****	0	0	0	1	**9****

Significantly different from the control using Fisher's exact probability test: * $p < 0.05$; ** $p < 0.01$; Source: Katoh, 2018

Histopathologically, various life-threatening cardiovascular and/or renal lesions were observed and these were considered to be the cause of the animals' morbidity. The lesions included inflammation and mineralization in the aorta, degeneration/necrosis of arteries in the heart, inflammation and mineralization of arteries in various organs and tissues, necrosis/inflammatory cell infiltration in cardiac muscle of the heart, renal papillary necrosis, renal tubular cell necrosis and mineralization, and obstructive nephropathy. In addition, necrosis of the bone marrow (sternum and femur), fibro-osseous proliferation and/or increased bone resorption (sternum and femur), and atrophic lesions in the spleen and pancreas were noted as secondary consequences of the renal and systemic dysfunction.

In the surviving animals of the 1000 and 3000 ppm groups, treatment-related effects were observed mainly on clinical signs, body weight and food consumption, the urinary and cardiovascular systems, and the liver. Decreased spontaneous motor activity, bradypnea, tremors, emaciation, piloerection, loss of fur and pale-coloured fur were observed mainly at 3000 ppm. However, piloerection in both sexes and decreased spontaneous motor activity, bradypnea and tremors were also noted in some animals at 1000 ppm. The functional observational battery (FOB) revealed a decrease in motor activity and a decrease in grip strength of forelimbs and hindlimbs in both sexes at the top dose. In addition, males of the 1000 ppm group showed a decreasing trend in the grip strength of their hindlimbs. Males and females of the 3000 and 1000 ppm groups displayed significantly lower body weights and food consumption throughout the treatment period, and consequently food efficiency was also lower compared to controls. In the 3000 ppm group, two out of the four surviving males and two out of the six surviving females showed narrowing of the retinal vessels in the ophthalmological examination at week 13. Detailed body weight and food consumption data is shown below in Tables 51 and 52 .

Table 51. Intergroup comparison of mean body weights in a 90-day study of DH-04 toxicity in rats (mean ± standard deviation shown)

	Dose level (ppm)									
	Males					Females				
Time point	**0**	**100**	**300**	**1000**	**3000**	**0**	**100**	**300**	**1000**	**3000**
Week 0	171 ± 6	171 ± 6	171 ± 6	171 ± 6	171 ± 7	138 ± 5	138 ± 5	138 ± 5	138 ± 5	138 ± 5
Week 1	221 ± 8	223 ± 9	223 ± 8	**184** ± 12**	**158** ± 8**	161 ± 7	161 ± 7	158 ± 9	154 ± 7	**122** ± 7**
Week 2	271 ± 10	270 ± 12	268 ± 10	**222** ± 16**	**160** ± 14**	180 ± 6	181 ± 10	177 ± 11	171 ± 9	**123** ± 9**
Week 3	302 ± 13	303 ± 17	301 ± 13	**241** ± 21**	**162** ± 18**	193 ± 9	195 ± 12	187 ± 10	184 ± 10	**129** ± 14**
Week 4	328 ± 15	327 ± 21	327 ± 17	**253** ± 22**	**174** ± 12**	206 ± 9	209 ± 11	201 ± 13	195 ± 13	**135** ± 17**
Week 5	351 ± 19	348 ± 25	346 ± 17	**260** ± 22**	**177** ± 14**	218 ± 8	218 ± 11	208 ± 13	**202* ± 13**	**138** ± 17**
Week 6	371 ± 22	365 ± 31	368 ± 21	**271** ± 22**	**185** ± 13**	226 ± 8	223 ± 10	214 ± 12	**207** ± 15**	**144** ± 16**
Week 7	385 ± 24	377 ± 34	384 ± 24	**280** ± 24**	**192** ± 13**	231 ± 8	228 ± 14	218 ± 12	**210** ± 14**	**150** ± 19**
Week 8	394 ± 23	390 ± 35	397 ± 25	**287** ± 24**	**194** ± 17**	237 ± 11	236 ± 13	225 ± 11	**214** ± 15**	**159** ± 20**

	Dose level (ppm)									
	Males					Females				
Time point	0	100	300	1000	3000	0	100	300	1000	3000
Week 9	404 ± 27	401 ± 36	406 ± 27	**296** ± 24**	**204** ± 16**	242 ± 10	239 ± 13	231 ± 12	**217** ± 18**	**160** ± 21**
Week 10	415 ± 28	413 ± 37	417 ± 29	**305** ± 24**	**206** ± 14**	245 ± 9	242 ± 13	233 ± 14	**219** ± 16**	**164** ± 22**
Week 11	425 ± 28	424 ± 39	426 ± 29	**308** ± 23**	**209** ± 18**	247 ± 10	244 ± 14	238 ± 11	**221** ± 14**	**165** ± 22**
Week 12	436 ± 30	432 ± 37	434 ± 29	**317** ± 23**	**212** ± 24**	250 ± 12	247 ± 14	241 ± 12	**222** ± 16**	**168** ± 25**
Week 13	441 ± 30	437 ± 35	441 ± 31	**320** ± 22**	**214** ± 27**	254 ± 10	248 ± 13	243 ± 13	**224** ± 18**	**163** ± 26**

* Significantly different from the control using Dunnett's test (or Dunnett-type test): $p < 0.05$; Source: Katoh, 2018

** Significantly different from the control using Dunnett's test (or Dunnett-type test): $p < 0.01$

Table 52. Intergroup comparison of food consumption in a 90-day study of DH-04 toxicity in rats (mean ± standard deviation shown)

	Dose level (ppm)									
	Males					Females				
Time point	0	100	300	1000	3000	0	100	300	1000	3000
Week 1	19.8 ± 1.6	20.7 ± 1.1	21.5 ± 1.4	**11.6** ± 1.5**	**6.9** ± 2.1**	15.9 ± 1.1	16.1 ± 1.1	15.4 ± 0.9	12.1 ± 2.6	**3.5** ± 0.4**
Week 2	23.0 ± 1.2	23.5 ± 0.7	23.3 ± 1.3	**18.3** ± 3.0**	**9.0** ± 1.5**	16.6 ± 1.6	16.4 ± 1.3	16.4 ± 1.5	17.1 ± 0.9	**8.3** ± 1.3**
Week 3	23.0 ± 0.9	23.8 ± 1.0	24.7 ± 2.3	**19.5** ± 2.2*	**9.3** ± 2.4**	16.8 ± 1.6	16.9 ± 1.5	16.0 ± 1.3	16.9 ± 2.2	**8.5** ± 2.5**
Week 4	23.0 ± 1.0	23.7 ± 1.3	23.8 ± 2.1	**18.5** ± 2.5**	**8.9** ± 1.9**	16.9 ± 0.8	17.2 ± 1.1	15.9 ± 1.7	16.5 ± 2.1	**8.6** ± 1.3**
Week 5	22.6 ± 0.8	22.8 ± 1.5	22.4 ± 1.3	**15.8** ± 3.3**	**9.8** ± 0.9**	16.6 ± 0.7	16.7 ± 1.3	15.8 ± 1.6	16.2 ± 1.6	**9.5** ± 1.3**
Week 6	22.9 ± 0.9	22.3 ± 2.1	23.5 ± 1.2	**18.2** ± 1.7**	**10.4** ± 1.2**	16.7 ± 0.5	16.7 ± 1.3	15.9 ± 1.4	16.0 ± 1.7	**8.9** ± 1.2**
Week 7	22.1 ± 0.7	21.2 ± 2.1	22.6 ± 1.5	**17.6** ± 1.7**	**11.2** ± 2.2**	16.7 ± 1.2	17.0 ± 1.1	16.1 ± 1.5	16.0 ± 1.5	**10.0** ± 0.8**
Week 8	21.8 ± 0.8	22.1 ± 1.7	23.1 ± 1.4	**17.7** ± 1.4**	**12.3** ± 1.7**	16.5 ± 0.7	16.5 ± 0.4	16.3 ± 1.5	16.3 ± 2.4	**9.1** ± 1.7*
Week 9	21.4 ± 0.7	21.9 ± 1.5	22.1 ± 1.2	**17.9** ± 2.0**	**12.5** ± 1.9**	16.2 ± 0.7	15.4 ± 1.4	15.7 ± 1.6	15.8 ± 1.6	**10.8** ± 1.4**
Week 10	21.0 ± 0.7	22.0 ± 2.0	22.5 ± 1.8	**18.5** ± 2.0	**11.3** ± 2.2**	16.2 ± 0.9	15.6 ± 1.3	15.5 ± 0.9	15.5 ± 1.5	**9.4** ± 1.7**
Week 11	21.3 ± 0.8	21.6 ± 1.4	22.1 ± 2.0	**18.0** ± 1.1*	**11.8** ± 2.3*	15.7 ± 1.6	16.3 ± 1.7	16.2 ± 1.2	16.0 ± 1.6	**9.8** ± 1.6**
Week 12	21.6 ± 1.0	21.7 ± 1.4	22.5 ± 1.4	**18.6** ± 1.4**	**12.6** ± 1.1**	15.9 ± 1.4	15.7 ± 0.5	16.1 ± 1.2	14.8 ± 2.3	**9.6** ± 1.0**
Week 13	21.4 ± 1.0	20.8 ± 0.8	22.5 ± 1.3	18.2 ± 1.2	**11.2** ± 6.0*	15.8 ± 1.2	15.0 ± 1.0	15.9 ± 1.7	15.9 ± 1.0	**10.0** ± 0.8**

* Significantly different from the control using Dunnett's test (or Dunnett-type test): $p < 0.05$; Source: Katoh, 2018

** Significantly different from the control using Dunnett's test (or Dunnett-type test): $p < 0.01$

Haematological changes indicative of anaemia and changes in clinical chemistry parameters indicative of liver, kidney and generalized systemic toxicity (for example, increases in ALP, GTP, creatinine, BUN, cholesterol, calcium, Phosphorus, potassium and decreases in total protein, albumin, glucose and bilirubin) were seen in both sexes at 1000 ppm and above. Urinalysis parameters (increases in protein, urine volume, decreases in specific gravity, ketones, pH and urobilinogen) were also affected in both sexes at 1000 ppm and above.

Table 53. Selected haematological parameters in a 90-day study of DH-04 toxicity in rats (mean ± standard deviation shown)

	Dose level (ppm)									
	Males					Females				
Parameter	**0**	**100**	**300**	**1000**	**3000**	**0**	**100**	**300**	**1000**	**3000**
Haematocrit (%)	44.7 ± 1.0	44.0 ± 0.8	44.4 ± 0.7	**38.6** ± 1.7**	**23.5** ± 0.9**	42.4 ± 1.7	41.9 ± 0.7	42.1 ± 1.5	40.7 ± 2.0	**27.7** ± 3.4**
Haemoglobin (g/dL)	15.2 ± 0.3	15.2 ± 0.2	15.3 ± 0.3	**13.1** ± 0.7**	**7.4** ± 0.9**	14.4 ± 0.6	14.3 ± 0.3	14.3 ± 0.6	13.9 ± 0.7	**8.5** ± 1.1**
Red blood cells (10^6/µL)	8.83 ± 0.26	8.85 ± 0.31	8.97 ± 0.23	**7.58** ± 0.55**	**4.10** ± 0.24**	7.78 ± 0.30	7.87 ± 0.27	7.83 ± 0.38	7.91 ± 0.40	**4.80** ± 0.66**
MCV (fL)	50.7 ± 1.5	49.7 ± 1.5	49.6 ± 1.3	51.0 ± 1.8	**57.2** ± 3.7**	54.5 ± 1.5	53.2 ± 1.5	53.8 ± 2.0	**51.5** ± 1.6**	**57.9** ± 2.9**
MCH (pg)	17.3 ± 0.4	17.2 ± 0.5	17.1 ± 0.5	17.3 ± 0.4	17.9 ± 1.3	18.5 ± 0.5	18.2 ± 0.4	18.3 ± 0.5	**17.6** ± 0.4**	**17.8* ± 0.7**
MCHC (g/dL)	34.1 ± 0.4	34.6 ± 0.4	34.4 ± 0.4	33.9 ± 0.5	**31.3** ± 0.6**	34.0 ± 0.2	34.1 ± 0.4	34.0 ± 0.6	34.2 ± 0.4	**30.7* ± 0.5**
Platelets (10^3/µL)	1021 ± 85	966 ± 63	971 ± 38	1076 ± 108	**753** ± 78**	954 ± 66	992 ± 70	934 ± 79	**1142** ± 77**	951 ± 99
Reticulocytes (10^9/L)	302.9 ± 32.9	298.9 ± 30.5	312.9 ± 35.7	269.0 ± 26.5	**424.1** ± 47.4**	286.7 ± 34.8	281.5 ± 49.7	301.9 ± 45.3	318.1 ± 29.2	**381.6** ± 43.8**
PT (seconds)	13.6 ± 0.9	13.1 ± 1.6	14.2 ± 1.2	**9.9** ± 0.4**	**9.2** ± 0.3**	9.8 ± 0.2	10.2 ± 1.3	9.9 ± 0.4	9.7 ± 0.8	9.8 ± 1.6
APTT (seconds)	21.1 ± 1.0	20.8 ± 1.0	21.3 ± 0.8	**16.3** ± 1.1**	**12.7** ± 0.7**	15.8 ± 1.5	16.1 ± 1.1	16.3 ± 1.8	15.6 ± 0.8	**12.0** ± 1.0**
White blood cells (10^3/µL)	4.53 ± 1.30	4.38 ± 1.10	4.64 ± 0.54	5.47 ± 1.47	3.46 ± 1.01	2.19 ± 0.66	2.50 ± 0.90	2.36 ± 0.54	**4.42** ± 1.07**	**3.61* ± 1.14**
Lymphocytes (10^3/µL)	3.71 ± 1.17	3.53 ± 1.07	3.67 ± 0.51	4.09 ± 1.34	2.94 ± 0.78	1.78 ± 0.58	2.02 ± 0.69	1.82 ± 0.47	**3.26** ± 0.96**	**2.96* ± 1.11**
Neutrophils (10^3/µL)	0.61 ± 0.11	0.65 ± 0.09	0.76 ± 0.45	**1.19** ± 0.30**	0.46 ± 0.26	0.31 ± 0.11	0.37 ± 0.19	0.44 ± 0.23	**0.99** ± 0.33**	**0.60* ± 0.10**
Monocytes (10^3/µL)	0.12 ± 0.06	0.12 ± 0.06	0.14 ± 0.05	0.09 ± 0.04	**0.04* ± 0.03**	0.05 ± 0.02	0.06 ± 0.04	0.06 ± 0.01	0.09 ± 0.03	0.03 ± 0.01
Eosinophils (10^3/µL)	0.09 ± 0.04	0.07 ± 0.03	0.08 ± 0.03	0.10 ± 0.06	**0.02* ± 0.01**	0.05 ± 0.02	0.04 ± 0.01	0.05 ± 0.02	**0.08* ± 0.02**	0.02 ± 0.01
NCBM (10^5/µL)	24.6 ± 4.6	24.8 ± 2.0	24.9 ± 2.8	24.0 ± 2.1	**13.5** ± 1.6**	23.2 ± 3.2	22.2 ± 1.7	23.2 ± 3.6	25.6 ± 2.7	**17.4** ± 5.3**

MCV: Mean corpuscular volume; MCH: Mean corpuscular haemoglobin Source: Katoh, 2018

MCHC: Mean corpuscular haemoglobin concentration; PT: Prothrombin time;

APTT: Activated partial thromboplastin time;

* Significantly different from the control using Dunnett's test (or Dunnett-type test): $p < 0.05$;

** Significantly different from the control using Dunnett's test (or Dunnett-type test): $p < 0.01$

Table 54. Selected clinical chemistry parameters in a 90-day study of DH-04 toxicity in rats (mean ± standard deviation shown)

	Dose level (ppm)									
	Males					Females				
Parameter	**0**	**100**	**300**	**1000**	**3000**	**0**	**100**	**300**	**1000**	**3000**
Alkaline phosphatase (U/L)	210 ± 30	208 ± 20	216 ± 28	222 ± 31	**271** ± 34**	74 ± 11	73 ± 13	102 ± 13	95 ± 23	**206** ± 48**
γ-gutamyl transpeptidase (U/L)	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	**1.5** ± 0.4**	**2.0** ± 0.3**	0.8 ± 0.4	0.8 ± 0.2	0.8 ± 0.3	1.0 ± 0.1	**1.7** ± 0.7**
Creatinine (mg/dL)	0.29 ± 0.03	0.29 ± 0.01	0.29 ± 0.03	**1.20** ± 0.25**	**2.75** ± 0.37**	0.34 ± 0.05	0.37 ± 0.06	0.37 ± 0.06	**0.65** ± 0.9**	**1.87** ± 0.32**
Blood urea nitrogen (mg/dL)	18.2 ± 1.9	19.0 ± 2.0	20.6 ± 1.7	**80.0** ± 15.1**	**185.0** ± 26.5**	19.4 ± 3.0	21.0 ± 4.0	21.0 ± 4.2	**45.3** ± 5.8**	**142.6** ± 49.9**
Total protein (g/dL)	6.12 ± 0.16	6.08 ± 0.24	6.15 ± 0.20	**5.84* ± 0.19**	**5.28** ± 0.31**	6.23 ± 0.21	6.25 ± 0.37	6.20 ± 0.29	**6.32 ± 0.25**	**5.38** ± 0.51**
Albumin (A) (g/dL)	4.08 ± 0.16	4.12 ± 0.12	4.15 ± 0.20	3.96 ± 0.20	**3.61** ± 0.19**	4.78 ± 0.22	4.84 ± 0.36	4.62 ± 0.40	**4.40* ± 0.18**	**3.63** ± 0.29**
Globulin (G) (g/dL)	2.03 ± 0.15	1.96 ± 0.20	2.00 ± 0.08	1.89 ± 0.21	**1.67** ± 0.14**	1.46 ± 0.13	1.42 ± 0.14	1.58 ± 0.16	**1.93** ± 0.20**	**1.76* ± 0.34**
A:G ratio	2.02 ± 0.20	2.13 ± 0.24	2.08 ± 0.15	2.13 ± 0.30	2.17 ± 0.10	3.31 ± 0.41	3.44 ± 0.40	2.97 ± 0.46	**2.31** ± 0.27**	**2.11** ± 0.36**
Glucose (mg/dL)	172 ± 23	173 ± 22	176 ± 15	**144** ± 10**	**128** ± 11**	135 ± 10	136 ± 17	132 ± 13	130 ± 15	**113* ± 10**
Total cholesterol (mg/dL)	54 ± 6	55 ± 10	55 ± 7	**107** ± 22**	**129** ± 6**	55 ± 11	51 ± 11	53 ± 7	65 ± 6	**119** ± 32**
Triglycerides (mg/dL)	41 ± 13	54 ± 24	55 ± 23	44 ± 15	39 ± 20	21 ± 9	20 ± 4	27 ± 14	33 ± 14	**51** ± 30**
Total bilirubin (mg/dL)	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	**0.04** ± 0.01**	**0.03** ± 0.01**	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	**0.05** ± 0.01**	**0.03** ± 0.01**
Calcium (mg/dL)	9.8 ± 0.2	9.8 ± 0.3	9.8 ± 0.3	**10.8** ± 0.3**	**11.9** ± 0.3**	9.8 ± 0.3	9.8 ± 0.4	9.6 ± 0.3	10.2 ± 0.2	**11.5** ± 0.8**
Phosphorus (mg/dL)	4.2 ± 0.8	4.3 ± 0.6	4.3 ± 0.7	**6.1** ± 1.0**	**10.2** ± 1.3**	4.0 ± 0.9	3.8 ± 0.7	4.0 ± 1.0	4.1 ± 1.0	**8.3** ± 2.2**
Potassium (mEquiv./L)	3.66 ± 0.05	3.61 ± 0.22	3.65 ± 0.12	3.86 ± 0.16	**4.15* ± 0.34**	3.31 ± 0.19	3.28 ± 0.26	3.42 ± 0.11	3.38 ± 0.15	**3.94** ± 0.37**
Chloride (mEquiv./L)	108.2 ± 1.2	107.9 ± 1.6	108.1 ± 1.0	107.2 ± 2.1	**105.8* ± 2.0**	109.4 ± 1.4	109.0 ± 1.8	109.3 ± 1.2	109.6 ± 1.2	108.7 ± 4.0

* Significantly different from the control using Dunnett's test (or Dunnett-type test): $p < 0.05$; Source: Katoh, 2018

** Significantly different from the control using Dunnett's test (or Dunnett-type test): $p < 0.01$

Table 55. Selected urinalysis parameters in a 90-day study of DH-04 toxicity in rats

Parameter		Dose level (ppm)									
		Males					Females				
		0	**100**	**300**	**1000**	**3000**	**0**	**100**	**300**	**1000**	**3000**
Specific gravity [a] (mean ± SD)		1.039 ± 0.014	1.035 ± 0.016	1.045 ± 0.011	**1.013** ± 0.001**	**1.012** ± 0.001**	1.040 ± 0.024	1.039 ± 0.017	1.034 ± 0.015	**1.017* ± 0.003**	**1.012** ± 0.002**
Ketones [b] (Number of animals out of 10 examined)	−	1	2	–	10**	5**	6	7	8	9	6
	±	3	4	2	–	–	4	3	2	–	–
	+	6	4	8	–	–	–	–	–	–	–
	++	–	–	–	–	–	–	–	–	–	–
	+++	–	–	–	–	–	–	–	–	–	–
	++++	–	–	–	–	–	–	–	–	–	–
pH [b] (Number of animals out of 10 examined)	5.5	–	–	–	–	–	–	–	–	–	–
	6.0	–	–	–	2	–	1	–	3	3	–
	6.5	–	–	–	4	–	1	2	1	1	2
	7.0	–	–	1	2	2	1	1	1	2	1
	7.5	–	–	–	**2****	**3****	2	–	2	3	3
	8.0	2	3	4	–	–	1	4	–	–	–
	8.5	8	7	5	–	–	4	3	3	–	–
Protein [b] (Number of animals out of 10 examined)	−	–	–	–	–	–	4	3	5	5	–
	±	2	3	–	–	–	2	2	3	2	–
	+	7	6	8	8	–	3	4	2	2	–
	++	1	1	2	1	**5****	1	1	–	–	5
	+++	–	–	–	1	–	–	–	–	–	**1****
	++++	–	–	–	–	–	–	–	–	–	–
Urobilinogen [b] (Number of animals out of 10 examined)	Ehrlich unit/dL										
	0.1	4	7	1	**10***	5	4	4	6	**9***	**6***
	1.0	6	3	9	–	–	5	6	4	–	–
	2.0	–	–	–	–	–	1	–	–	–	–
	4.0	–	–	–	–	–	–	–	–	–	–
	8.0	–	–	–	–	–	–	–	–	–	–
Appearance [b] (Number of animals out of 10 examined)	Colour-less	–	–	–	–	–	–	–	–	–	–
	Pale yellow	–	–	–	**10****	**5****	–	–	–	4	6**
	Yellow	10	10	10	–	–	10	10	10	5	–
	Yellow brown	–	–	–	–	–	–	–	–	–	–
	Brown	–	–	–	–	–	–	–	–	–	–
Urine volume [a] [mL/day] (mean ± SD)		15.7 ± 3.7	14.9 ± 5.2	19.3 ± 7.2	**49.9 ± 8.7****	36.5 ± 19.6	14.6 ± 4.1	12.9 ± 3.4	16.9 ± 4.9	**29.2 ± 8.5****	**28.8 ± 8.8***

SD: Standard deviation; Source: Katoh, 2018

* Significantly different from the control at $p < 0.05$: [a] using Dunnett's test; [b] a Dunnett-type test;

** Significantly different from the control at $p < 0.01$: [a] using Dunnett's test; [b] a Dunnett-type test

At necropsy, macroscopic lesions of the kidney similar to those seen with the parent substance were seen in both sexes at 1000 ppm and above, and some 300 ppm males also revealed the same findings (depressed area). In addition, macroscopic lesions were observed in the heart at 1000 ppm and above.

Table 56. Selected gross necropsy parameters in a 90-day study of DH-04 toxicity in rats; (number of rats out of 10 affected; unscheduled and terminal sacrifice)

		Dose level (ppm)									
		Males					Females				
Parameter		**0**	**100**	**300**	**1000**	**3000**	**0**	**100**	**300**	**1000**	**3000**
Kidneys	Atrophy	0	0	0	0	1	0	0	0	0	0
	Coarse surface	0	0	0	**10****	**6****	0	0	0	**9****	**6****
	Depressed area	0	0	1	0	0	0	0	0	0	0
	Enlargement	0	0	0	0	**4***	0	0	0	0	0
	Pale in colour	0	0	0	**10****	**8****	0	0	0	0	**8****
	Pelvic dilatation	1	1	1	2	0	0	2	1	0	1
	Spot(s)	0	0	0	0	1	0	0	0	0	0

* Significantly different from the control using Fisher's exact probability test: $p < 0.05$; Source: Katoh, 2018

** Significantly different from the control using Fisher's exact probability test: $p < 0.01$

Significant changes in organ weights were noted for the liver, kidney, adrenals and heart of rats in the 1000 ppm group. Decreases in the weight of brain, thymus, spleen, testes, epididymides, ovaries and uterus observed at 1000 ppm and above were considered a secondary consequence of the low terminal body weights recorded at these dose levels.

Table 57. Selected organ weights in a 90-day study of DH-04 toxicity in rats (mean ± standard deviation shown)

Parameter		**Dose level (ppm)**									
		Males					**Females**				
		0	**100**	**300**	**1000**	**3000**	**0**	**100**	**300**	**1000**	**3000**
Body weight (g)		422 ± 29	421 ± 33	424 ± 31	**306**** **± 23**	**203**** **± 23**	240 ± 9	237 ± 14	232 ± 11	**213**** **± 15**	**158**** **± 23**
Brain (mg)	A	2009 ± 55	1976 ± 67	1963 ± 63	**1871**** **± 63**	**1701**** **± 50**	1832 ± 47	1830 ± 62	1807 ± 40	1798 ± 57	**1678**** **± 84**
	R	0.48 ± 0.03	0.47 ± 0.04	0.47 ± 0.02	**0.62**** **± 0.04**	**0.85**** **± 0.10**	0.77 ± 0.04	0.78 ± 0.05	0.78 ± 0.04	**0.85*** **± 0.06**	**1.08*** **± 0.13**
Heart (mg)	A	1089 ± 127	1055 ± 64	1088 ± 87	985 ± 65	1086 ± 26	714 ± 68	711 ± 74	722 ± 103	734 ± 49	769 ± 80
	R	0.26 ± 0.02	0.25 ± 0.02	0.26 ± 0.01	**0.32**** **± 0.03**	**0.55**** **± 0.07**	0.30 ± 0.03	0.30 ± 0.03	0.31 ± 0.03	**0.35**** **± 0.03**	**0.49**** **± 0.06**
Thymus (mg)	A	385 ± 93	359 ± 95	352 ± 64	**229**** **± 30**	**110**** **± 38**	315 ± 47	317 ± 53	293 ± 60	293 ± 76	**165**** **± 86**
	R	0.091 ± 0.022	0.085 ± 0.022	0.083 ± 0.011	0.075 ± 0.013	**0.053**** **± 0.013**	0.131 ± 0.017	0.134 ± 0.022	0.126 ± 0.023	0.137 ± 0.028	0.100 ± 0.044
Liver (g)	A	10.41 ± 1.13	10.40 ± 1.05	10.57 ± 0.81	**7.32**** **± 0.48**	**6.70**** **± 0.81**	5.85 ± 0.62	5.72 ± 0.46	5.72 ± 0.37	5.71 ± 0.55	5.46 ± 0.52
	R	2.46 ± 0.15	2.47 ± 0.11	2.50 ± 0.13	**2.40 ± 0.12**	**3.31**** **± 0.12**	2.43 ± 0.22	2.42 ± 0.16	2.47 ± 0.11	2.69 ± 0.22	**3.50**** **± 0.47**
Kidneys (mg)	A	2510 ± 192	2527 ± 155	2605 ± 285	2630 ± 283	**1516**** **± 311**	1641 ± 149	1638 ± 164	1631 ± 232	**1914*** **± 303**	1684 ± 235
	R	0.60 ± 0.04	0.60 ± 0.03	0.62 ± 0.05	**0.86**** **± 0.07**	**0.74*** **± 0.09**	0.68 ± 0.05	0.69 ± 0.05	0.70 ± 0.08	**0.90**** **± 0.10**	**1.08**** **± 0.17**
Spleen (mg)	A	648 ± 55	605 ± 68	620 ± 61	652 ± 91	**427**** **± 63**	436 ± 57	416 ± 44	444 ± 46	**514*** **± 57**	387 ± 75
	R	0.15 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	**0.21**** **± 0.02**	**0.21**** **± 0.01**	0.18 ± 0.02	0.18 ± 0.02	0.19 ± 0.03	**0.24**** **± 0.04**	**0.25**** **± 0.03**

Adrenals	A	63.8 ± 4.0	63.2 ± 10.7	62.8 ± 8.8	68.7 ± 5.2	57.1 ± 7.2	74.2 ± 11.6	73.2 ± 8.5	71.9 ± 10.0	70.2 ± 12.4	**51.0** ± 5.2**
	R	0.015 ± 0.001	0.015 ± 0.002	0.015 ± 0.002	**0.023** ± 0.002**	**0.028** ± 0.004**	0.031 ± 0.005	0.031 ± 0.004	0.031 ± 0.004	0.033 ± 0.005	0.033 ± 0.006
Testes/ovaries (mg)	A	3504 ± 143	3401 ± 303	3246 ± 756	3232 ± 299	**1705** ± 707**	104.2 ± 14.6	113.4 ± 18.8	102.2 ± 14.1	98.1 ± 20.1	**46.7** ± 18.4**
	R	0.83 ± 0.06	0.81 ± 0.11	0.77 ± 0.19	**1.06** ± 0.08**	**0.85 ± 0.34**	0.043 ± 0.005	0.048 ± 0.007	0.044 ± 0.006	0.046 ± 0.008	**0.029** ± 0.009**
Epididymides (mg)	A	1223 ± 74	1154 ± 179	1099 ± 220	**1036** ± 96**	**461** ± 157**	-	-	-	-	-
	R	0.29 ± 0.02	0.28 ± 0.05	0.26 ± 0.05	0.34 ± 0.03	0.23 ± 0.08	-	-	-	-	-
Uterus (mg)	A	-	-	-	-	-	703 ± 246	667 ± 189	603 ± 167	637 ± 278	**284** ± 202**
	R	-	-	-	-	-	0.29 ± 0.10	0.28 ± 0.07	0.26 ± 0.08	0.30 ± 0.12	0.17 ± 0.10

A: Absolute weight; R: Relative weight change; organ weight/body weight Source: Katoh, 2018

* Significantly different from the control using Dunnett's test (or Dunnett-type test): $p < 0.05$;

** Significantly different from the control using Dunnett's test (or Dunnett-type test): $p < 0.01$

The histopathology examination revealed effects in numerous organs: bone marrow (decreased haematopoiesis), bone (resorption and fibro-osseous proliferation), thymus (atrophy), heart (artery degeneration/necrosis and mineralization, perivascular fibrosis, cardiac muscle necrosis), aorta (inflammation and mineralization), glandular stomach (mineralization), testis (atrophy), epididymides (oligospermia), adrenals (hypertrophy zona glomerulosa) and kidneys (crystals in renal tubules and pelvis, dilatation of renal tubules, obstructive nephropathy and urothelium hyperplasia). These lesions showed the highest incidences at the top dose of 3000 ppm, but some (in bone marrow, bone, thymus, heart, aorta, glandular stomach) were also present at much lower incidences in the 1000 ppm groups. Crystals in the renal pelvis, by contrast, were also seen in both sexes in the 300 ppm groups, and obstructive nephropathy was apparent in the 300 ppm males. Detail of histopathology findings is shown below in Table 58.

Table 58. Selected histopathological findings in a 90-day study of DH-04 toxicity in rats; number affected rats from terminal and unscheduled sacrifice, original groups of 10 rats

Parameter	**Dose level (ppm)**									
	Males					**Females**				
	0	**100**	**300**	**1000**	**3000**	**0**	**100**	**300**	**1000**	**3000**
Bone marrow (sternum)										
Decreased haematopoiesis	0	0	0	0	**6****	0	0	0	0	**5***
Bone marrow (femur)										
Decreased haematopoiesis	0	0	0	0	**7****	0	0	0	0	**6****
Necrosis	0	0	0	0	**1**	0	0	0	**1**	**4***
Thymus										
Atrophy	0	0	0	0	**7****	0	0	0	**1**	**3**
Bone (femur)										
Resorption, bone, increased	0	0	0	0	**0**	0	0	0	1	**4***
Proliferation, fibro-osseous	0	0	0	0	**4***	0	0	0	0	0

Parameter	Dose level (ppm)									
	Males					Females				
	0	100	300	1000	3000	0	100	300	1000	3000
Heart										
Degeneration/necrosis, artery	0	0	0	0	**6****	0	0	0	1	**8****
Fibrosis, perivascular	0	0	0	0	**2**	0	0	0	0	**1**
Infiltration, mononuclear cell	2	2	1	0	1	0	0	0	0	0
Inflammation, artery	0	0	0	0	**2**	0	0	0	0	0
Mineralization, artery	0	0	0	0	**5***	0	0	0	1	**10****
Necrosis/inflammatory cell infiltrate, cardiac muscle	0	0	0	0	**7****	0	0	0	1	**4***
Artery (aorta)										
Inflammation, artery	0	0	0	0	**3**	0	0	0	0	**4***
Mineralization, artery	0	0	0	0	**4***	0	0	0	1	**9****
Glandular stomach										
Oedema, submucosa	0	0	0	0	0	0	0	1	0	0
Mineralization	0	0	0	0	**4***	0	0	0	1	**5***
Kidneys										
Basophilic change, renal tubule	1	1	2	0	0	1	1	1	0	0
Calculi	0	0	0	0	0	1	1	0	0	0
Crystal(s), luminal, renal tubule	0	0	0	**10****	**10****	0	0	0	**9****	**9****
Crystal(s), pelvis	0	0	2	**6****	**4***	0	0	1	**5***	**3**
Dilatation, luminal, renal tubule	0	0	0	**10****	**10****	1	0	0	**9****	**6***
Dilatation, pelvis	3	1	2	2	**5**	0	2	1	2	1
Mineralization, artery	0	0	0	0	**4***	0	0	0	0	0
Mineralization, fornix	0	0	0	0	0	1	0	0	0	0
Mineralization, renal tubular cell, outer medulla, outer stripe	0	0	0	0	0	0	0	0	1	2
Necrosis, papillary	0	0	0	0	**4***	0	0	0	0	0
Necrosis, renal tubular cell, outer medulla, outer stripe	0	0	0	0	0	0	0	0	**1**	**4***
Nephropathy, obstructive	0	0	1	**10****	**6****	0	0	0	**9****	**6****
Hyperplasia, urothelium	0	0	0	**1**	**6****	0	0	0	2	1
Testes										
Atrophy, seminiferous tubule	0	0	2	0	**4***	-	-	-	-	-
Epididymides										
Granuloma	1	0	0	0	0	-	-	-	-	-
Oligospermia	0	0	1	0	**4***	-	-	-	-	-
Adrenals										
Hypertrophy, zona glomerulosa cell	0	0	0	0	**5***	0	0	0	**1**	**7****

* Significantly different from the control using Fisher's exact probability test: $p < 0.05$; Source: Katoh, 2018

** Significantly different from the control using Fisher's exact probability test: $p < 0.01$

In conclusion, dietary administration of metabolite DH-04 (or M4) to rats for 13 weeks was highly toxic at the top dose of 3000 ppm causing mortality, moribundity, clinical signs of toxicity, effects on FOB, body weight, food consumption and generalized systemic toxicity, characterized by anaemia, liver toxicity, kidney toxicity and effects on numerous other organs such as the heart, aorta, bone marrow,

bone, adrenals, thymus and testes. Similar effects, although milder and at a lower incidence were also seen at 1000 ppm. Some kidney effects (crystals in the renal pelvis, obstructive nephropathy and depressed areas) were also present in some animals of both sexes at 300 ppm. There were no treatment-related effects at 100 ppm. The kidney effects are considered not relevant to humans (see section 2.6 Special studies). Overall, the NOAEL was 300 ppm (19.6 mg/kg bw per day) based on one death, clinical signs of toxicity (piloerection, decreased motor activity, bradypnea, tremors and decreased grip strength of hindlimbs), decreased body weight and food consumption, haematological changes indicative of anaemia, clinical chemistry changes indicative of liver toxicity and generalized systemic toxicity, changes in the weights of the liver, adrenals and heart and low incidence of histopathological changes of the bone marrow, bone, thymus, heart, aorta and glandular stomach at the LOAEL of 1000 ppm (65.2 mg/kg bw per day) (Katoh, 2018).

Summary of data on metabolite DH-04 or M4 and comparison with parent

Metabolite DH-04 (or M4) was negative in an Ames test and an in vitro micronucleus assay, was of low acute oral toxicity, with a LD_{50} of > 2000 mg/kg bw in rats, but caused severe toxicity in a dietary 90-day rat study where a NOAEL of 19.6 mg/kg bw per day was identified based on one death, clinical signs of toxicity (piloerection, decreased motor activity, bradypnea, tremors and decreased grip strength of hindlimbs), decreased body weights and food consumption, haematological changes indicative of anaemia, clinical chemistry changes indicative of liver toxicity, changes in the weights of the liver, adrenals and heart and low incidence of histopathological changes of the bone marrow, bone, thymus, heart, aorta and glandular stomach at the LOAEL of 1000 ppm (65.2 mg/kg bw per day).

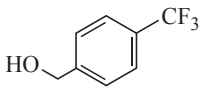
In the equivalent dietary 90-day study in rats with the parent, benzpyrimoxan, a NOAEL of 18.7 mg/kg bw per day (300 ppm) was identified based on liver toxicity (increased weight and changes in clinical chemistry parameters) at the LOAEL of 64.2 mg/kg bw per day (1000 ppm). Although the NOAEL and LOAEL numerical values are comparable, the toxicity observed at the LOAEL with metabolite DH-04 was much more severe than that reported at the LOAEL with the parent substance. Therefore, DH-04 appears to be quantitatively more toxic than its parent substance. At the top dose (three times higher than the LOAEL for both the parent substance and DH-04), the effects observed in the parent's 90-day study were generally similar to the those observed at the LOAEL in the 90-day study with DH-04 (see Table 59 below). Therefore, it is possible to conclude that DH-04 is approximately three times more toxic than benzpyrimoxan.

Table 59. Comparison of data for DH-04 (M4) and parent, benzpyrimoxan

DH-04 (or M4)		Parent benzpyrimoxan	
Ames test	Negative	**Ames test**	Negative
In vitro micronucleus test	Negative	**In vitro chromosome aberration and in vivo micronucleus tests**	Negative
Acute oral rat	LD_{50} > 2000 mg/kg bw Kidney findings at necropsy	**Acute oral rat**	LD_{50} > 2000 mg/kg bw Kidney findings at necropsy
90-day dietary rat	NOAEL: 19.6 mg/kg bw/d (300 ppm) LOAEL: 65.2 mg/kg bw/d (1000 ppm) based on one death, clinical signs of toxicity, decreased body weight and food consumption, haematological changes indicative of anaemia, clinical chemistry changes indicative of liver toxicity, changes in the weights of the liver, adrenals and heart and low incidence of histopathological changes to various organs Top dose was 168 mg/kg bw/d (3000 ppm): caused severe toxicity	**90-day dietary rat**	NOAEL: 18.7 mg/kg bw/d (300 ppm) LOAEL: 64.2 mg/kg bw/d (1000 ppm) based on liver toxicity (1000 ppm) Top dose was 194 mg/kg bw/d (3000 ppm): caused decreased body weight, body weight gain, food consumption, food efficiency, haematological changes indicative of anaemia, clinical chemistry changes indicative of liver toxicity, changes in liver and spleen weight, liver histopathology

Studies on metabolite DH-102 (or M11)

Figure 8. Chemical structure of metabolite DH-102 (or M11)



Ames test

An Ames test (OECD 471) was conducted with metabolite DH-102 (or M11) (purity 99.95%) in *Salmonella typhimurium* strains TA100, TA1535, TA98 and TA1537, and *Escherichia coli* strain WP2 *uvr*A with and without metabolic activation (S9 mix) by the pre-incubation method. The vehicle used for the test article was DMSO. Triplicate plates were used in the range-finding test and in the two main tests.

The range-finding test was conducted with concentrations between 19.5 and 5000 µg/plate. From the results of the range-finding test, the minimum concentration which showed growth inhibition was selected as the maximum concentration for the main tests, which were conducted at six concentrations ranging from 156 to 5000 µg/plate in *S. typhimurium* TA98 and *E. coli* WP2 *uvr*A, and at six concentrations ranging from 39.1 to 1250 µg/plate in *S. typhimurium* TA100, TA1535 and TA1537.

No precipitation was observed at any concentration with or without metabolic activation. Growth inhibition was observed at 1250 µg/plate and above in all strains without S9, at 2500 µg/plate and above in *S. typhimurium* TA98 and *E. coli* WP2 *uvr*A with S9, and at 1250 µg/plate and above in *S. typhimurium* TA100, TA1535 and TA1537 with S9. In the two main tests there was neither an increase in the number of revertant colonies of two-fold or more in comparison with that of the negative control group, nor was a dose–response relationship apparent for any strains with or without S9. The positive control substances gave the expected responses.

Overall, metabolite DH-102 (or M11) was negative in a GLP and guideline Ames study up to concentrations causing cytotoxicity (Minegawa, 2017).

Genotoxicity QSAR analysis

In addition to the Ames test, the sponsor also submitted a genotoxicity QSAR analysis (using DEREK, OECD QSAR Toolbox, Vega and ToxTree) for DH-102 (M11) and the parent substance. The results shown in Table 60 were obtained.

Table 60. Summary of genotoxicity information for DH-102 (M11) and parent

Substances	Gene mutation	Structural chromosome aberration	Numerical chromosome aberration	Final evaluation
Parent (benzpyrimoxan)	EXP + QSAR **Negative**	EXP + QSAR **Negative**	EXP + QSAR **Negative**	**Non-genotoxic**
DH-102 or M11	EXP + QSAR + Literature + Comparative tox profiling with parent **Negative**	QSAR + Comparative tox profiling with parent **Negative**	QSAR + Comparative tox profiling with parent **Negative**	**Non-genotoxic**

EXP: Experimental data; Source: Minegawa, 2017

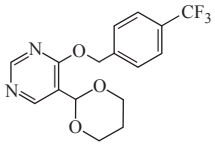
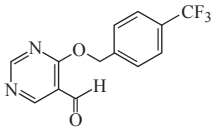
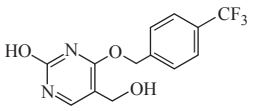
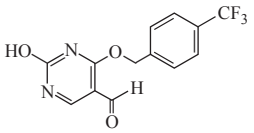
Summary of toxicological information on metabolite DH-102 or M11

Based on the negative Ames test and the results of the genotoxicity QSAR analysis with a number of different models, it can be concluded that metabolite DH-102 (M11) is unlikely to be genotoxic. In the absence of further data, and taking into account that DH-102 is not a major rat metabolite, if a dietary risk assessment were to be required, the TTC Cramer class III value of 1.5 µg/kg bw per day should be used.

Studies on metabolites DH-03 (M3), DH-06 (M6), DH-07 (M7)

A genotoxicity QSAR analysis (using DEREK, OECD QSAR Toolbox, Vega and ToxTree) was submitted by the sponsor for DH-03 (M3), DH-06 (M6), DH-07 (M7) and the parent substance.

Table 61. Details of molecules selected for genotoxicity QSAR analysis

IDs/Substance name	Structure	SMILES notation	MW	log K_{ow}
Parent Benzpyrimoxan NNI-1501 5-(1,3-dioxan-2-yl)-4-{[4-(trifluoromethyl)phenyl] methoxy}pyrimidine		FC(F)(F)c1ccc(cc1)COc2ncncc2C3OCCCO3	340	3.42 (experimental) 2.97 (calculated)
DH-03 or M3 NNI-1501-aldehyde 4-{[4-(trifluoromethyl) phenyl] methoxy}pyrimidine-5-carbaldehyde		O=Cc2cncnc2OCc1ccc(cc1)C(F)(F)F	282	2.86 (calculated)
DH-06 or M6 NNI-1501-CH_2OH-2-OH 5-(hydroxymethyl)-4-{[4-(trifluoromethyl)phenyl] methoxy}pyrimidin-2-ol		Oc2nc(OCc1ccc(cc1)C(F)(F)F)c(cn2)CO	300	2.5 (calculated)
DH-07 or M7 NNI-1501-aldehyde-2-OH 2-hydroxy-4-{[4-(trifluoromethyl)phenyl] methoxy}pyrimidine-5-carbaldehyde		Oc2nc(OCc1ccc(cc1)C(F)(F)F)c(cn2)C=O	298	3.13 (calculated)

MW: Molecular weight (g/mole);
log K_{ow}: Logarithm of the ratio of the concentration of a solute between water and octanol

A read-across evaluation of these three metabolites in relation to the parent was also performed. To support the read-across, the profiling, data gathering and categorization tools of the OECD QSAR Toolbox v4.4 were used. Simple considerations based on structural similarity, molecular weight and log K_{ow} were taken into account first. The table below summarizes the thresholds for these three parameters, which affect molecular similarity.

Table 62: Parameters for simple read-across

Parameter	Threshold
Molecular weight	Maximum difference: ± 20%
log K_{ow}	Maximum difference: ± 1 unit
Structural similarity [a]	At least 70%

[a] Dice/atom centred structural similarity, calculated with the OECD QSAR Toolbox.

In a second step, molecular initiating events of relevance to genotoxicity, such as interaction with DNA and/or proteins, were compared. Several profilers are available in the OECD QSAR Toolbox (v. 4.4), which include structural alerts relevant for these type of interactions.

To evaluate structural similarity, organic functional group profilers can be applied in parallel to the structural alerts identified, with the aim of investigating if the functional groups could have an impact on the genotoxicity of the substances.

The following results were obtained and are summarized below in Table 63.

Table 63. Summary of genotoxicity information for DH-03 (M3), DH-06 (M6), DH-07 (M7) and parent benzpyrimoxan

	Gene mutation	Structural chromosome aberration	Numerical chromosome aberration	Final evaluation
Substances	**Grouping for read-across evaluation**			
Parent (benzpyrimoxan)	EXP+QSAR **Negative**	EXP+QSAR **Negative**	EXP+QSAR **Negative**	Nongenotoxic
DH-03 or M3	QSAR+RA (Parent, DH-04) **Negative**	QSAR+RA (Parent, DH-04) **Negative**	QSAR+RA (Parent, DH-04) **Negative**	Nongenotoxic
DH-06 or M6	QSAR+RA (Parent, DH-04) **Negative**	QSAR+RA (Parent, DH-04) **Negative**	QSAR+RA (Parent, DH-04) **Negative**	Nongenotoxic
DH-07 or M7	QSAR+RA (Parent, DH-04) **Negative**	QSAR+RA (Parent, DH-04) **Negative**	QSAR+RA (Parent, DH-04) **Negative**	Nongenotoxic

EXP: experimental data; RA: Read-across; QSAR: Quantitative structure–activity relationship analysis

Overall genotoxicity effects were not expected for metabolites DH-03, DH-06 or DH-07 based on the QSAR analysis and the read-across evaluation.

Summary of toxicological information on metabolites DH-03 (M3), DH-06 (M6) and DH-07 (M7)

Based on the genotoxicity QSAR analysis and read-across evaluation, DH-03, DH-06 and DH-07 are unlikely to be genotoxic. Metabolites DH-03, DH-06 and DH-07 are not found in the rat or are present only at low levels. Therefore, if a dietary risk assessment were to be required, the TTC Cramer class III value of 1.5 µg/kg bw per day should be used.

The sponsor claimed that DH-06 was the unique upstream metabolite of DH-205 (M14) in the rat (see Fig. 5), a major metabolite. However, the Meeting agreed that DH-205 could be generated not only from DH-06, but also from DH-05 and DH-01. On this basis, DH-06 cannot be regarded as a major rat metabolite covered by parent.

Metabolites present in rat kinetic studies

Metabolites DH-01, DH-02, DH-05, DH-101 and DH-402 were considered to be major rat metabolites.

Metabolite DH-01 (M1)

This was present in plasma at levels of 21–32% TRR at the high dose of 100 mg/kg bw (see Table 6) in males and females in the rat kinetic study (phenyl label) by Yasunaga (2018a) . It was also present in the same study in urine (see Table 5) in both sexes at levels of 0.16–0.47% of AD, that is 0.2–0.6% of the absorbed dose (AbD) assuming 80% oral absorption, in the liver at levels of 0.9–1.73% TRR (see Table 7) and in the kidneys at levels of 2.0–2.48% TRR (see Table 8), which are the key target organs of toxicity of benzpyrimoxan. Similar levels in each matrix were found in the study by Yasunaga (2018b) (pyrimidinyl label). It is also noted that DH-01 is structurally very similar to DH-05, which is clearly a major rat metabolite.

Metabolite DH-02 (M2)

This was present in plasma at levels of 15.5% TRR at the high dose of 100 mg/kg bw (see Table 14) in males in the rat kinetic study (pyrimidinyl label) by Yasunaga (2018b) . It was also present in the same study in urine (see Table 13) in both sexes at levels of 0.12–0.22% of the administered dose (0.15–0.28% of AbD; 80% oral absorption), in the liver at levels of 29.8–52.85% TRR (see Table 15) and in the kidneys at levels of 10.69–37.78% TRR (see Table 16). It should also be noted that the downstream conjugates of DH-02 (DH-02-glucuronide and sulfate) were present in urine (see Table 5) up to levels of 4.39% of the administered dose (5.5% of AbD; 80% oral absorption) and in bile (see Table 18) up to levels of 14.08% of the administered dose (17.6% of AbD; 80% oral absorption).

Metabolite DH-05 (M5)

This was present in urine at levels of 18–28% of the administered dose (22.5–35% of AbD; 80% oral absorption) in males and females at the low dose of 1 mg/kg bw (see Table 5) in the rat kinetic study (phenyl label) by Yasunaga (2018a). It was also present in plasma at levels up to 11% TRR (see Table 14) in the rat kinetic study (pyrimidinyl label) by Yasunaga (2018b)and in bile up to 12% of the administered dose (15% of AbD) at the low dose of 1 mg/kg bw (see Table 18) in the rat kinetic study (bile duct-cannulated) by Yasunaga (2018c).

Metabolite DH-101 (M10)

This was present in plasma at levels of 79–86% TRR at the low dose of 1 mg/kg bw (see Table 6) in males and females in the rat kinetic study (phenyl label) by Yasunaga (2018a). It was also present in the same study in urine (see Table 5) in both sexes at levels of 1.10–2.66% of the administered dose (1.4–3.3% of AbD; 80% oral absorption), in the liver at levels of 12.39–13.82% TRR (see Table 7) and in the kidneys at levels of 17.45–26.05% TRR (see Table 8). It should also be noted that the downstream conjugates of DH-101 (DH-101-glucuronide and sulfate) were present in urine (see Table 5) up to levels of 6.10% of the administered dose (7.6% of AbD; 80% oral absorption).

Metabolite DH-402 (M16)

This was present in urine at levels of 4–7% of the administered dose (5–9% of AbD; 80% oral absorption) at the low dose of 1 mg/kg bw (see Table 5) in males and females in the rat kinetic study (phenyl label) by Yasunaga (2018a). In the same study, it was also present in kidneys at levels of 3–4% TRR at the low dose of 1 mg/kg bw (see Table 8) in males and females, and in plasma at levels of 0.2% TRR in males at the low dose of 1 mg/kg bw (see Table 6). In addition, DH-402 is the glycine derivative of DH-101, a major rat metabolite. It was considered most likely that the addition of glycine will not increase the toxicity of DH-402 compared to DH-101. Therefore, there is sufficient evidence based on the levels found in the rat and by read-across from a major rat metabolite (DH-101), to conclude that DH-402 is covered by the parent.

Overall, the above metabolites are considered covered by the parent and are potential candidates for inclusion in the residue definition for risk assessment. The dietary reference values of the parent can therefore be used in the risk assessment of DH-01, DH-02, DH-05, DH-101 and DH-402, if required.

Conjugates

The glucuronides of DH-02 and DH-06 were also identified in livestock. These conjugates are easily cleaved in the human GI tract to release the aglycones, DH-02 and DH-06. DH-02 is considered a major rat metabolite, and hence, covered by the parent, therefore DH-02-glucuronide is also covered by parent. It is also noted that the glucuronide of DH-02 was present in rat urine (see Table 5) up to 3.5% of AD (4.4% of AbD; 80% oral absorption) and in rat bile (see Table 18) up to 3.83% of the AD (4.8% of AbD; 80% oral absorption). The dietary reference values for the parent benzpyrimoxan can therefore be used in the risk assessment of DH-02-glucuronide, if required.

Metabolite DH-06

Metabolite DH-06 is not a major rat metabolite, but based on its lack of genotoxicity it was assigned the TTC Cramer class III value of 1.5 µg/kg bw/day. Therefore, DH-06-glucuronide should also be risk assessed against the TTC Cramer class III value, if required.

Overall summary of the toxicological characterization of plant/livestock metabolites for the purposes of the residue definition for risk assessment

The metabolites found in crops and livestock and/or after high-temperature hydrolysis were DH-01 (M1), DH-02 (M2), DH-02-glucuronide, DH-03 (M3), DH-04 (M4), DH-05 (M5), DH-06 (M6), DH-06-glucuronide, DH-07 (M7), DH-08 (M8), DH-101 (M10), DH-102 (M11) and DH-402 (M16).

Metabolites DH-01, DH-02, DH-05 and DH-101 are major rat metabolites and hence considered covered by the parent.

Metabolite DH-402, based on the levels found in the rat and by read-across from a major rat metabolite (DH-101) is considered covered by the parent.

Metabolite DH-02-glucuronide is easily cleaved in the human GI tract to release the aglycone, DH-02. The aglycone is considered a major rat metabolite, hence,covered by the parent. Therefore DH-02-glucuronide is also covered by the parent.

Metabolite DH-06-glucuronide is easily cleaved in the human GI tract to release the aglycone, DH-06. Metabolite DH-06 is not a major rat metabolite, but based on its lack of genotoxicity it has been assigned the TTC Cramer class III with a value of 1.5 µg/kg bw per day. Therefore, DH-06-glucuronide should also be risk assessed against the TTC Cramer class III value, if required.

Metabolites DH-03, DH-06, DH-07 and DH-102 are not found in the rat or are present at low levels, but genotoxicity QSAR analysis, limited genotoxicity experimental data (for example Ames test for DH-102) and genotoxicity read-across evaluation indicate that they are unlikely to be genotoxic. Therefore, if a dietary risk assessment were to be required for DH-03, DH-06, DH-07 or DH-102, the TTC Cramer class III value of 1.5 µg/kg bw per day should be used.

Metabolite DH-04 has been tested in genotoxicity and general toxicity studies. It was negative in an Ames test and an in vitro micronucleus assay, was of low acute oral toxicity with an LD_{50} of greater than 2000 mg/kg bw in rats, but caused appreciable toxicity in a dietary 90-day study in rats where a NOAEL of 19.6 mg/kg bw per day was identified based on one death, clinical signs of toxicity (piloerection, decreased motor activity, bradypnea, tremors and decreased grip strength of hindlimbs), decreased body weight and food consumption, haematological changes indicative of anaemia, clinical chemistry changes indicative of liver toxicity and generalized systemic toxicity, changes in the weights of the liver, adrenals and heart and low incidence of histopathological changes of the bone marrow, bone, thymus, heart, aorta and glandular stomach at the LOAEL of 1000 ppm (equal to 65.2 mg/kg bw per day). In the equivalent dietary 90-day study in rats with benzpyrimoxan, a NOAEL of 18.7 mg/kg bw per day (300 ppm) was identified based on liver toxicity (increased weight and changes in clinical chemistry parameters) at the LOAEL of 64.2 mg/kg bw per day (1000 ppm). Although the NOAEL and LOAEL numerical values are comparable, the toxicity observed at the LOAEL with DH-04 was much more severe than that reported at the LOAEL with the parent substance. Therefore, DH-04 appears to be quantitatively more toxic than the parent substance. At the top dose (three times higher than the LOAEL for both the parent substance and DH-04), the effects observed in the parent's 90-day study were generally similar to the those observed at the LOAEL in the 90-day study with DH-04. Therefore, it was concluded that DH-04 is approximately three times more toxic than benzpyrimoxan.

No information was available for metabolite DH-08, therefore, if a dietary risk assessment were to be required for DH-08, the genotoxicity TTC value of 0.0025 µg/kg bw per day should be used.

Table 64 below provides an overall summary overview of the toxicological characterization of the metabolites discussed above.

Table 64. Summary overview of toxicological characterization of plant/livestock metabolites

Compound (codes)	Rat ADME; toxicity covered by toxicological properties of parent compound (level in rat biofluids >10% AbD)?	Genotoxicity assessment (data, QSAR, read-across)	General toxicity
Benzpyrimoxan (NNI-1501)	Parent - not applicable	Unlikely to be genotoxic (data)	Full data set available
Benzpyrimoxan-acid (DH-01 or M1)	**Yes** (high levels in plasma)	Unlikely to be genotoxic as covered by parent	Covered by parent
Benzpyrimoxan-CH_2OH (DH-02 or M2 and its glucuronide)	**Yes** (high levels in plasma)	Unlikely to be genotoxic as covered by parent	Covered by parent
Benzpyrimoxan-aldehyde (DH-03 or M03)	**No** (not found in rat)	Unlikely to be genotoxic (QSAR and RA)	No information available
Benzpyrimoxan-2-OH (DH-04 or M4)	**No** (not found in rat)	Unlikely to be genotoxic (data)	Three-times more toxic than parent based on 90-day study
Benzpyrimoxan-acid-2-OH (DH-05 or M5)	**Yes** (high levels in urine)	Unlikely to be genotoxic as covered by parent	Covered by parent
Benzpyrimoxan-CH_2OH-2-OH (DH-06 or M6 and its glucuronide)	**No** (low levels in rat)	Unlikely to be genotoxic (QSAR and RA)	No information available
Benzpyrimoxan-aldehyde-2-OH (DH-07 or M7)	**No** (not found in rat)	Unlikely to be genotoxic (QSAR and RA)	No information available
Benzpyrimoxan-enamine-aldehyde (DH-08 or M8)	**No** (not found in rat)	No information available	No information available
Benzpyrimoxan-benzoic acid (DH-101 or M10)	**Yes** (high levels in plasma)	Unlikely to be genotoxic as covered by parent	Covered by parent
Benzpyrimoxan-benzyl alcohol (DH-102 or M11)	**No** (not found in rat or at very low levels)	Unlikely to be genotoxic (Ames and QSAR)	No information available
Benzpyrimoxan-benzoyl glycine (DH-402 or M16)	**Yes** (high levels in urine and kidneys; similar to major rat metabolite DH-101)	Unlikely to be genotoxic as covered by parent	Covered by parent

ADME: Absorption, distribution, metabolism and excretion; AbD: Absorbed dose;

3. Observations in humans

As this is a new substance, no data are available.

4. Microbial aspects

The possible impact of benzpyrimoxan residues on the human intestinal microbiome was considered for evaluation. A search of the literature available in the public domain did not identify information describing any direct or indirect experimental evidence which addressed the impact of benzpyrimoxan residues on the human intestinal microbiome. No experimental data was submitted by the sponsor in this regard.

Comments

Biochemical aspects

The absorption, distribution, metabolism, excretion and toxicokinetic properties of benzpyrimoxan have been investigated in the rat using two different radiolabelled test materials (on the phenyl and pyrimidinyl rings) administered orally. A study in bile duct-cannulated rats with the two labels was also available. In addition an in vitro comparative metabolism study was submitted.

Following oral administration of ^{14}C-labelled benzpyrimoxan as a single low dose of 1 mg/kg body weight (bw) to rats, the compound was rapidly and extensively absorbed. In bile duct-cannulated rats, oral absorption was approximately 76–83% (mean 80%) of administered radioactivity (AR) at the low dose of 1 mg/kg bw based on the sum of radioactivity excreted in bile (29–46% AR) and urine (36–48% AR), and residual radioactivity in the gastrointestinal (GI) tract and liver (0.1% AR) by 72 hours after dosing (Yasunaga, 2018c). At the high single dose of 100 mg/kg bw, absorption was only slightly slower and less. At the low dose, radioactivity in blood and plasma reached maximum concentrations (T_{max}) at 1–9 hours post dose, then rapidly decreased in a biphasic pattern. At the high dose, the area under the curve (AUC) was approximately 50–80 times higher (that is, slightly less than dose-proportional) when compared to the low dose, and the T_{max} was in the range of 6–12 hours post dosing.

No significant sex-related, dose-related or label-related differences were noted in the distribution profile of benzpyrimoxan. At around the T_{max} the highest level of radioactivity was found in the GI contents, followed by the liver, plasma, GI tract itself, kidneys and adrenals. By 24 hours post dose, radioactivity concentrations in all organs and tissues had significantly decreased and by 168 hours post dose were nearly negligible, suggesting that no organs or tissues specifically retained benzpyrimoxan and/or its metabolites.

The absorbed radioactivity was rapidly excreted via urine, faeces and expired air. Radioactivity in expired air was confirmed as ^{14}C carbon dioxide. At the low dose, radioactivity in urine, faeces and expired air accounted for 39–65%, 33–44% and 6% AR respectively by 168 hours post dosing. Almost all the radioactivity excreted in faeces resulted from biliary excretion. At the high dose, radioactivity in urine, faeces and expired air accounted for 36–45%, 54–58% and 2.4% AR respectively by 168 hours post dosing. These data indicate that at the high dose of 100 mg/kg bw more radioactivity was eliminated via faeces, possibly resulting from unabsorbed material.

Benzpyrimoxan was extensively metabolized, with up to nine metabolites identified in plasma, 15 in urine and six in bile. Unchanged parent compound was detected only in faeces, in small amounts (0.19–0.39% AR) at the low dose but at much higher levels (24–29% AR) with the high dose. Neither sex nor dose level had any significant impact on metabolism. Metabolite DH-05 was the only major metabolite in urine, accounting for 14–28% of administered dose (AD) at the low dose, whilst the DH-02 conjugates were the only major metabolites in bile, accounting for 13–14% AD at the low dose. In plasma, at the low dose, DH-01 was a major metabolite, accounting for 79–85% of the total radioactive residue (TRR) in this matrix at six hours post dosing (around the T_{max}). At the high dose, DH-01 and DH-101 were significant metabolites, accounting for 21–32% and 23–30% respectively of the plasma TRR at nine hours post dosing (around the T_{max}) (Yasunaga, 2018a, b).

Overall, no significant differences between phenyl-labelled and pyrimidine-labelled benzpyrimoxan were seen in the absorption, distribution, metabolism, excretion or toxicokinetic profiles of rats.

In an in vitro comparative metabolism study with rat, mouse, dog and human liver microsomes, the main metabolites detected in all species were DH-01, DH-02, DH-05, DH-22 and DH-101. There were no qualitative differences between species in metabolite profiles and no significant unique human metabolite was detected (Yasunaga, 2019).

Toxicological data

The acute oral median lethal dose (LD_{50}) of benzpyrimoxan in rats was greater than 2000 mg/kg bw (Tsukushi, 2016a) and the dermal LD_{50} in rats was greater than 2000 mg/kg bw (Yoshida, 2016). The inhalation median lethal concentration (four-hour LC_{50}) of a benzpyrimoxan aerosol in rats was greater than 3.9 mg/L, the maximum attainable concentration (van Huygevoort, 2017). Benzpyrimoxan was a very slight irritant to rabbit skin (Munechika 2017a). It was non-irritant in an in vitro eye irritation test (Munechika, 2017b) and slightly irritant to the rabbit eye in vivo (Tsukushi, 2017a). Benzpyrimoxan was sensitizing to skin in a Guinea pig maximization test when tested at concentrations up to 50% in olive oil (Takehara, 2016), but was negative in a local lymph node assay (LLNA) at up to 10% in acetone/olive oil (Fujishima, 2016).

The short-term toxicity of benzpyrimoxan was investigated by the dietary route in studies with mice, rats and dogs. The main target organs of toxicity in all species were the liver and the haematological system, with associated effects on spleen and/or bone marrow. In addition, effects on the kidney and urinary bladder, due to the deposition of crystals, with ensuing obstructive nephropathy were noted in mice and rats. Mice appeared the least sensitive species to the toxicity of benzpyrimoxan, with rats and dogs showing similar sensitivity.

The effects on the urinary system caused by benzpyrimoxan in rats and mice, but not in dogs were considered unlikely to be relevant to humans. A mechanistic study showed that the different responses observed in rodents and dogs appeared to be due to differences in the critical concentration at which the main constituent of the crystals (metabolite DH-05) crystallizes in the glomerular filtrate when it is concentrated. The effect was not due to species differences in the formation of DH-05, as this metabolite was formed in vitro not only in rats and mice, but also in dogs and humans. Rather the cause lay in a number of species differences in urinary concentration rates and urine composition.

In the subsequent 90-day dietary study in mice, benzpyrimoxan was administered at concentrations of 0, 400, 2000 or 4000 (males)/6000 (females) ppm (equal to 0, 56, 282 and 523 mg/kg bw per day for males, 0, 66, 327 and 971 mg/kg bw per day for females). The NOAEL was 400 ppm (equal to 56 mg/kg bw per day) based on liver (hypertrophy) and spleen (extramedullary haemopoiesis) histopathological changes associated with changes in organ weights and/or haematological and clinical chemistry findings (increased alanine and aspartate aminotransferase [ALT and AST] activities) at the LOAEL of 2000 ppm (equal to 282 mg/kg bw per day) (Coleman, 2017).

In a 90-day dietary study in rats, benzpyrimoxan was administered at concentrations of 0, 100, 300, 1000 or 3000 ppm (equal to 0, 6.26, 18.7, 64.2 and 194 mg/kg bw per day for males, 0, 7.41, 22.2, 78.1 and 227 mg/kg bw per day for females). The NOAEL was 300 ppm (equal to 18.7 mg/kg bw per day) based on clinical chemistry (increased plasma γ-glutamyl transpeptidase [GGTP] and total cholesterol in females and increased AST in males) and increased liver weight at the LOAEL of 1000 ppm (equal to 64.2 mg/kg bw per day) (Ohtsuka, 2018a).

In a 90-day dietary study in dogs, benzpyrimoxan was administered at concentrations of 0, 500, 2500 or 10 000 ppm, (equal to 0, 17, 79 and 302 mg/kg bw per day for males, 0, 16, 81 and 246 mg/kg bw per day for females). The NOAEL was 500 ppm (equal to 16 mg/kg bw per day) based on liver toxicity (increased weight, hypertrophy and increased levels of ALP, total cholesterol, triglycerides and phospholipids) and bone marrow histopathology (hypocellularity of femoral bone marrow in females) at the LOAEL of 2500 ppm (equal to 79 mg/kg bw per day) (Ishii, 2016).

In a one-year dietary study in dogs, benzpyrimoxan was administered at concentrations of 0, 100, 500 or 2500 ppm (equal to 0, 2.9, 14.6 and 71 mg/kg bw per day for males, 0, 2.7, 14.3 and 67 mg/kg bw per day for females). The NOAEL was 500 ppm (equal to 14.3 mg/kg bw per day) based on increased platelet count in both sexes, liver toxicity (increased relative weight in both sexes), hepatocyte brown pigmentation (lipofuscin) in both sexes, hypertrophy in males, increased levels of total cholesterol, triglycerides and phospholipids in males and increased levels of ALP in females, at the LOAEL of 2500 ppm (equal to 67 mg/kg bw per day) (Ishii, 2018).

The overall NOAEL for the dog was 500 ppm (equal to 16 mg/kg bw per day) on the basis of the 90-day and one-year study, with an overall LOAEL of 2500 ppm (equal to 67 mg/kg bw per day).

In a carcinogenicity dietary study in mice, benzpyrimoxan was administered at concentrations of 0, 80, 400 or 2000 (males)/1500 (females) ppm, (equal to 0, 7.7, 40 and 195 mg/kg bw per day for males, 0, 8.9, 44 and 163 mg/kg bw per day for females). The carcinogenicity NOAEL was 1500 ppm (equal to 163 mg/kg bw per day), the highest dose tested. The NOAEL for chronic toxicity was 400 ppm (equal to 40 mg/kg bw per day) based on decreased body weight gain, gall bladder calculi and liver effects in both sexes at the LOAEL of 1500 ppm (equal to 163 mg/kg bw per day) (Coleman, 2018).

In a chronic toxicity/carcinogenicity study in rats, benzpyrimoxan was administered in the diet for one year (chronic toxicity group) or two years (carcinogenicity group), at dose levels of 0, 60, 300 or 1500 ppm (equal to 0, 2.3, 12 and 59 mg/kg bw per day for males, 0, 2.9, 15 and 78 mg/kg bw per day for females in the chronic phase: in the carcinogenicity phase this was 0, 2.7, 14 and 69 mg/kg bw per day for males, 0, 3.6, 17.5 and 90 mg/kg bw per day for females). The carcinogenicity NOAEL was 1500 ppm (equal to 69 mg/kg bw per day), the highest dose tested. The top dose caused significant toxicity. The NOAEL for chronic toxicity was 300 ppm (equal to 14 mg/kg bw per day) based on decreased body weights and body weight gains, changes in some haematological and clinical chemistry parameters, and increased weights of liver with associated histopathological changes at the LOAEL of 1500 ppm (equal to 69 mg/kg bw per day) (Ohtsuka, 2018b).

The Meeting concluded that benzpyrimoxan is not carcinogenic in mice or rats.

The genotoxic potential of benzpyrimoxan was investigated in an adequate range of in vitro (Oguma, 2014; Tsukushi, 2017b; Verspeek-Rip, 2017) and in vivo tests (Tsukushi, 2015). No evidence indicating induction of gene mutation or structural chromosomal aberrations was found. Benzpyrimoxan induced polyploidy in vitro in Chinese hamster lungs cells at precipitating concentrations following short-term exposure but not after long-term treatment, where the likelihood of such an effect is much greater. Therefore, the finding was considered a chance observation. Benzpyrimoxan was negative in vivo in a bone marrow micronucleus study.

The Meeting concluded that benzpyrimoxan is unlikely to be genotoxic.

In view of the lack of carcinogenicity in mice and rats and the fact that the compound is unlikely to be genotoxic, the Meeting concluded that benzpyrimoxan is unlikely to pose a carcinogenic risk to humans.

In a two-generation reproduction toxicity study, benzpyrimoxan was given in the diet to rats at concentrations of 0, 60, 300 or 2000 ppm (equal to 0, 2.5, 12 and 85 mg/kg bw per day for males, 0, 4.7, 24 and 156 mg/kg bw per day for females). The NOAEL for parental toxicity was 300 ppm (equal to 12 mg/kg bw per day), based on effects on body weight, body weight gain, feed consumption and toxicity to the liver and thyroid at the LOAEL of 2000 ppm (equal to 85 mg/kg bw per day). The NOAEL for reproductive toxicity was 300 ppm (equal to 12 mg/kg bw per day) based on a slight reduction in the gestation index (number of females with live pups/number of pregnant females) for the F1 generation and increased post-implantation loss in both generations at a LOAEL of 2000 ppm (equal to 85 mg/kg bw per day). The NOAEL for offspring toxicity was also 300 ppm (equal to 12 mg/kg bw per day) based on reduced pup body weight and reduced viability on lactation days 0, 4 and 14 at the LOAEL of 2000 ppm (equal to 85 mg/kg bw per day) (Hojo, 2018).

In a rat prenatal developmental toxicity study, benzpyrimoxan was administered by gavage from gestation day (GD) 6–19 at 0, 10, 50 or 250 mg/kg bw per day. The NOAEL for maternal toxicity was 50 mg/kg bw per day based on effects on body weight, body weight gains, feed consumption and placental weight at 250 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 250 mg/kg bw per day, the highest dose tested (Fujii, 2017a).

In a rabbit prenatal developmental toxicity study, benzpyrimoxan was administered by gavage from GDs 6 to 27 at 0, 3, 10 or 30 mg/kg bw per day. The NOAEL for maternal toxicity was 10 mg/kg bw per day based on effects on body weight, body weight gains, food consumption, gravid uterine weight and abortions at 30 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 10 mg/kg bw per day, based on reduced fetal weight at 30 mg/kg bw per day. It is noted that fetal effects were most likely secondary, unspecific consequence of maternal toxicity (Fujii, 2017b).

The Meeting concluded that benzpyrimoxan is not teratogenic.

In an acute neurotoxicity study benzpyrimoxan was administered to rats by single gavage dose at 0, 500, 1000 or 2000 mg/kg bw. The NOAEL for acute neurotoxicity and general toxicity was 2000 mg/kg bw, the highest dose tested (Namiki, 2017). No specific neurotoxicity study with repeated administration was available but functional neurological examinations in the 90-day rat study and histopathological examination of the brain, spinal cord and sciatic nerve in short-term and long-term studies in rats and mice showed no evidence of neurotoxicity.

The Meeting concluded that benzpyrimoxan is not neurotoxic.

No specific immunotoxicity study was available, but no concern was identified from the available studies. The Meeting concluded that benzpyrimoxan is unlikely to be immunotoxic.

Toxicological data on metabolites and/or degradates

The metabolites found in crops and livestock, and/or after high temperature hydrolysis, were DH-01, DH-02, DH-02-glucuronide, DH-03, DH-04, DH-05, DH-06, DH-06-glucuronide, DH-07, DH08, DH-101, DH-102 and DH-402.

Metabolites DH-01, **DH-02**, **DH-05** and **DH-101** are major rat metabolites and hence considered covered by the parent.

Metabolite DH-02-glucuronide is easily cleaved in the human gastrointestinal tract to release the aglycone, DH-02. The aglycone is considered a major rat metabolite and hence, covered by the parent. Therefore DH-02-glucuronide is also covered by parent.

Metabolite DH-402: based on the levels found in the rat and by read-across from the major rat metabolite DH-101, DH-402 is considered covered by parent.

Metabolites DH-03, **DH-06**, **DH-07** and **DH-102** are either not present in the rat or are not major rat metabolites. However, genotoxicity QSAR analysis, limited genotoxicity experimental data (Ames test for DH-102) and genotoxicity read-across evaluation indicated that they are unlikely to be genotoxic. Therefore, if a dietary risk assessment were to be required for DH-03, DH-06, DH-07 or DH-102, the TTC Cramer class III value of 1.5 µg/kg bw per day should be used.

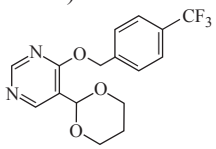
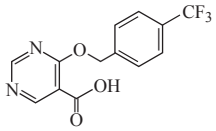
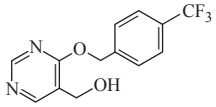
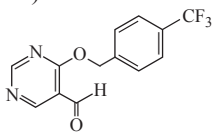
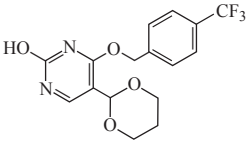
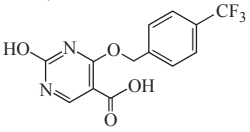
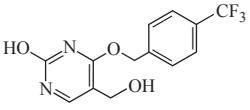
Metabolite DH-06-glucuronide is easily cleaved in the human gastrointestinal tract to release the aglycone DH-06. Since the aglycone is unlikely to be genotoxic, in the absence of further information it has been assigned the TTC Cramer class III value. Therefore, DH-06-glucuronide is also assigned the TTC Cramer class III value of 1.5 µg/kg bw per day.

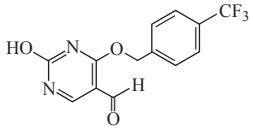
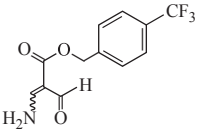
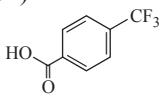
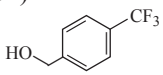
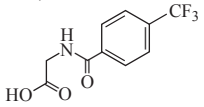
Metabolite DH-04 has been tested in genotoxicity and general toxicity studies. Metabolite DH-04 was negative in an Ames test and an in vitro micronucleus assay, was of low acute oral toxicity with an LD_{50} of greater than 2000 mg/kg bw in rats, but caused appreciable toxicity in a dietary 90-day study in rats where a NOAEL of 19.6 mg/kg bw per day was identified based on the occurrence of considerable adverse effects at the LOAEL of 65.2 mg/kg bw per day. A comparison of these results with those obtained in the equivalent 90-day rat study performed with the parent substance indicate that DH-04 is approximately three times more toxic than benzpyrimoxan.

Metabolite DH-08; no information was available for this metabolite, therefore if a dietary risk assessment were to be required the genotoxicity TTC value of 0.0025 µg/kg bw per day should be used.

The table below provides an overall summary overview of the toxicological characterization of these metabolites.

Summary of benzpyrimoxan and its metabolites

Compound, code and structure	Rat ADME Toxicity covered by toxicological properties of parent compound (content in rat biofluids > 10% of absorbed dose)?	Genotoxicity assessment (data, QSAR, read-across)	General toxicity	Reference value for dietary risk assessment
Benzpyrimoxan (NNI-1501) 	Not applicable	Unlikely to be genotoxic (data)	Full data set	ADI: 0.1 mg/kg bw per day
NNI-1501-acid, (DH-01) 	Yes (high levels in plasma)	Unlikely to be genotoxic as covered by parent	Covered by parent	Parent ADI
NNI-1501-CH_2OH (DH-02) and its glucuronide 	Yes (high levels in plasma)	Unlikely to be genotoxic as covered by parent	Covered by parent	Parent ADI
NNI-1501-aldehyde (DH-03) 	No (not found in rat)	Unlikely to be genotoxic (QSAR and RA)	No information	TTC Cramer class III value: 1.5 µg/kg bw per day
NNI-1501-2-OH (DH-04) 	No (not found in rat)	Unlikely to be genotoxic (data)	Three times more toxic than parent based on 90-day study	Approximately three times more toxic than parent
NNI-1501-acid-2-OH (DH-05) 	Yes (high levels in urine)	Unlikely to be genotoxic as covered by parent	Covered by parent	Parent ADI
NNI-1501-CH_2OH-2-OH (DH-06) and its glucuronide 	No (low levels in rat)	Unlikely to be genotoxic (QSAR and RA)	No information	TTC Cramer class III value: 1.5 µg mg/kg bw per day

Compound, code and structure	Rat ADME Toxicity covered by toxicological properties of parent compound (content in rat biofluids > 10% of absorbed dose)?	Genotoxicity assessment (data, QSAR, read-across)	General toxicity	Reference value for dietary risk assessment
NNI-1501-aldehyde-2-OH (DH-07) 	No (not found in rat)	Unlikely to be genotoxic (QSAR and RA)	No information	TTC Cramer class III value: 1.5 µg/kg bw per day
NNI-1501-enamine-aldehyde, (DH-08) 	No (not found in rat)	No information available	No information available	TTC for genotoxicity: 0.0025 µg/kg bw per day
NNI-1501-benzoic acid (DH-101) 	Yes (high levels in plasma)	Unlikely to be genotoxic as covered by parent	Covered by parent	Parent ADI
NNI-1501-benzyl alcohol (DH-102) 	No (not found in rat or at very low levels)	Unlikely to be genotoxic (Ames + QSAR)	No information available	TTC Cramer class III value: 1.5 µg/kg bw per day
NNI-1501-benzoyl glycine (DH-402) 	Yes (high levels in urine and kidneys + similar to major rat metabolite DH-101)	Unlikely to be genotoxic as covered by parent	Covered by parent	Parent ADI

QSAR Quantitative structure–activity relationship RA Read-across; TTC: Threshold of toxicological concern

Microbiological data

There was no information available in the public domain and no experimental data were submitted that addressed the possible impact of benzpyrimoxan residues on the human intestinal microbiome.

Human data

No information was available from the sponsor since benzpyrimoxan is a new compound. A literature search provided no information.

The Meeting concluded that the existing database on benzpyrimoxan was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

Toxicological evaluation

The Meeting established an ADI for benzpyrimoxan of 0–0.1 mg/kg bw based on the NOAEL of 10 mg/kg bw per day from the rabbit developmental study. A safety factor of 100 was applied. The rabbit NOAEL is supported by the NOAEL of 12 mg/kg bw per day for parental, offspring and reproductive toxicity in the two-generation rat study, the chronic NOAEL of 14 mg/kg bw per day from the rat two-year study, and the NOAEL of 14.3 mg/kg bw per day from the one-year dog study.

The parent compound's ADI applies also to DH-01, DH-05 and DH-04 (multiplied by three).

The Meeting concluded that it was not necessary to establish an acute reference dose (ARfD) for benzpyrimoxan in view of its low acute oral toxicity and the absence of developmental toxicity, neurotoxicity or any other toxicological effects that would be likely to be elicited by a single dose.

Levels relevant to risk assessment of benzpyrimoxan

Species	Study	Effect	NOAEL	LOAEL
Mouse	78-week study of carcinogenicity [a]	Toxicity	400 ppm, equal to 40 mg/kg bw per day	1500 ppm, equal to 163 mg/kg bw per day
		Carcinogenicity	1500 ppm, equal to 163 mg/kg bw per day [c]	
Rat	Acute neurotoxicity study [b]	Neurotoxicity General toxicity	2000 mg/kg bw [c] 2000 mg/kg bw [c]	
	Two-year studies of toxicity and carcinogenicity [a]	Toxicity	300 ppm, equal to 14 mg/kg bw per day	1500 ppm, equal to 69 mg/kg bw per day
		Carcinogenicity	1500 ppm, equal to 69 mg/kg bw per day [c]	
	Two-generation study of reproductive toxicity [a]	Reproductive toxicity	300 ppm, equal to 12 mg/kg bw per day	2000 ppm, equal to 85 mg/kg bw per day
		Parental toxicity	300 ppm, equal to 12 mg/kg bw per day	2000 ppm, equal to 85 mg/kg bw per day
		Offspring toxicity	300 ppm, equal to 12 mg/kg bw per day	2000 ppm, equal to 85 mg/kg bw per day
	Developmental toxicity study [b]	Maternal toxicity	50 mg/kg bw per day	250 mg/kg bw per day
		Embryo/fetal toxicity	250 mg/kg bw per day [c]	
Rabbit	Developmental toxicity study [b]	Maternal toxicity	10 mg/kg bw per day	30 mg/kg bw per day
		Embryo/fetal toxicity	10 mg/kg bw per day	30 mg/kg bw per day
Dog	13-week and one-year studies of toxicity [d]	Toxicity	500 ppm, equal to 16 mg/kg bw per day	2500 ppm, equal to 67 mg/kg bw per day
Metabolite DH-04				
Rat	13-week study of toxicity [a]	Toxicity	300 ppm, equal to 19.6 mg/kg bw per day	1000 ppm, equal to 65.2 mg/kg bw per day

[a] Dietary administration

[b] Gavage administration

[c] Highest dose tested

[d] Two or more studies combined

Acceptable daily intake (ADI), applies to benzpyrimoxan, DH-01, DH-05 and DH-04 (multiplied by 3)

0–0.1 mg/kg bw

Acute reference dose (ARfD)

Not necessary

Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure.

Critical end-points for setting guidance values for exposure to benzpyrimoxan

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid and extensive; 80% at 1 mg/kg bw
Dermal absorption	No data
Distribution	Wide; highest concentrations in liver, plasma, GI tract, kidney and adrenals
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	Rapid and nearly complete by 72 hours post dose via bile and urine
Metabolism in animals	Extensively metabolized
Toxicologically significant compounds in animals and plants	Parent, DH-01, DH-04 and DH-05
Acute toxicity	
Rat, LD_{50}, oral	> 2000 mg/kg bw
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	> 3.9 mg/L
Rabbit, dermal irritation	Mildly irritating
Rabbit, ocular irritation	Mildly irritating
Guinea pig, dermal sensitization	Sensitizing (Magnussen & Kligmann)
Short-term studies of toxicity	
Target/critical effect	Liver weight and histopathology with associated clinical chemistry
Lowest relevant oral NOAEL	16 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	200 mg/kg bw per day (rat)
Lowest relevant inhalation NOAEC	0.1 mg/L (rat)
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Decreased body weight, body weight gain, feed consumption, haematology and liver toxicity
Lowest relevant NOAEL	14 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic
Genotoxicity	Unlikely to be genotoxic
Reproductive toxicity	
Target/critical effect	**Parental**: decreases in body weight, body weight gain and feed consumption; toxicity to liver and thyroid **Offspring**: decreased pup body weight, low viability index days 0, 4 and 14 **Reproductive**: decreased gestation index in F1 generation and increased post implantation loss in F1 and F2
Lowest relevant parental NOAEL	12 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	12 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	12 mg/kg bw per day (rat)

Developmental toxicity	
Target/critical effect	**Maternal:** decreased body weight, body weight gain, feed consumption and gravid uterine weight, two abortions **Embryo/fetal:** reduced fetal weight
Lowest relevant maternal NOAEL	10 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	10 mg/kg bw per day (rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	> 2000 mg/kg bw, highest dose tested (rat).
Subchronic neurotoxicity NOAEL	No evidence from routine studies
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity	No evidence from routine studies
Studies on toxicologically relevant metabolites	
Metabolite DH-04	Acute oral LD_{50}: > 2000 mg/kg bw (rat) 90-day NOAEL: 19.6 mg/kg bw per day (rat) Not genotoxic (Ames, micronucleus in vitro)
Microbiological data	No information available
Human data	No clinical cases or poisoning incidents have been recorded

Summary

	Value	Study	Safety factor
ADI	0–0.1 mg/kg bw [a]	Rabbit developmental study	100
ARfD	Unnecessary		

[a] ADI applies to benzpyrimoxan, DH-01, DH-05 and DH-04 (multiplied by 3)

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Broflanilide

First draft prepared by

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Explanation

Broflanilide is the common name approved by the International Organization for Standardization (ISO) for *N*-[2-bromo-4-(perfluoropropan-2-yl)-6-(trifluoromethyl)phenyl]-2-fluoro-3-(*N*-methylbenzamido) benzamide (IUPAC), with the Chemical Abstracts Service number 1207727-04-5.

It is a meta-diamide pro-insecticide that exerts its pesticidal mode of action (MOA) by binding to an inter-subunit allosteric site on the ionotropic γ-aminobutyric acid (GABA) receptor, resulting in a blocking of inhibitory neurotransmission.

Broflanilide has not previously been evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and was reviewed by the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR).

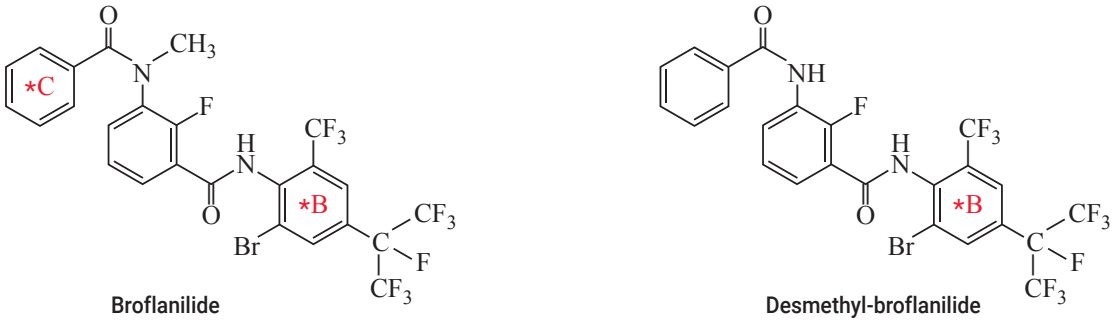
All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with relevant national or international test guidelines, unless otherwise specified. No additional information from a literature search was identified that complemented the toxicological information submitted for the current assessment.

Evaluation for acceptable daily intake

1. Biochemical aspects

The absorption, distribution, metabolism and excretion (ADME) of broflanilide (code names used include MCI-8007 and BAS 450 I) were investigated in rats following the administration of broflanilide in a single oral low or high dose, to intact and bile duct-cannulated rats. The test item was labelled with ^{14}C, incorporated either in either the B-ring or C-ring (see Fig. 1). Broflanilide radiolabelled in the B-ring was administered intravenously in a single low dose, and orally for up to 14 days. In addition, a comparative in vitro metabolism study was conducted with human, mouse and rat hepatocytes using broflanilide and its metabolite desmethyl-broflanilide (DM-8007, M11) radiolabelled with ^{14}C in the B-ring.

Figure 1. Position of radiolabels in broflanilide (MCI-8007) and its metabolite desmethyl-broflanilide (DM-8007) used in ADME studies



*B denotes [B-ring(U)^{14}C] radiolabelled material

*C denotes [C-ring(U)^{14}C] radiolabelled material

(Redrawn from Rabe, 2020a, b)

1.1 Absorption, distribution and excretion

(a) Oral route

In a toxicokinetic study to investigate whether absorption or bioavailability saturation effects occur at high dose levels, four Han Wistar rats of each sex per group were administered [*B-ring-U*-^{14}C]broflanilide (purity 98.2%) at a single oral dose of 20, 100 or 500 mg/kg bw. The vehicle was 1.0% (w/v) carboxymethyl cellulose (CMC). Animals were evaluated for mortality and clinical signs of toxicity

twice a day and for clinical signs once a day. Blood was sampled at 0.5, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144 and 168 hours after dosing. Radioactivity was measured by liquid scintillation counting (LSC).

No mortality or clinical signs were observed.

In plasma, the data indicated that the peak concentration (C_{max}) and the area under the concentration–time curve (AUC) did increase with increasing dose, the increase seen being less than dose proportional. The C_{max} and AUC values increased by a factor of around 14–16 between 20 and 500 mg/kg bw, compared with an expected factor of 25. No substantial differences were observed between sexes, with male to female ratios of C_{max} and AUC ranging from 1.0 to 1.4. Concentrations of total radioactivity present in the red blood cells (RBCs) were lower than in plasma during the first part of their concentration–time curves, but after approximately 72–96 hours the concentrations in plasma and RBCs were similar, or indeed the concentrations in RBCs were even higher than in plasma, with blood/plasma values of 1and greater. This result together with the longer half life ($t_{½}$) of elimination values suggests that the labelled broflanilide was significantly distributed towards erythrocytes and only slowly released.

In RBCs, the C_{max} and AUC values did increase with increasing dose, however this increase was less than dose proportional, as was seen for plasma. The C_{max} and AUC values increased by a factor of around 10–19 between 20 to 500 mg/kg bw, compared to an expected factor of 25. No substantial differences were observed between sexes, with male to female ratios of C_{max} and AUC ranging from 0.8 to 1.6 (Wenker, 2012).

Table 1. Toxicokinetic parameters of [B-ring-U-^{14}C]broflanilide in plasma and red blood cells

Medium	Dose (mg/kg bw)	C_{max} (mg/L)		T_{max} (h)		Terminal half-life (h)		AUC_{last} (h × mg/L)		AUC_{∞} (h × mg/L)	
		male	female	male	female	male	female	male	female	male	female
Plasma	20	1.23	1.08	4–8	4–8	51.8	90.1	61.3	71.8	68.3	98.3
	100	5.18	3.59	4–8	2–4	52.3	81.1	247	204	275	271
	500	17.1	15.0	8	2–4	57.6	63.7	945	892	1080	1061
RBC	20	0.47	0.29	1–8	24–120	86.3	285	35.7	40.5	54.3	118
	100	1.58	1.45	8	2–8	131	206	134	142	212	328
	500	4.41	5.52	8–48	4–48	93.8	205	475	627	738	1375

RBC: Red blood cell; h: Hours; $t_{½}$: Terminal elimination half-life; Source: Wenker, 2012
C_{max}: Maximum concentration; AUC_{last}: Area under the concentration–time curve until last measurement;
T_{max}: Time taken to reach C_{max}; AUC_{∞}: Area under the concentration–time curve until infinity

In a pharmacokinetics, absorption, distribution and excretion study, male and female Han Wistar rats were orally (gavage) administered [*C-ring-U*-^{14}C]broflanilide (purity 95.2%–97.7%), or [*B-ring-U*-^{14}C]broflanilide (purity 97.0%–99.2%), giving a single dose of 5 mg/kg bw (B- and C-labelled) and 500 mg/kg bw (C-labelled only), or a single intravenous (i.v.) dose at 1.6 mg/kg bw (B-labelled only). The vehicle for the oral doses was 1.0% (w/v) CMC. The intravenous vehicle was a dimethyl sulfoxide : ethanol : saline mixture, in the volume ratios of 2 : 4 : 4. In each group (oral and i.v. administration) blood samples were taken over predetermined time intervals up to seven days after administration. Excretion samples were obtained over a seven-day period, with expired air collected over the first 24 hours following oral doses. Residual radioactivity was measured in selected tissues/organs and in the remaining carcasses. Radioactivity was measured by LSC.

Following a single oral dose of [*C-ring-U*-^{14}C]broflanilide at 5 mg/kg bw the majority of radioactivity was excreted in faeces (0–168 hours) accounting for 91.2% and 78.3% of administered dose (AD) in males and females, respectively; most of this was excreted during 0–48 hours (87.2% and 70.0%, of AD respectively). Radioactivity excreted in faeces during 0–72 hours in males and females was 89.1% and 73.0% of AD respectively. Radioactivity recovered in urine during 0–168 hours accounted for 7.6% and 13.6% of AD in males and females, respectively, the majority of which was excreted during 0–48 hours (6.4% and 11.0%, of AD respectively). Radioactivity excreted in urine during 0–72 hours in males and females was 6.8% and 12.0% of AD respectively. Radioactivity remaining in the carcass

at 168 hours accounted for 0.98% or less of AD. Overall recoveries were 100.1% and 93.8% of AD for the males and females, respectively. The total radioactivity retained in tissues accounted for 0.71% and 1.45% of AD in males and females respectively. The highest concentrations were measured in fat (0.345 and 0.770 µg equiv./g in males and females respectively). Concentrations of 0.023–0.093 µg equiv./g were measured in the kidney, liver, pancreas and adrenal gland in both sexes, epididymis in males and thyroid, spleen, ovaries and uterus in females.

Following a single oral dose of [*C-ring-U*-^{14}C]broflanilide at 500 mg/kg bw the majority of radioactivity was excreted in faeces, accounting for 96.5% and 100.4% of AD in males and females respectively, during 0–168 hours. Most of the radioactivity in faeces was excreted during 0–48 hours (90.4 and 98.1%, of AD respectively). Radioactivity excreted in faeces during 0–72 hours in males and females was 95.6% and 99.7% of AD respectively. Radioactivity recovered in urine during 0–168 hours accounted for 1.53% and 1.41% of AD in males and females respectively, the majority of which was excreted during 0–48 hours (1.4 and 1.3%, of AD respectively). Radioactivity excreted in urine during 0–72 hours in males and females was 1.44% and 1.31% of AD respectively. Radioactivity remaining in the carcass at 168 hours accounted for 0.1% of AD. Overall recoveries were 98.3% and 102.0% of AD for the males and females respectively. The total radioactivity retained in tissues accounted for 0.10% of dose in both sexes. The highest concentrations were measured in fat (4.00 and 6.55 µg equiv./g in males and females respectively). Concentrations of 0.187–1.53 µg equiv./g were measured in the kidney, liver and pancreas in both sexes, epididymis in males and heart, lungs, adrenal glands, ovaries and uterus in females.

Following a single oral dose of [*B-ring-U*-^{14}C]broflanilide at 5 mg/kg bw the majority of radioactivity was excreted in faeces, accounting for 93.8% and 99.5% of AD in males and females respectively, during 0–168 hours. Most of the radioactivity in faeces was excreted during 0–48 hours (87.9% and 93.0%, of AD respectively). Radioactivity excreted in faeces during 0–72 hours in males and females was 90.5% and 95.7% of AD respectively. Radioactivity recovered in urine during 0–168 hours accounted for 0.27% and 0.47% of AD in males and females respectively, the majority of which was excreted during 0–48 hours (0.19% and 0.32%, of AD respectively). Radioactivity excreted in urine during 0–72 hours in males and females was 0.22% and 0.37% of AD respectively. Radioactivity remaining in the carcass at 168 hours accounted for 0.9% or less of AD. Overall recoveries were 94.9% and 101.3% of AD for the males and females respectively. The total radioactivity retained in tissues accounted for 0.33% and 0.49% of AD in male and female rats respectively. The highest concentrations were measured in fat (0.314 and 0.630 µg equiv./g in males and females respectively). Concentrations of 0.035–0.126 µg equiv./g were measured in the kidney, liver, pancreas, adrenal gland and thyroid in both sexes, epididymis in males and lungs, spleen, ovaries and uterus in females.

Table 2. Excretion balance (percentage of administered radioactivity) and tissue concentration (all values shown are µg equiv./g) in rats administered C- and B-ring ^{14}C radiolabelled broflanilide

Position of ^{14}C label		C-ring label				B-ring label	
Dose groups		5 mg/kg bw		500 mg/kg bw		5 mg/kg bw	
Sex		Male	Female	Male	Female	Male	Female
Number of animals		4	4	4	4	4	4
Matrix	**Time (h)**						
Urine	0–6	3.33	4.41	0.78	0.72	0.03	0.07
	6–12	0.97	2.84	0.27	0.23	0.05	0.08
	12–24	1.05	1.87	0.18	0.18	0.06	0.09
	24–48	1.01	1.89	0.14	0.13	0.05	0.08
	48–72	0.48	1.02	0.07	0.05	0.03	0.05
	72–96	0.32	0.67	0.04	0.04	0.02	0.04
	96–120	0.21	0.43	0.02	0.03	0.01	0.03
	120–144	0.17	0.27	0.01	0.02	0.01	0.03
	144–168	0.08	0.17	0.02	0.01	0.01	0.02
	0–168	7.62	13.57	1.53	1.41	0.27	0.47

Position of ^{14}C label		C-ring label				B-ring label	
Dose groups		5 mg/kg bw		500 mg/kg bw		5 mg/kg bw	
Sex		Male	Female	Male	Female	Male	Female
Number of animals		4	4	4	4	4	4
Matrix	**Time (h)**						
Faeces	0–12	16.49	6.90	14.42	19.63	26.13	14.12
	12–24	50.47	34.09	48.8	44.3	49.25	64.82
	24–48	20.28	29.03	27.22	34.16	12.48	14.02
	48–72	1.83	3.00	5.14	1.59	2.66	2.75
	72–96	0.98	2.01	0.73	0.44	1.38	1.56
	96–120	0.56	1.52	0.08	0.07	0.98	1.04
	120–144	0.34	0.97	0.04	0.06	0.60	0.75
	144–168	0.28	0.79	0.04	0.11	0.35	0.52
	0–168	91.22	78.31	96.47	100.37	93.83	99.51
Aqueous cage wash	0–168	0.41	0.37	0.11	0.05	0.04	0.05
Ethanol cage wash	0–168	0.20	0.32	0.07	0.02	0.02	0.04
Subtotal cage washes	0–168	0.61	0.69	0.18	0.08	0.05	0.09
Expired air	0–24	ND	0.02	0.01	ND	<0.01	ND
Residual carcasses	168	0.53	0.98	0.07	0.10	0.48	0.87
Liver	168	0.02	0.04	<0.01	0.01	0.09	0.10
GI tract and contents	168	0.12	0.23	0.01	0.02	0.14	0.26
Total recovery	0–168	100.12	93.84	98.26	101.98	94.87	101.32
Carcass	168	0.031	0.070	0.417	0.622	0.029	0.059
Plasma	168	0.004	0.011	ND	ND	0.009	0.016
Whole blood	168	0.004	0.009	ND	ND	0.012	0.022
Blood cells	168	0.003	0.005	ND	ND	0.017	0.030
Brain	168	ND	0.004	ND	ND	0.006	0.013
Heart	168	0.006	0.016	ND	0.187	0.012	0.029
Kidney	168	0.037	0.046	0.580	0.431	0.050	0.056
Liver	168	0.023	0.059	0.416	0.811	0.092	0.126
Lungs	168	0.011	0.022	ND	0.221	0.018	0.037
Pancreas	168	0.030	0.074	0.347	0.661	0.035	0.086
Spleen	168	0.012	0.028	ND	ND	0.019	0.052
Adrenal glands	168	0.026	0.068	ND	0.745	0.041	0.097
Pituitary gland	168	ND	ND	ND	ND	ND	ND
Thyroid	168	ND	0.049	ND	ND	0.074	0.104
Epididymis	168	0.093	0.056	1.53	1.15	0.081	0.121
Testes/ovary/uterus	168	0.003	0.029	0.167	0.347	0.009	0.081
Bone	168	ND	ND	ND	ND	ND	ND
Bone marrow	168	0.006	0.023	ND	ND	ND	0.030
Fat (abdominal)	168	0.345	0.770	4.00	6.550	0.314	0.630
Muscle (skeletal)	168	0.006	0.012	ND	ND	0.011	0.019
GI tract and contents	168	0.047	0.131	0.466	0.769	0.061	0.121

GI: Gastrointestinal; ND: Not detected (less than twice background value); Source: Townley, 2017a

Following a single oral administration of [*C-ring-U*-^{14}C]broflanilide at a dose level of 5 mg/kg bw, peak mean plasma radioactivity concentrations in males and females were reached at two hours and one hour respectively. At the higher dose level of 500 mg/kg bw mean plasma concentrations were reached at one hour and 0.5 hours respectively. The time to peak concentration (T_{max}) was the same for whole blood and plasma at both dose levels. At the dose level of 5 mg/kg bw, $t_{½}$ was similar in males (45.4 hours) and females (42.0 hours). The rate and extent of systemic exposure to the administered radioactivity in rats, as reflected by the parameters C_{max} and AUC_t (AUC until the last quantifiable sample) in plasma, increased with increasing dose of [*C-ring-U*-^{14}C]broflanilide from 5 to 500 mg/kg bw. However, these increases were less than proportionate dose administered. Systemic exposure to radioactivity in plasma and whole blood in females was higher than in males. However, at the higher 500 mg/kg bw dose level systemic exposure in males and females was broadly similar, with the exception of the plasma AUC_s values at the 500 mg/kg bw dose level, which were lower in females. The levels of radioactivity for individual animals at the high dose, in which quantifiable values were obtained, were comparable between females and males, however the numbers of animals reported as lower than the limit of quantification (<LOQ) was more frequent in females than males.

Results indicated that relatively little distribution of radioactivity into red blood cells occurred.

Following intravenous bolus administration of labelled broflanilide at a nominal dose of 1.6 mg/kg bw, the T_{max} of radioactivity in plasma and whole blood was 0.25 hours, that is, the first sampling time. The $t_{½}$ of radioactivity after intravenous administration therefore appeared to be broadly similar to that for oral administration. No remarkable sex differences were observed in the parameters for systemic exposure (C_{max} and AUC_t or AUC).

Following a single oral administration of [*B-ring-U*-^{14}C]broflanilide at a dose level of 5 mg/kg bw peak mean plasma concentrations of 0.223 µg equiv./g (males) and 0.148 µg equiv./g (females) were reached at four hours, which was later than observed following administration the C-ring-labelled broflanilide (1–2 hours). Peak mean whole blood concentrations of 0.132 µg equiv./g (males) and 0.095 µg equiv./g (females) were reached at 4 and 12 hours respectively. Systemic exposure to radioactivity in plasma and whole blood in males and females was broadly similar.

Overall, the whole blood to plasma ratios (calculated using AUC_t) indicated that relatively little distribution of radioactivity into RBCs occurred at either dose level, with either label. Following administration of the [*B-ring-U*-^{14}C]broflanilide (5 mg/kg bw, low dose), the C_{max} values in male plasma and whole blood were similar to the values for rats to whom [*C-ring-U*-^{14}C]broflanilide had been administered at the low dose level, although AUC_t values were slightly higher. In females, the C_{max} values were lower than those administered [*C-ring-U*-^{14}C]broflanilide, but the AUC_t values were similar. Following oral gavage administration of [*B-ring-U*-^{14}C]broflanilide the oral bioavailability (*F*) of total radioactivity, based on plasma AUC and the nominal administered doses (5 mg/kg bw oral and 1.6 mg/kg bw intravenous), was 16.1% in males and 12.7% in females (Townley, 2017a).

Table 3. Pharmacokinetic parameters of radioactivity in plasma and whole blood following a single administration (oral and i.v.) of C-ring and B-ring labelled broflanilide, at different dose levels

Position of ^{14}C label	**C-ring label**				**B-ring label**			
Dose groups	**5 mg/kg bw (oral)**		**500 mg/kg bw (oral)**		**1.6 mg/kg bw (intravenous)**		**5 mg/kg bw (oral)**	
Sex	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Number of rats	**12**	**12**	**12**	**12**	**12**	**12**	**12**	**12**
				Plasma				
C_0 (µg equiv./g)	NA	NA	NA	NA	2.51	1.99	NA	NA
C_{max} (µg equiv./g)	0.230	0.470	3.25	3.32	1.69	1.30	0.223	0.148
T_{max} (hours)	2	1	1	0.5	0.25	0.25	4	4
AUC_t (µg equiv.h/g)	7.67	12.9	120	23.9	22.2	22.8	10.2	9.61
AUC_∞ (µg equiv.h/g)	8.18	13.7	[162]	[42.0]	23.8	28.4	[12.0]	11.3
K (per hour)	0.0153	0.0165	[0.0121]	[0.0684]	0.0152	0.0092	[0.0088]	0.0112

Position of ^{14}C label	C-ring label				B-ring label			
Dose groups	5 mg/kg bw (oral)		500 mg/kg bw (oral)		1.6 mg/kg bw (intravenous)		5 mg/kg bw (oral)	
Sex	Males	Females	Males	Females	Males	Females	Males	Females
Number of rats	12	12	12	12	12	12	12	12
$t_{½}$ (hours)	45.4	42.0	[57.5]	[10.1]	45.7	75.3	[78.5]	62.0
				Whole blood				
C_0 (µg equiv./g)	NA	NA	NA	NA	1.62	1.05	NA	NA
C_{max} (µg equiv./g)	0.135	0.271	2.18	2.29	0.973	0.667	0.132	0.095
T_{max} (hours)	2	1	1	0.5	0.25	0.25	4	12
AUC_t (µg equiv.h/g)	4.37	7.80	17.0	15.6	16.0	17.7	6.09	7.30
AUC (µg equiv.h/g)	[5.10]	8.37	[28.8]	[25.1]	18.4	[27.1]	6.94	[10.8]
K (per hour)	[0.0134]	0.0157	[0.0752]	[0.0828]	0.0117	[0.0061]	0.0132	[0.0065]
$t_{½}$ (hours)	[51.8]	44.2	[9.2]	[8.4]	59.1	[114.5]	52.4	[106.7]

NA: Not applicable; $t_{½}$: Half-life of terminal elimination; Source: Townley, 2017a

[bracketed values] indicate that 50% or more of the samples were below limit of quantification (<LOQ);

C_0: Concentration at $t = 0$; C_{max}: Maximum concentration; T_{max}: Time taken to reach C_{max};

AUC_t: The area under the concentration–time curve up to the time of the last quantifiable sample;

AUC_∞: The area under the concentration–time curve until infinity

In an absorption, distribution (liver and GIT) and excretion study, [*B-ring-U*-^{14}C]broflanilide (purity 97.2%) and [*C-ring-U*-^{14}C]broflanilide (purity 97.2–97.6%) were administered by gavage to male and female bile duct-cannulated Han Wistar rats as a single dose of 5 mg/kg bw and also [*C-ring-U*-^{14}C]broflanilide as a single dose of 500 mg/kg bw. Bile, urine and faeces were collected at predetermined intervals up to 48 hours after dosing. The vehicle used was 1.0% (w/v) CMC. Radioactivity was measured by LSC.

After single oral doses of either radiolabelled broflanilide, generally more than 85% of the AD was excreted within 48 hours. Excretion was mainly via the faeces and this accounted for 71%–80% of AD at 5 mg/kg bw and 98% of AD at 500 mg/kg bw. Urinary excretion during the period 0–48 hours accounted for 8%–10% of AD at the low dose and 1% of AD at the high dose level when the C-ring label was employed. By contrast recovery with the B-ring label at the low dose was less than 3% (females) and 1% (males) of AD, indicating that in urine there were metabolites specific with regard to the C-ring. Biliary excretion when the C-ring was employed accounted for 3–4% of AD at the low dose and less than 1% of AD at the high dose; with B-ring-labelled recovery in bile was 9%–10% of AD at the low dose level, indicating that in bile there were metabolites specific with regard to the B-ring. There were no major differences in the patterns of excretion between sexes.

Absorption was assessed in bile duct-cannulated rats after administration of single oral doses and calculated as the sum of the percentages of AD recovered in bile, urine, liver and carcass. The total absorbed with the C-ring label was 14–19% of AD at 5 mg/kg bw and 2% of AD at the 500 mg/kg bw; with the B-label total absorbed was 16–23% of AD at the 5 mg/kg bw dose level (Stroud, 2017). A summary of results is shown below in Table 4.

Table 4. Excretion balance (percentage of administered radioactivity) and tissue concentration (µg equiv./g) in bile duct-cannulated rats administered C- and B-ring ^{14}C radiolabelled broflanilide

Position of ^{14}C label		C-ring label				B-ring label	
Dose groups		5 mg/kg bw		500 mg/kg bw		5 mg/kg bw	
Sex		M	F	M	F	M	F
Number of animals		**4**	**4**	**5**	**0**	**5**	**5**
Matrix	**Time (hours)**						
Bile	0–3	0.29	0.15	0.11	-	1.03	0.74
	3–6	0.52	0.41	0.08	-	1.27	1.24
	6–9	0.47	0.29	0.05	-	1.08	0.84
	9–12	0.43	0.48	0.03	-	1.00	1.20
	12–24	1.05	0.98	0.06	-	2.64	2.20
	24–48	0.97	0.84	0.06	-	3.04	2.70
	Total (0–48)	**3.73**	**3.14**	**0.37**	-	**10.06**	**8.92**
Urine	0–12	5.49	6.38	1.26	-	0.26	0.90
	12–24	1.15	1.71	0.13	-	0.09	0.82
	24–48	1.07	1.95	0.10	-	0.09	1.40
	Total (0–48)	**7.71**	**10.03**	**1.49**	-	**0.44**	**3.12**
Faeces	0–12	7.95	6.29	14.86	-	25.78	8.81
	12–24	59.1	33.77	58.74	-	43.28	45.59
	24–48	13.16	31.26	24.02	-	10.16	19.41
	Total (0–48)	**80.20**	**71.32**	**97.62**	-	**79.21**	**73.81**
Aqueous cage wash (0–48)	0.04	0.03	0.02	-	0.03	0.16	
Ethanol cage wash (0–48)	0.12	0.08	0.02	-	0.02	0.14	
Total cage washes (0–48)	0.16	0.11	0.04	-	0.05	0.30	
Residual carcass	2.46	5.32	0.35	-	5.32	9.79	
Liver	0.10	0.27	0.02	-	0.40	0.80	
GI tract and contents	1.36	1.57	1.25	-	1.25	2.32	
Total recovery	95.71	91.76	101.14	-	96.85	99.00	
Total absorption	13.99	18.76	2.24	-	16.21	22.63	

M: Male; F: Female; GI: Gastrointestinal; Source: Stroud, 2017

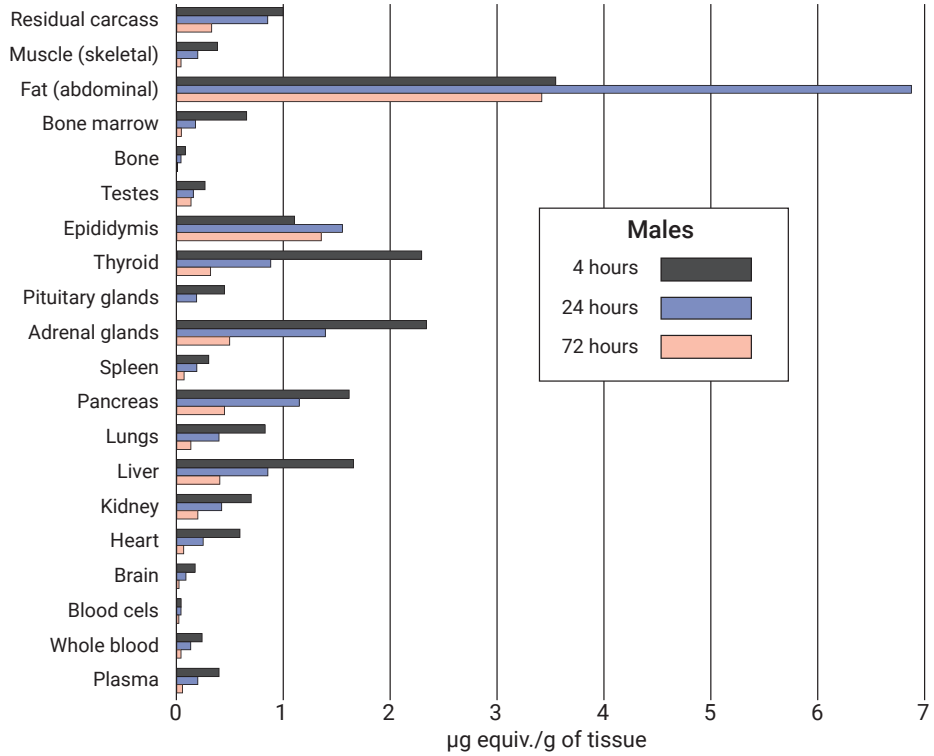
Study 1

In a tissue depletion study, 12 male and 12 female Han Wistar rats per dose group were administered, as a single oral (gavage) dose, [*B-ring-U*-^{14}C]broflanilide (purity 95.1%–95.5%) at 5 mg/kg bw or [*C-ring-U*-^{14}C]broflanilide (purity 97.5%–97.6%) at 500 mg/kg bw. Groups of eight rats (four of each sex) were sacrificed at 4, 24 and 72 hours post dose for B-ring groups, and similarly eight rats from the C-ring groups, but these at 1, 8 and 24 hours post dose. The vehicle used was 1.0% (w/v)CMC. Residual radioactivity was measured in selected tissues/organs and carcass remains using LSC.

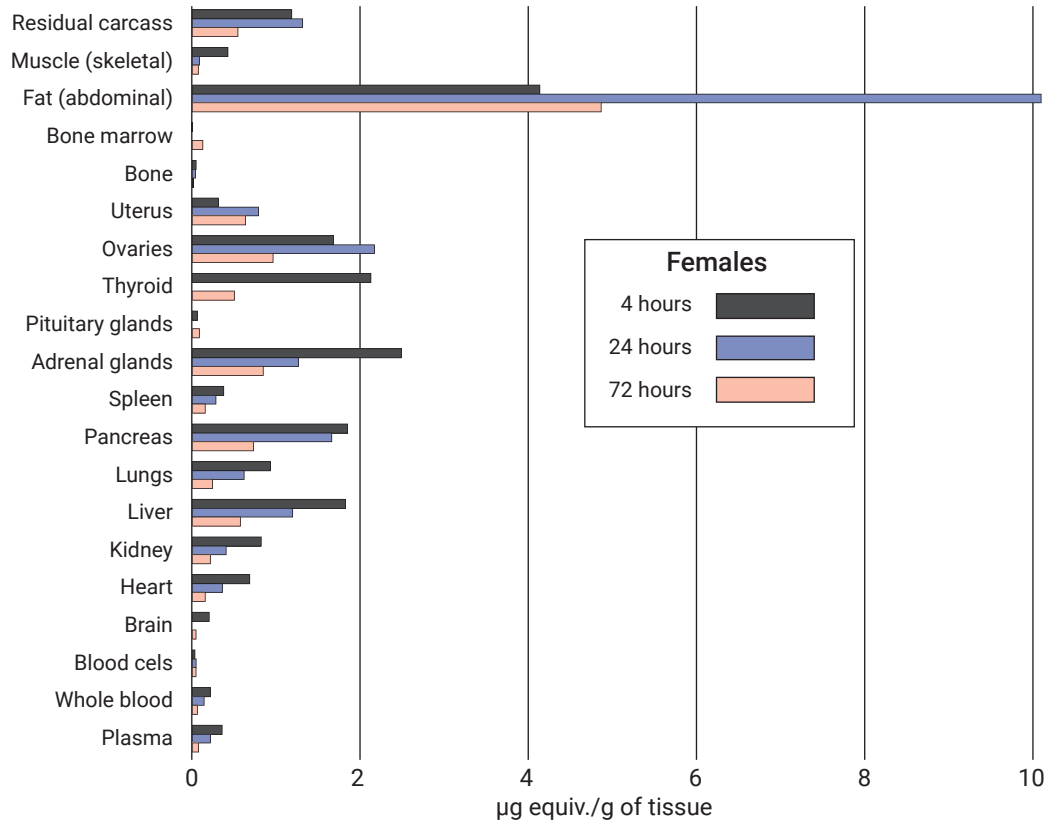
After a single dose of [*B-ring-U*-^{14}C]broflanilide at 5 mg/kg bw, peak tissue concentrations generally occurred at four hours, with greatest concentrations (excluding the GI tract) in the fat, followed by liver, pancreas, adrenal glands, thyroid, epididymis and ovaries. With the exception of whole blood, blood cells, brain, spleen (males only), testes, bone and bone marrow (females only), concentrations of radioactivity in tissues were similar to, or greater than, that measured at the same time in plasma. At 24 hours the concentration in fat had increased approximately two-fold, and concentrations in epididymides and ovaries also increased, but to a lesser extent than in fat. Concentrations in most other tissues remained similar to those at four hours or had declined. At 72 hours, tissue radioactivity had significantly declined in the majority of tissues, in a pattern similar to earlier time points. There was no notable sex difference in the distribution of radioactivity or the observed concentrations.

Figure 2. Mean tissue concentration of radioactivity (in mg/kg tissue) after single oral administration of [B-ring-U-^{14}C]broflanilide at a dose level of 5 mg/kg bw at 4, 24 and 72 hours.

(a) Males



(b) Females

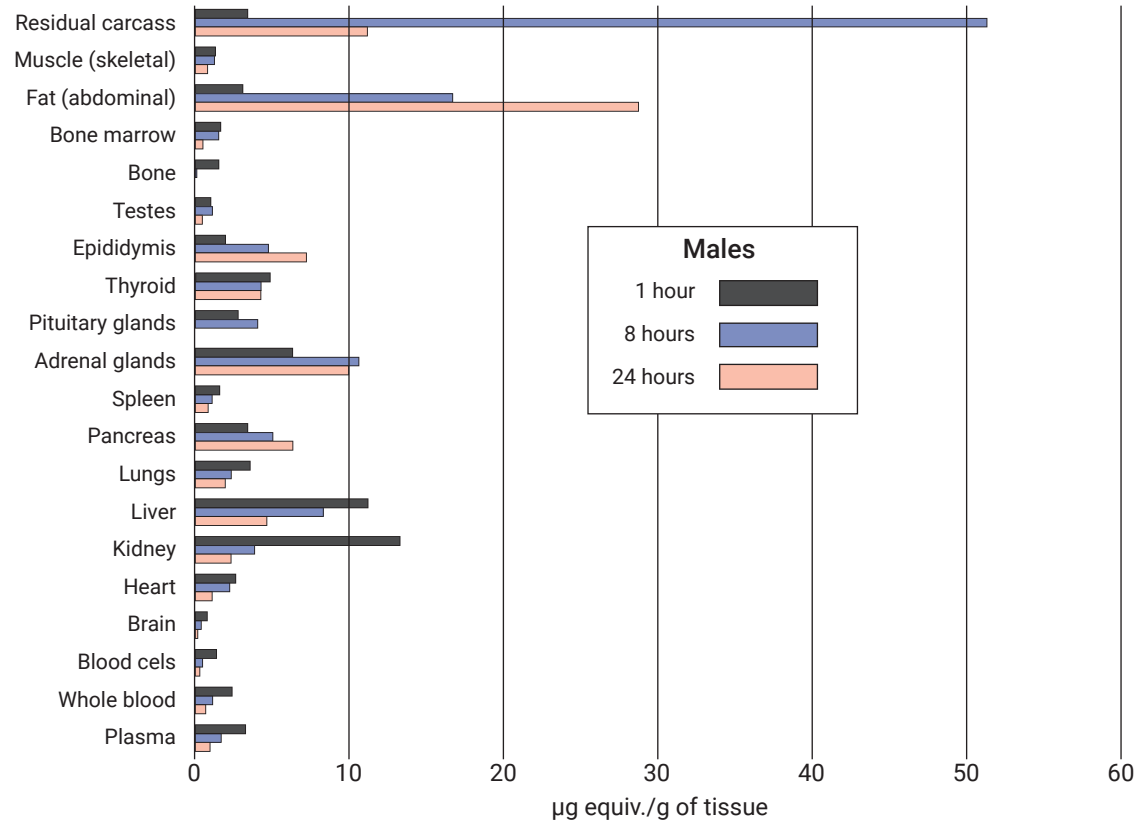


(Redrawn from Townley, 2017b)

After a single dose of [*C-ring-U*-^{14}C]broflanilide at 500 mg/kg bw peak tissue concentrations generally occurred at one hour post dose with the highest concentrations (excluding GI tract) in the kidney and liver, followed by plasma (males), pancreas, adrenal glands, lungs, fat (male), thyroid, and ovaries. At eight hours, concentrations in fat had increased approximately five-fold, and concentrations in male adrenal glands, pituitary gland and testes also increased, but to a lesser extent than in fat. Concentrations in most other tissues declined but, with the exception of female pituitary gland, were above the limit of detection. At 24 hours tissue radioactivity had significantly declined in the majority of tissues with a pattern similar to that observed at eight hours post dose. While there was no notable sex difference in the distribution of radioactivity, concentrations of radioactivity in male tissues appeared to be higher, particularly at the later time points (Townley, 2017b).

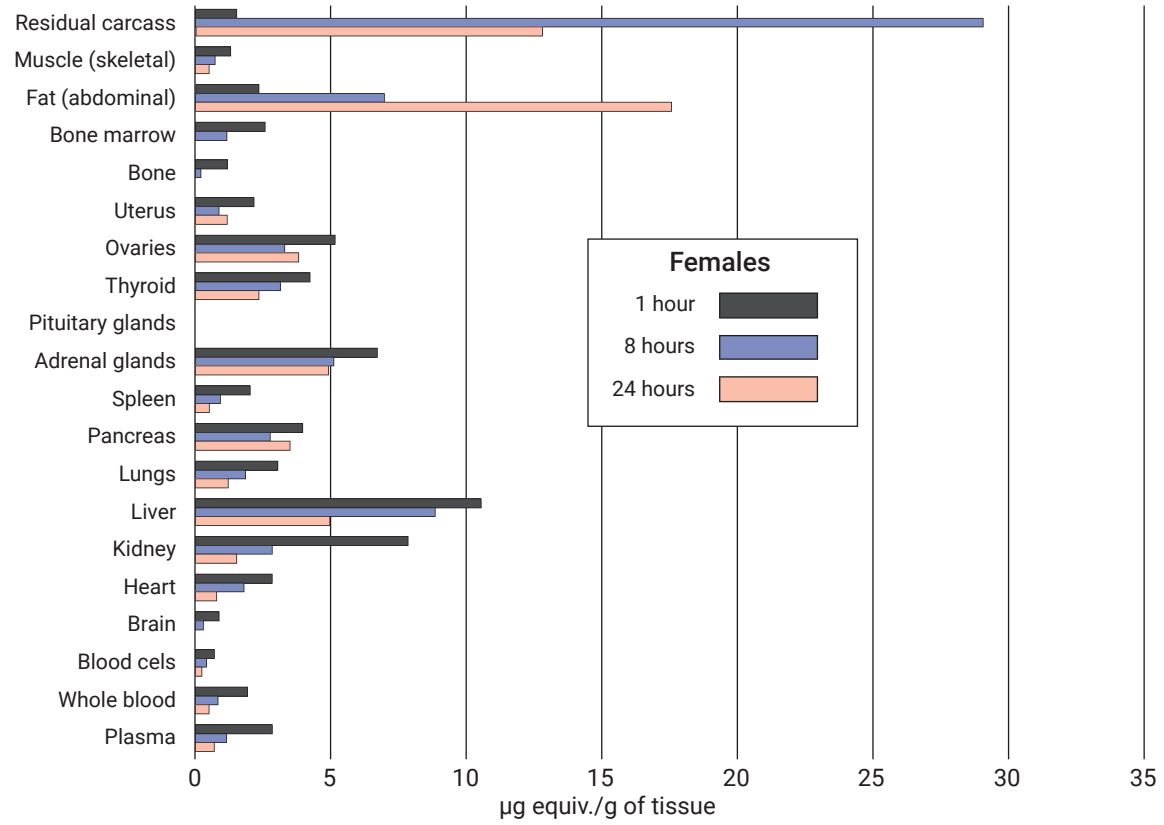
Figure 3. Mean tissue concentration of radioactivity (in mg/kg tissue) after single oral administration of [C-ring-U-^{14}C]broflanilide at a dose level of 500 mg/kg bw at 1, 8 and 24 hours.

(a) Males



(Redrawn from Townley, 2017b)

(b) Females



(Redrawn from Townley, 2017b)

Study 2

In a pharmacokinetic, absorption, excretion and tissue distribution study male and female Han Wistar rats received a daily oral dose (gavage) of [*B-ring-U*-^{14}C]broflanilide (purity 95.0%–96.8%) at a dose of 5 mg/kg bw for up to 14 days. The vehicle was 1.0% (w/v) CMC. Urine was collected during the 24 hours after the first and seventh doses and during the periods 0–6, 6–12, 12–24 hours and at 24-hour intervals thereafter until 168 hours after the day 14 dose. Faeces were collected during the periods 0–12 and 12–24 hours after the first and seventh doses and then after the day 14 dose during the periods 0–12, 12–24 hours after dosing and at 24-hour intervals for 168 hours. The interiors of the metabolism cages were washed well with around 100 mL of ethanol and around 100 mL of distilled water at intervals of 24 hours during collection of excreta, and the washings also retained. Residual radioactivity was measured in selected tissues/organs and in the remaining carcasses. Blood samples were taken over predetermined time intervals up to 168 hours after the last dose days. Radioactivity was measured by LSC.

Radioactivity excreted in urine during the 24 hours after the first dose or after the seventh dose accounted for 0.09%–0.12% and 0.21%–0.32% of the daily dose in males and females respectively. Radioactivity in faeces excreted during the 24 hours after the first dose or after the seventh dose accounted for 74.8%–88.9% and 57.0%–86.4% in males and females respectively. During 168 hours after the last dose (after the 14 daily doses) faecal excretion was 87.2% and 89.5% in males and females respectively. Following sacrifice at 168 hours after administration of the final dose, radioactivity remaining in the carcass and tissues accounted for 5.3% and 8.8% of daily dose in males and females respectively. Overall recoveries at 168 hours after administration of the final dose were 92.7% in males and 99.3% in females.

Pharmacokinetic parameters evaluated for the period of 168 hours following 14 daily doses showed values of C_{max} that were similar between plasma and RBCs, and between the sexes, and occurring in all cases at four hours after the final dose. Mean plasma concentration was quantifiable up to 96 hours, while whole blood concentrations were quantifiable up to 168 hours post dose in both sexes. While the

AUC_t values were slightly higher in females, the whole blood to plasma ratios, calculated using AUC_t (1.0 and 1.1 in males and females respectively), indicated that radioactivity concentrations in plasma and red blood cells were similar for both males and females. It was not always possible to determine the parameters K and $t_{½}$ in accordance with the acceptance criteria since 50% or more of the samples fell below the limit of quantification for total radioactivity in plasma in males, or in whole blood in either males or females. However, the half-life of excretion total radioactivity in plasma in females was 34.7 hours and appeared to be approximately 45.9 hours in males. The half-life in whole blood appeared to approximately 110 and 150 hours in males and females respectively.

Table 5. Mean retention and excretion in rats of radioactivity[a] 24 hours after doses on days 1 and 7, and during the 168 hours after the last of 14 daily oral doses of [B-ring-U-^{14}C]broflanilide

	[*B-ring-U*-^{14}C]broflanilide at 5 mg/kg bw per day					
Time interval	**Day 1 + 24 hours**		**Day 7 + 24 hours**		**Day 14 + 168 hours**	
Sex [number of rats]	**Males [4]**	**Females [4]**	**Males [4]**	**Females [4]**	**Males [4]**	**Females [4]**
Source of sample						
Urine	0.09	0.21	0.12	0.32	0.25	0.80
Faeces	88.9	57.0	74.8	86.4	87.2	89.5
Cage wash 1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.07
Cage wash 2	0.04	0.02	0.02	0.06	0.03	0.13
Residual carcass/tissues	NA	NA	NA	NA	5.3	8.8
Total	**89.0**	**57.3**	**74.9**	**86.8**	**92.7**	**99.3**

[a] Results expressed as percentage of dose administered per stated time interval; Source: Townley, 2017c

NA: Not applicable; <LOQ: Below the limit of quantification (less than twice background value)

Following 14 consecutive daily oral doses of [*B-ring-U*-^{14}C]broflanilide the highest tissue concentration (excluding the GI tract and its contents) was recovered from fat (14.7 and 15.1 µg equiv./g in males and females, respectively). At 24 hours, concentrations of 1.41–4.16 µg equiv./g were measured in the liver, pancreas, adrenal glands, thyroid, epididymis and ovaries. Concentrations in all other tissues, except male pituitary glands (not determined), were equal to 0.922 µg equiv./g or less, and were above the limit of detection. With the exception of whole blood, blood cells (males only), brain, testes, skeletal muscle (males only) and bone, the concentrations of radioactivity in tissues were greater than that in plasma.

At the 96 hours the concentrations of radioactivity in all tissues had declined, with a distribution pattern similar to that observed at 24 hours post dose. The greatest concentrations were observed in fat (4.56 and 6.26 µg equiv./g in males and females respectively) and concentrations in the remaining tissues ranging from 0.057 to 1.51 µg equiv./g. Concentrations of radioactivity in the pituitary gland and bone were below the limit of detection.

At the time of final sacrifice, 168 hours, tissue concentrations in the majority of tissues had declined significantly. Concentrations, where measurable, generally ranged between 0.013 and 2.86 µg equiv./g. The pattern of distribution was similar to that seen with earlier sacrifices. Concentrations of radioactivity in the pituitary, testes and bone were below the limit of detection.

There was no notable sex difference in the distribution of radioactivity, while slightly higher concentrations were generally observed in females (Townley, 2017c).

Table 6. Mean concentrations of radioactivity in rats after the last of 14 daily, oral doses of [B-ring-U-^{14}C]broflanilide at 5 mg/kg per day (all results expressed as µg equiv./g)

	[*B-ring-U*-^{14}C]broflanilide at 5 mg/kg bw per day					
	Males			Females		
Time after administration	24 hours	96 hours	168 hours	24 hours	96 hours	168 hours
Matrix						
Plasma	0.346	0.101	0.040	0.344	0.139	0.081
Whole blood	0.282	0.098	0.087	0.333	0.159	0.154
Blood cells	0.204	0.095	0.144	0.377	0.188	0.259
Brain	0.164	0.057	0.014	0.251	0.109	0.062
Heart	0.476	0.159	0.066	0.693	0.305	0.178
Kidney	0.903	0.415	0.244	0.922	0.509	0.280
Liver	1.90	0.912	0.422	2.01	1.17	0.693
Lungs	0.529	0.267	0.097	0.638	0.294	0.194
Pancreas	1.89	0.758	0.202	2.17	0.893	0.346
Spleen	0.400	0.223	0.140	0.724	0.444	0.460
Adrenal glands	2.49	0.766	0.223	2.13	0.965	0.458
Pituitary gland	ND	ND	ND	0.551	ND	ND
Thyroid	2.02	0.848	0.642	1.41	0.789	0.740
Epididymis/ovaries	4.16	1.51	0.455	1.86	1.12	0.378
Testes/uterus	0.238	0.089	ND	0.830	0.414	0.213
Bone	0.055	ND	ND	0.054	ND	ND
Bone marrow	0.384	0.171	0.013	0.479	0.194	0.177
Fat (abdominal)	14.7	4.56	1.63	15.1	6.26	2.86
Muscle (skeletal)	0.336	0.118	0.035	0.446	0.205	0.099
Gastrointestinal tract and contents	8.93	0.948	0.335	10.3	1.05	0.629
Residual carcass	-	-	0.181	-	-	0.292

ND: Not detected; Source: Townley, 2017c

1.2 Biotransformation

Biotransformation of broflanilide (purity greater than 95%) was investigated in a number of studies using Han Wistar rats.

Rats, some of which were bile duct-cannulated, were placed in groups each containing four animals of each sex. They were administered [*B-ring-U*-^{14}C]broflanilide or [*C-ring-U*-^{14}C]broflanilide as a single oral (gavage) low dose of 5 mg/kg bw, or [*C-ring-U*-^{14}C]broflanilide as a single high dose of 500 mg/kg bw. In an additional biotransformation study uncannulated rats (four rats of each sex) were administered, by daily oral gavage, low doses of [*B-ring-U*-^{14}C]broflanilide at 5 mg/kg bw for fourteen consecutive days. The vehicle used was 1.0% (w/v) CMC. Radioactivity was measured in urine and faeces samples pooled by sex and time period. In uncannulated rats given a single low or high dose of broflanilide, samples of urine and faeces were collected at six hour intervals up to 96 hours post dose, except urine samples in animals given a single high dose of broflanilide from which urine and faeces were collected for the first 12 hours post dose. In bile duct-cannulated rats given a single low or high dose, samples of urine (only for C-ring, low dose group), faeces and bile were collected at 12 hours intervals up to 48 hours post dose. In the repeat-dose study urine samples were collected at 24 hours after each of the 14 consecutive daily oral doses, and faeces were collected at 24 hours after the first and seventh dose, and at 12 hours intervals up to 96 hours after the last of the 14 consecutive doses. Metabolites were also identified in plasma, kidney, liver and fat sampled from animals given a single oral low and high dose

at four hours and one hour post dose, respectively. Metabolites in urine, faeces and bile were identified by liquid chromatography–mass spectrometry (LC-MS) and liquid chromatography with tandem mass spectrometry (LC-MS/MS) using a system with ultraviolet and radioactivity detection; metabolites in plasma, kidney, liver and fat samples were identified by LSC and subjected to high-performance liquid chromatography (HPLC).

Following a single oral dose of [*C-ring-U*-^{14}C]broflanilide at 5 mg/kg bw or 500 mg/kg bw, the most abundant radioactive component in urine was hippuric acid which, on and AD basis, accounted for a total of 6.4% (males) and 11.3% (females) after the low dose, and 0.8% (males) and 0.7% (females) after the high dose. In the same animals, the major radioactive component in faeces was broflanilide, accounting for 74.9% (males) and 51.6% (females) of AD at the low dose and 91.0% (male) and 94.0% (female) dose at the high dose. Other significant metabolites in faeces were: DM-(C-H_2O)-8007-cys conjugate (M4) which accounted for 2.1% and 5.6% of dose in males and females respectively, and DM-8007 (desmethyl-broflanilide, M11) which accounted for 3.4% and 4.5% respectively.

Following a single oral dose of [*B-ring-U*-^{14}C]broflanilide at 5 mg/kg bw, due to low levels of radioactivity it was not possible to accurately quantify or identify radioactive components present in urine from either males or females. Following a single oral dose of [*B-ring-U*-^{14}C]broflanilide at 5 mg/kg bw, broflanilide was the major component in faeces (66.5% in males; 75.1% in females). Two other significant metabolites were present in faeces that were identified by mass spectrometry as DM-(C-H_2O)-8007-cys conjugate (M4) which accounted for 3.9% and 2.3% of dose in males and females respectively, and DM-8007 (desmethyl-broflanilide, M11) which accounted for 5.4% and 5.2% respectively.

Minor components in the faeces of animals that received B-ring or C-ring broflanilide also included: hydroxylated metabolites of DM-8007 (DM-(A,C-diOH)-8007 (that is M9A), DC-DM-(A-OH)-8007 (M9B) and DM-(A,C-OH)-8007) (that is M10) and subsequent conjugates, DC-DM-(A-OH)-8007-cys conjugate (M1) and S(PFP-OH)-8007 (M8).

Following consecutive daily oral doses of [*B-ring-U*-^{14}C]broflanilide at 5 mg/kg bw for up to 14 days, it was not possible to accurately quantify or identify radioactive components present in urine samples from males or females due to the low radioactivity concentration. In faecal samples of these animals broflanilide was the most abundant component, accounting from 61.4%–75.0% in males and from 52.9%–76.6% in females in samples collected during the period 0–24 hours after a single oral dose and after seven daily oral doses, or 0–96 hours after fourteen daily oral doses. Other metabolites identified in faeces included DM-8007 (M11), which accounted from 2.5%–4.9% in males and from 2.0%–3.0% in females in samples collected during the period 0–24 hours after a single oral dose and after seven daily oral doses, or 0–96 hours after fourteen daily oral doses. Also present as minor components in male and female faeces and each accounting for 3.0% or less of administered daily dose were:

- DM-(C-H_2O)-8007-cys conjugate (M4),
- DM-(A,C-diOH)-8007/DC-DM-(A-OH)-8007 (M9A/M9B), and
- DM-(A,C-OH)-8007 (M10).

In all cases, the sum of unidentified components detected in all analyzed samples accounted for a maximum of 9% of the administered dose (Townley, 2017a, c). See Table 7 below for a detailed summary of the results.

Table 7. Proportions of radioactive components in extracts of urine and faeces from uncannulated rats after single and repeated oral doses (14 daily) of B-ring labelled broflanilide (5 mg/kg bw per day), or after a single oral dose of C-ring labelled broflanilide (5 mg/kg bw or 500 mg/kg bw) (All results expressed as percentage of administered dose)

Label position	B-ring label					C-ring label			
Dose regime	Single dose		Repeat-dose (14 days)			Single dose			
Dose level	5 mg/kg bw		5 mg/kg bw			5 mg/kg bw		500 mg/kg bw	
Matrix	Urine	Faeces	Faeces day 1	Faeces day 7	Faeces day 14	Urine	Faeces	Urine	Faeces
[time in hours]	[0–96]	[0–96]	[0–24]	[0–24]	[0–96]	[0–96]	[0–96]	[0–12]	[0–96]
Males									
Metabolite									
Broflanilide (parent)	NA	66.5	75.0	61.4	64.7	–	74.9	–	91.0
Hippuric acid	NA	-	-	-	-	6.4	-	0.8	-
DC-DM-(AOH)-8007-cys conjugate (M1)	NA	0.3	-	-	0.4	-	-	-	-
DM-(C-H_2O)-8007-cys conjugate (M4)	NA	3.9	1.9	1.2	2.0	-	2.1	-	-
S(PFP-OH)-8007 (M8)	NA	1.6	-	-	0.6	-	1.6	-	1.9
DM-(A,C-diOH)-8007 (M9A)	NA	0.7 [b]	1.7	1.5 [b]	1.1	-	1.7	-	-
DM-(A,C-OH)-8007 (M10)	NA	1.2	0.9	1.6	1.8	-	-	-	-
DM-8007 (M11)	NA	5.4	4.9	2.9	2.5	-	3.4	-	0.9
Others [a]	NA	8.7	3.7	4.4	4.6	0.3	1.5	0.1	1.1
Females									
Broflanilide	NA	75.0	52.9	76.6	56.9	-	51.6	-	94.0
Hippuric acid	NA	-	-	-	-	11.3	-	0.7	-
DC-DM-(A-OH)-8007-cys conjugate (M1)	NA	0.1	-	-	0.3	-	-	-	-
DM-(C-H_2O)-8007-cys conjugate (M4)	NA	2.3	-	1.2	3.0	-	5.6	-	-
S(PFP-OH)-8007 (M8)	NA	1.8	-	-	0.5	-	2.6	-	1.8
DM-(A,C-diOH)-8007 (M9A)	NA	0.3 [b]	-	1.3 [b]	3.4	-	0.3	-	-
DM-(A,C-OH)-8007 (M10)	NA	2.0	-	1.7	2.7	-	3.3	-	-
DM-8007 (M11)	NA	5.2	2.0	2.8	3.0	-	4.5	-	2.2
Others [a]	NA	7.5	1.6	0.8	7.7	0.3	3.3	0.1	0.9

NA no data available; - : Not detected Source: Townley, 2017a, c

[a] Total sample radioactivity not accounted for by the discrete components;

[b] Combination of DM-(A,C-diOH)-8007 (M9A) and DC-DM-(A-OH)-8007 (M9B)

In bile duct-cannulated rats, given a single oral dose of [*C-ring-U*-^{14}C]broflanilide at 5 mg/kg bw, broflanilide was not detected in urine samples. Broflanilide was metabolized in both sexes to the major metabolite hippuric acid, which accounted for 6.9% and 8.6% in males and females respectively. Hippuric acid is a metabolite specific to the C-ring of broflanilide, hence the higher proportion of administered radioactivity found following administration of C-ring-labelled test compound, cleavage of the parent resulting in labelled hippuric acid. In faecal samples of these animals the major radioactive component was broflanilide which accounted for 70.9% and 61.3% of AD in males and females respectively. Three other metabolites were identified in faecal samples: DM-(C-H_2O)-8007-cys conjugate (M4), DM-(A,C-diOH)-8007 (M9A) and DM-8007 (M11). Each of these components constituted less than 5% of AD. In bile, five minor metabolites were quantified, accounting for a maximum of 1% each of AD; these were DM-(C-H_2O)-8007-cys-glyc conjugate (M2), DM-(C-H_2O)-8007-cys conjugate (M4) and DM-(A,C-OH)-8007 glucuronide (M6).

In bile duct-cannulated rats, given a single oral dose of [*C-ring-U*-^{14}C]broflanilide at 500 mg/kg bw, broflanilide was not detected in urine samples, while it was the major radioactive component in faeces, accounting for 88.8% of AD, followed by metabolites DM-(A,C-diOH)-8007 (M9A) and DM-8007 (M11) which accounted for less than 4% of the dose.

In bile duct-cannulated rats, given a single oral dose of [*B-ring-U*-^{14}C]broflanilide at 5 mg/kg bw, the radioactive components identified in faeces were the same as those identified in the faeces of animals given the same dose level of B-ring labelled broflanilide, with no substantial quantitative differences. In bile samples of these animals seven metabolites were identified: DM-(C-H_2O)-8007-cys-glyc conjugate (M2), DM-S(C-Ac)-(A-OH)-8007 glucuronide (M3), DM-(C-H_2O)-8007-cys conjugate (M4), DC-DM-(A-OH)-8007-glucuronide conjugate (M5), DM-(A,C-OH)-8007-glucuronide (M6), DM-(A,C-OH,-OCH_3)-8007-glucuronide conjugate (M7) and DM-(A,C-OH)-8007 (M10). Each of these metabolites accounted for a maximum of 3% of AD.

Unidentified components were detected in all analyzed samples, accounting for a maximum each of 1% of AD, thus there were no major unidentified metabolites (Stroud, 2017).

Table 8. Proportions of radioactive components in extracts of urine and faeces from bile duct-cannulated rats after a single dose of B-ring labelled broflanilide (5 mg/kg bw per day), or after a single oral dose of C-ring labelled broflanilide (5 mg/kg bw or 500 mg/kg bw)
(All results expressed as percentage of administered dose)

Label position	**B-ring label**				**C-ring label**						
Dose level	**5 mg/kg bw**				**5 mg/kg bw**						**500 mg/kg bw**
Matrix	**Bile**		**Faeces**		**Urine**		**Faeces**		**Bile**		**Faeces**
Time period (hours)	**0–48**		**0–48**		**0–48**		**0–48**		**0–48**		**0–48**
Sex	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**
Metabolite											
Broflanilide	-	-	66.6	60.4	-	-	70.9	61.3	-	-	88.8
Hippuric acid [b]					6.9	8.6					
DM-(C-H_2O)-8007-cys-gly conjugate (M2)	1.4	1.3	-	-	-	-	-	-	1.0	0.9	-
DM-S(C-Ac)-(A-OH)-8007-gluc conjugate (M3)	2.7	1.2	-	-	-	-	-	-	-	-	-
DM-(C-H_2O)-8007-cys conjugate (M4)	0.7	0.7	2.9	2.1	-	-	1.8	0.3	0.6	0.7	-
DC-DM-(A-OH)-8007-gluc conjugate (M5)	1.8	3.0	-	-	-	-	-	-	-	-	-
DM-(A,C-OH)-8007-gluc conjugate (M6)	1.4	1.2	-	-	-	-	-	-	0.9	0.8	-
DM-(A,C-OH,-OCH3)-8007-gluc conjugate (M7)	0.1	-	-	-	-	-	-	-	-	-	-
DM-(A,C-diOH)-8007 (M9A)	-	-	1.0 [a]	0.9 [a]	-	-	1.2	2.0	-	-	2.4

Label position	B-ring label				C-ring label						
Dose level	5 mg/kg bw				5 mg/kg bw						500 mg/kg bw
Matrix	Bile		Faeces		Urine		Faeces		Bile		Faeces
Time period (hours)	0–48		0–48		0–48		0–48		0–48		0–48
Sex	M	F	M	F	M	F	M	F	M	F	M
DM-(A,C-OH)-8007 (M10)	0.4	0.2	-	-	-	-	-	-	-	-	-
DM-8007 (M11)	-	-	3.3	3.3	-	-	3.2	4.6	-	-	3.4
Others [c]	0.4	0.5	2.5 [d]	3.4 [d]	0.3	0.7	1.7 [d]	1.5 [d]	0.5	0.6	1.1

- : Not detected

Source: Stroud, 2017

[a] combination of DM-(A,C-diOH)-8007 (M9A) and DC-DM-(A-OH)-8007 (M9B);

[b] Not identified in this study but retention time of component matches hippuric acid, as confirmed in Townley, 2017a;

[c] Total sample radioactivity not accounted for by the discrete components;

[d] Minor components (described in the study report as non-common) or radioactivity distributed throughout regions of the chromatogram other than those specified and which did not contain any discrete radioactive components

Metabolite profiles at peak concentrations in plasma, liver, kidney (at four hours) and fat (at 24 hours) after a single oral dose of [*B-ring-U*-^{14}C]broflanilide (5 mg/kg bw), showed metabolite DM-8007 (M11) to be the major component, accounting for 42.1%–58.0% of total radioactivity, followed by metabolite DC-DM-8007 which accounted for 2.8%–17.2%, of total radioactivity. Other metabolites characterized and occurring at low levels were: DM-(C34-diOH)-8007 and S(PFP-OH)-8007 in all tissues (up to 13.1% of total radioactivity), and DM-(C4-OH)-8007 and S(Br-OH)-8007 in plasma, liver and kidney (up to 7.8% of total radioactivity).

Metabolites profile at peak concentrations in plasma, liver, kidney (at one hour) and fat (at 24 hours) after a single oral dose of [*C-ring-U*-^{14}C]broflanilide (500 mg/kg bw), showed that metabolite DM-8007 (M11) was the major component, accounting for 7.7%–49.6% of total radioactivity. Metabolites DM-(C34-diOH)-8007 and S(PFP-OH)-8007 in plasma, liver and kidney accounted for up to 11.4% of total radioactivity, and metabolites DM-(C4-OH)-8007 and S(Br-OH)-8007 in liver and kidney accounted for up to 15.3% of total radioactivity. In plasma and kidney one or more polar component accounted for up to 48.8% of total radioactivity.

In all cases there was no notable sex difference in the biotransformation of either [*B-ring-U*-^{14}C]broflanilide or [*C-ring-U*-^{14}C]broflanilide (Townley, 2017b).

The proposed metabolic pathways of broflanilide are shown in Figures 4a and b.

The metabolites identified comprise the following phase I and phase II conversions of the parent compound broflanilide:

(a) Broflanilide was metabolized to S(PFP-OH)-8007 (M8), S(Br-OH)-8007 or DM-8007 (desmethyl-broflanilide; M11).

(b) Metabolite DM-8007 (M11) was hydroxylated and conjugated to DM-(C-H_2O)-8007-cys-gly conjugate (M2) and DM-(C-H_2O)-8007-cys conjugate (M4).

(c) Metabolite DM-8007 (M11) was hydroxylated to (DM-(C4-OH)-8007) and DM-(C3,4-diOH)-8007.

(d) DM-8007 (M11) was also subject to hydrolysis of the amide bond (forming DC-DM-8007) followed by hydroxylation to DC-DM-(A-OH)-8007 (M9B) and conjugation to DC-DM-(A-OH)-8007-cys conjugate (M1), DC-DM-(A-OH)-8007-gluc conjugate (M5) and DM-S(C-Ac)-(A-OH)-8007-gluc conjugate (M3).

(e) Hydrolysis of DM-8007 (M11) also resulted in the formation of benzoic acid which was subsequently metabolized to hippuric acid.

(f) Metabolite DM-8007 (M11) was also hydroxylated to DM-(A,C-OH)-8007 (M10) and DM-(A,C-diOH)-8007 (M9A), as well being conjugated to form DM-(A,C-OH)-8007-gluc conjugate (M6) and DM-(A,C-OH,-OCH_3)-8007-gluc conjugate (M7).

Figure 4a. Proposed metabolic pathway of Broflanilide in rats

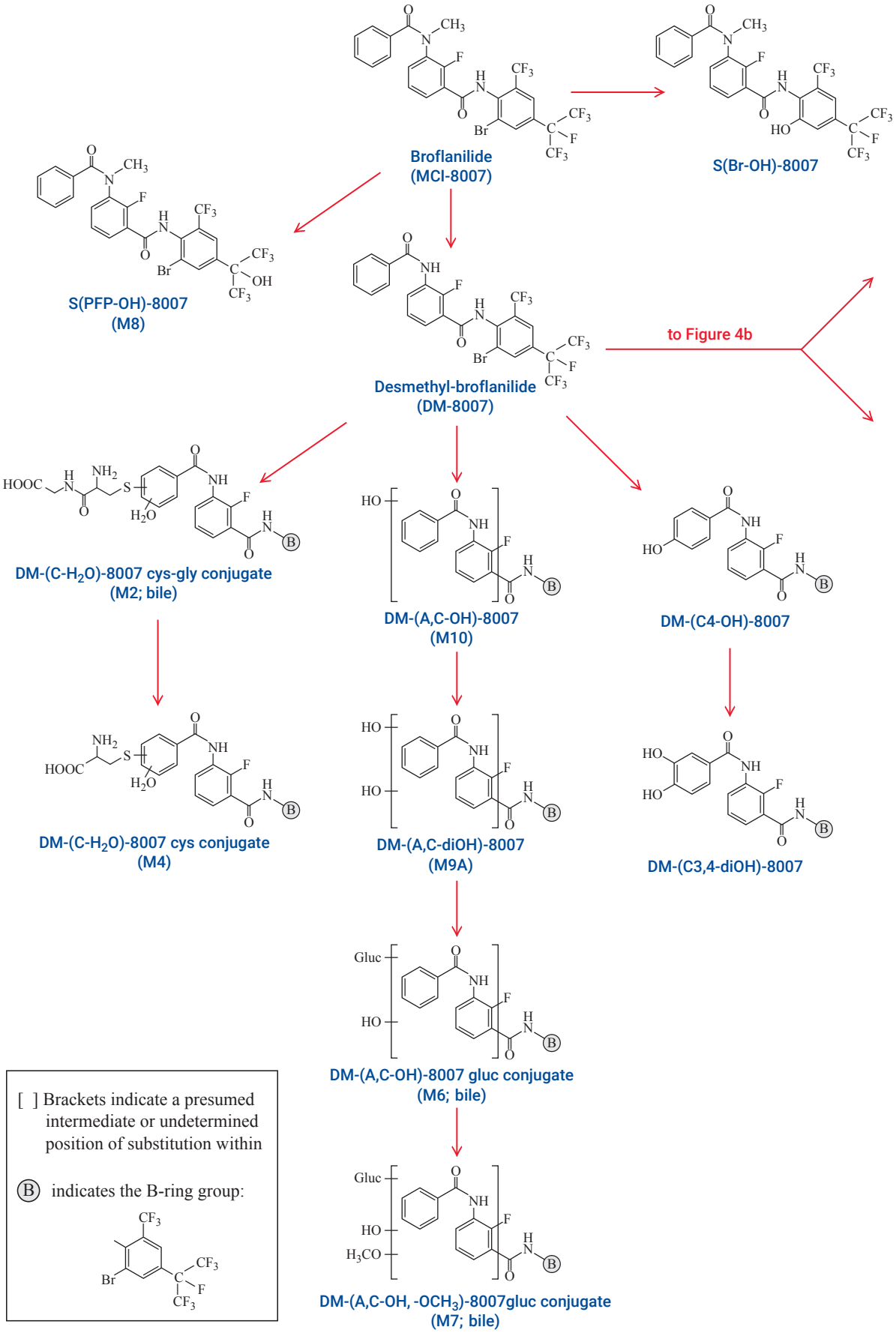
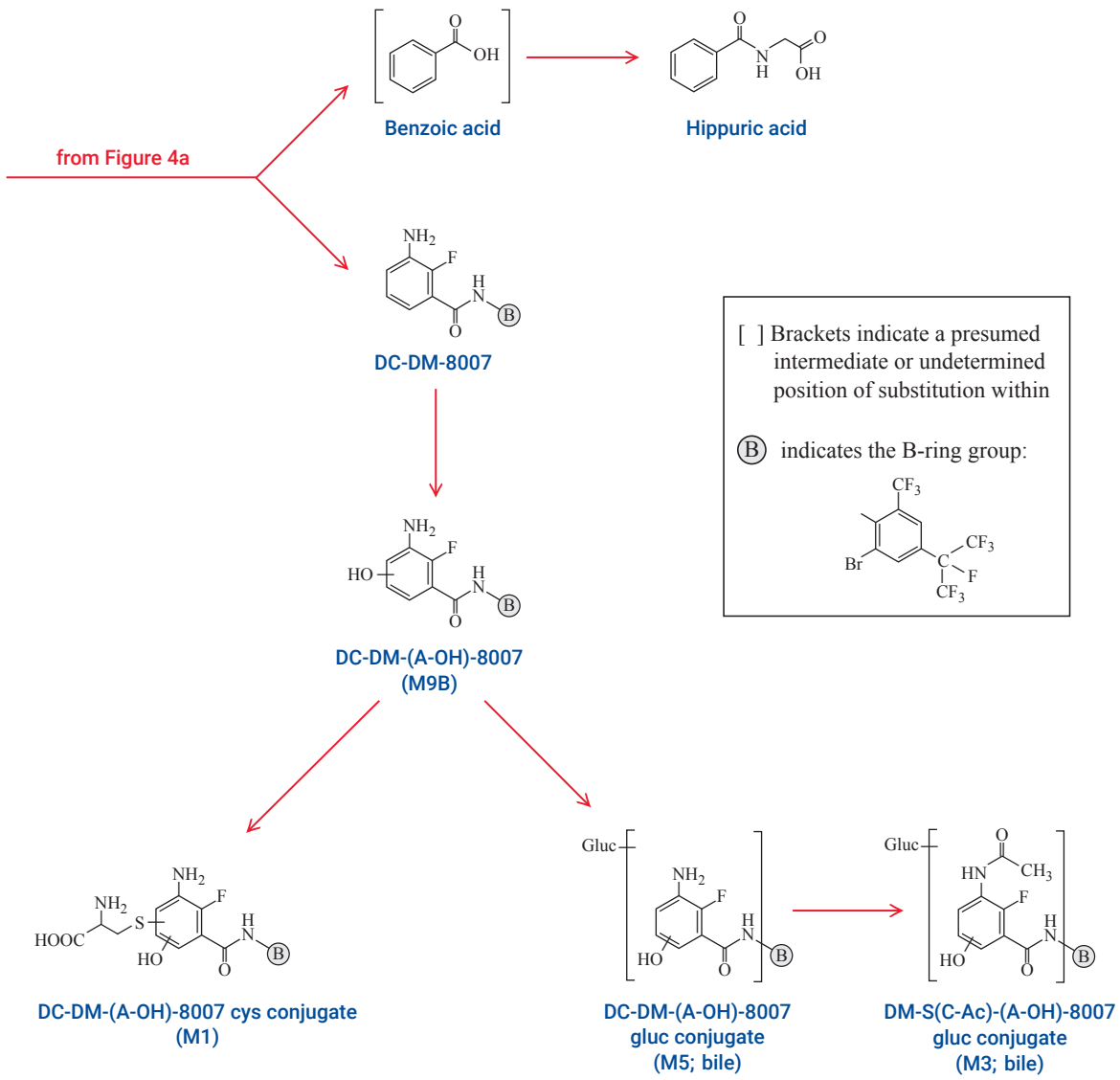
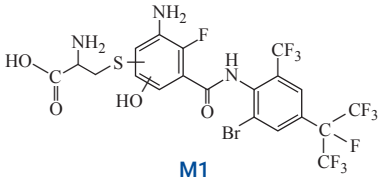


Figure 4b. Proposed metabolic pathway of Broflanilide in rats

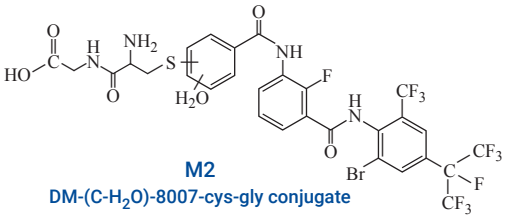


(Redrawn from Stroud, 2017 and Townley 2017a, b, c)

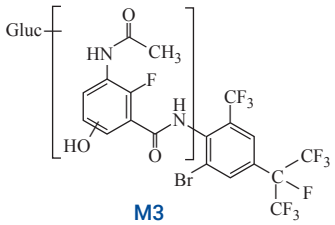
Table 9. Summary of identified and quantified metabolites



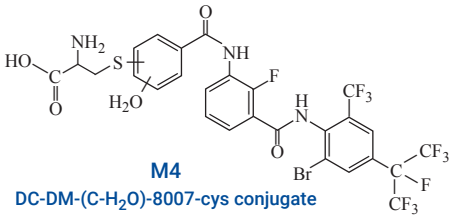
M1
DC-DM-(A-OH)-8007-cys conjugate



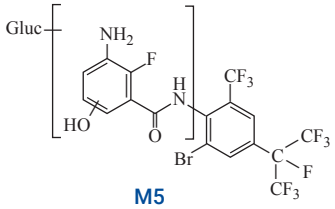
M2
DM-(C-H_2O)-8007-cys-gly conjugate



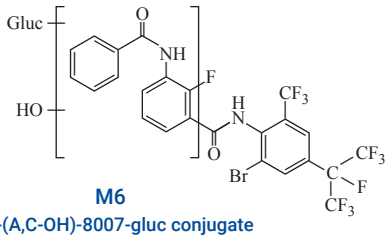
M3
DM-S(C-Ac)-(A-OH)-8007-gluc conjugate



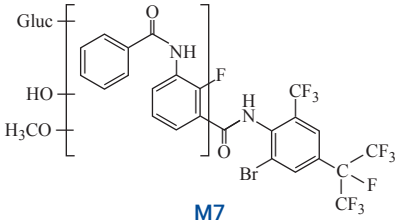
M4
DC-DM-(C-H_2O)-8007-cys conjugate



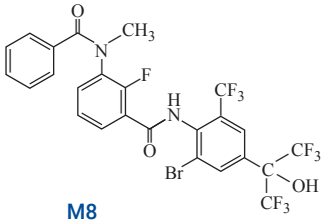
M5
DC-DM-(A-OH)-8007-gluc conjugate



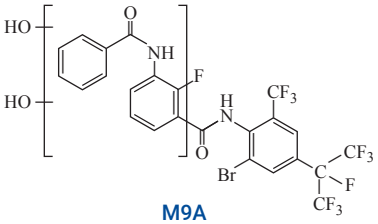
M6
DM-(A,C-OH)-8007-gluc conjugate



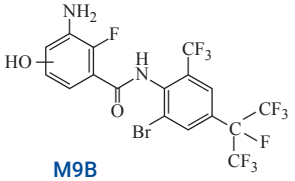
M7
DM-(A,C-OH -OCH_3)-8007-gluc conjugate



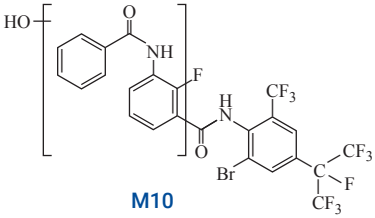
M8
S(PFP-OH)-8007-gluc conjugate



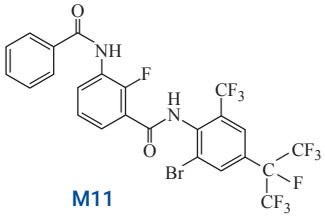
M9A
DM-(A,C-diOH-8007



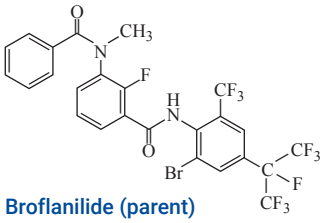
M9B
DC-DM-(A-OH)-8007



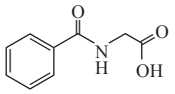
M10
DM-(A,C-OH)-8007



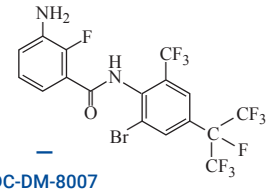
M11
DM-8007



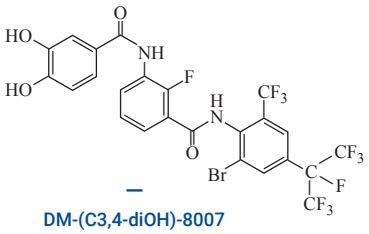
Broflanilide (parent)
MCI-8007



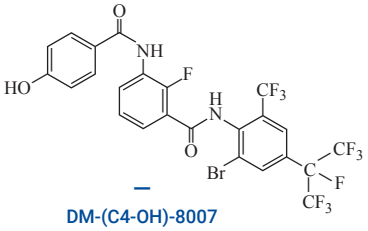
Hippuric acid



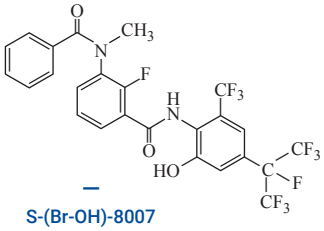
DC-DM-8007



DM-(C3,4-diOH)-8007



DM-(C4-OH)-8007



S-(Br-OH)-8007

[ ] Brackets indicate an undetermined position of substitution within

Source: Stroud (2017) and Townley (2017a, b, c)

1.3 Effects on enzymes and other biochemical parameters

A comparative in vitro metabolism study was performed with broflanilide and human, rat and mouse hepatocytes. Human, rat (Wistar) and mouse (ICR-CD1) hepatocytes (all of mixed sex origin) were incubated (at 37°C in 5% carbon dioxide) with [*B-ring-U*-^{14}C]broflanilide at a concentration of 10 µM for 0, 10, 30, 60 or 180 minutes. A stability control (without hepatocytes), two positive controls (radiolabelled testosterone and 7-ethoxycoumarin) and a blank control (application medium with dimethyl sulfoxide instead of the test item) were also included in the tests. Two experiments were performed with human hepatocytes from 200 donors of mixed sex. All in vitro experiments were performed in triplicate. Ice-cold ethanol was added to the incubation mixtures to stop the reaction. All triplicates were analyzed by HPLC, at least one replicate per triplicate was also analyzed with HPLC-MS and the resulting *m*/*z* values were assigned to any peaks that accounted for more than 5% of AR for at least one time point in samples of human hepatocytes.

Negative controls demonstrated that the test item can be considered stable under the given incubation conditions. Positive controls with the reference items testosterone and 7-ethoxycoumarin demonstrated adequate metabolic activity, phase I and phase II reactions. The viability of human 1, human 2, rat and mouse hepatocytes in the presence of 10 µM broflanilide ranged from 96%–109%, when compared to viability without the test item. Hence, incubation of the cells with 10 µM broflanilide would not significantly alter their viability.

With human hepatocytes the concentration of parent broflanilide decreased continuously from start of incubation to 180 minutes in both experiments. Biodegradation of broflanilide with mouse and rat hepatocyte was slightly faster in the period 0–60 minutes. After incubation for 180 minutes broflanilide was not detected any longer in rat and mouse hepatocyte preparations, and only in small amounts with human hepatocytes.

With human hepatocytes the concentration of metabolite DM-8007 increased continuously in both experiments from 0 minutes to 180 minutes. With rat or mouse hepatocytes the concentration of DM-8007 increased from 10 minutes to 180 minutes.

No human-specific metabolites of broflanilide were identified in this study (Rabe, 2020a).

Table 10. Comparison of broflanilide metabolites obtained with human, rat and mouse hepatocytes (all values are expressed as percentage of applied radioactivity)

		Sampling time (minutes)				
Significant peaks[a]	**Hepatocyte source**	**0**	**10**	**30**	**60**	**180**
Broflanilide	Human 1	107.92	96.80	86.16	63.70	4.96
	Human 2	97.30	NA	NA	NA	1.67
	Rat	90.29	71.61	48.27	21.92	-
	Mouse	88.20	81.66	66.95	35.95	-
DM-8007	Human 1	1.11	7.81	26.23	54.26	109.86
	Human 2	1.57	NA	NA	NA	96.67
	Rat	-	16.74	41.89	75.76	85.16
	Mouse	-	4.91	19.98	50.27	66.45
P16.0	Human 1	4.11	4.52	4.21	3.90	1.57
	Human 2	4.01	NA	NA	NA	0.96
	Rat	3.40	3.38	3.05	0.56	-
	Mouse	3.81	3.72	2.90	2.81	11.52
P17.1	Human 1	-	-	-	-	2.84
	Human 2	-	NA	NA	NA	2.59
	Rat	-	-	1.20	4.36	7.72
	Mouse	-	-	1.36	3.83	10.34

-: Not applicable and/or not detected; NA: Not applicable; Source: Rabe, 2020a

[a] Significant peak defined as representing more than 5% of AR: (sum of supernatant and acetonitrile/water extract) when discovered on at least one time point in human hepatocyte samples;

An comparative in vitro metabolism study very similar to the one above (Rabe, 2020a) was performed using hepatocytes from 200 human donors of mixed sex, from mice (48 mixed sex donors, pooled) and from rats (16 male donors pooled, and 18 female donors pooled) to determine if the metabolic profiles of metabolite DM-8007 (at 10 µM) were similar among species and if unique human metabolites occurred. The experimental protocol was the same as the one described above.

Negative controls demonstrated that the test item was stable under the given incubation conditions. Positive controls with the reference items 7-ethoxycoumarin and testosterone demonstrated adequate metabolic activity, phase I and phase II reactions.

With human hepatocytes the concentration of DM-8007 remained largely unchanged during the course of the experiment. Biodegradation of DM-8007 was visible with mouse hepatocytes and its concentration decreased between 30 minutes and 180 minutes. With rat hepatocytes the concentration decreased slightly between 30 minutes and 180 minutes.

In summary, no human-specific metabolites of DM-8007 were identified in the present study (Rabe, 2020b).

Table 11. Metabolite DM-8007- comparison of its onward biotransformation obtained with human, rat and mouse hepatocytes (all values are expressed as percentage of applied radioactivity)

Parameter	Hepatocyte source	Sampling time (minutes)				
		0	10	30	60	180
Metabolite DM-8007 (% of AR)	Human	84.72	90.89	93.03	94.66[a]	93.32[b]
	Rat	94.54	98.58	98.96	96.25	87.88
	Mouse 1	87.75	93.75	92.92[a]	80.69	43.60
	Mouse 2	94.68	NA	NA	NA	52.02

NA: Not applicable; AR: Applied radioactivity Source: Rabe, 2020b

[a] This value was calculated as the sum of the mean [% AR] value of three supernatants and the [% AR] value of only one acetonitrile extract because the pellets received from the other two incubations were not further extracted;

[b] This value was calculated as the sum of the mean [% AR] value of three supernatants and the mean [% AR] value of only two acetonitrile extracts because the pellet received from the other incubation was not further extracted

2. Toxicological studies

2.1 Acute toxicity

The results of acute oral, dermal and inhalation toxicity studies with broflanilide, along with the results of dermal and eye irritation, and skin sensitization studies, are summarized below in Table 12; the studies are described in more detail below. For all studies broflanilide purity was greater than 98%.

Table 12. Summary of acute toxicity studies with broflanilide

Species	Strain	Sex	Route	Purity	LD_{50} / LC_{50}	Reference
Rat	HsdHan™: WIST	F	Oral	99.67%	> 5000 mg/kg	Dreher, 2012a
Rat	HsdHan™: WIST	M + F	Dermal	99.67%	> 5000 mg/kg	Dreher, 2012b
Rat	Crl:WI(Han)	M + F	Inhalation[a]	99.67%	> 2.20 mg/L[b]	Wako, 2014
Rabbit	HsdIf:NZW	M	Skin irritation	99.67%	Non irritant	Dreher, 2012c
Rabbit	HsdIf:NZW	M	Eye irritation	99.67%	Non irritant	Dreher, 2012d
Mouse	CBA/J	F	Skin sensitization (LLNA)	99.67%	Non sensitizing	Remmele & Landsiedel (2012)
Mouse	CBA/CaCrl	F	Skin sensitization (LLNA)	99.67%	Non sensitizing	Dreher, 2012e
Guinea pig	Slc:Hartley	F	Skin sensitization (maximization test)	98.67%	Range-finding test	Ueda, 2014
Guinea pig	Slc:Hartley	F	Skin sensitization (maximization test)	98.67%	Non sensitizing	Ueda, 2015

F: Female; M: Male; LD_{50} : Median lethal dose; LC_{50} : Median lethal concentration;

[a] Dust aerosol-nose only;

[b] Maximum achievable concentration

(a) Lethal doses

In an acute oral toxicity study, five female fasted HsdHan®:WIST rats were given a single oral gavage dose of broflanilide in 1% aqueous methyl cellulose solution. Individual rats were first dosed at 0, 550, 1750 and 5000 mg/kg bw. As there were no deaths at 5000 mg/kg bw, two further animals were treated at this dose level. All animals were observed individually for clinical signs of toxicity for 15 days (last day of the observation period). The body weights of the animals were recorded on day −1 and days 1, 4, 8 and 15. At termination animals were subject to a full macroscopic necropsy examination.

Mortality was not observed at any of the dose levels. There were no clinical signs noted during the observation period, nor effects on body weight or gross findings.

Under the experimental conditions of this study, the oral median lethal dose (LD_{50}) of broflanilide was greater than 5000 mg/kg bw for females (Dreher, 2012a).

In an acute dermal study, five male and five female HsdHan®:WIST strain rats were dermally exposed to 5000 mg/kg bw of broflanilide moistened with water, which was applied to an area on the dorsum of the rat that was at least 10% of its total body surface. Test sites were covered with a semi-occlusive dressing for 24 hours. All animals were observed individually for clinical signs of toxicity for 15 days (the last day of the observation period). Body weights were recorded on day −1 and days 1, 4, 8 and 15. At termination animals were subject to a full macroscopic necropsy examination.

No mortality was observed. No clinical signs were noted during the observation period. One female underwent no change in body weight during the first week of the study. All the other rats gained weight during the first and second weeks of the study.

No dermal or macroscopic changes were reported.

Under the experimental conditions of this study, the dermal LD_{50} of broflanilide was determined to be greater than 5000 mg/kg bw (Dreher, 2012b).

In an acute inhalation toxicity study, one group (six males and six females) of Crl:WI(Han) rats was exposed nose-only via the inhalation route to broflanilide dust (purity 98.67%) for four hours at the maximally attainable concentration of 2.20 mg/L. The mass median aerodynamic diameter (MMAD) of the dust was measured after 10 minutes exposure and 220 minutes after the start of exposure at 4.9 µm and 4.5 µm, with geometric standard deviations of 2.9 and 2.5, respectively. The ratios of particles of diameter under 4 µm (which is considered the inhalable particle size for rats) were 42.4% and 45.4% respectively.

Animals were observed for clinical signs of toxicity for 14 days. Body weights were recorded before exposure on day 1 and on days 2, 4, 8 and 15. At termination animals were subject to a full macroscopic necropsy examination.

No mortality was observed. No clinical signs were noted during the observation period. Body weight loss was observed in males and females on day 2. Body weights of the males and females increased from day 4. No macroscopic changes were reported.

Under the experimental conditions of this study, the LC_{50} of broflanilide was greater than 2.20 mg/L (Wako, 2014).

(b) Dermal irritation

In a primary dermal irritation study, three male New Zealand White rabbits (HsdIf:NZW) were dermally exposed to 500 mg of broflanilide (purity 99.67%) which had been moistened with water. The treatment covered an area of 6 cm^2 on each rabbit's dorsal side and test sites were covered with a semi-occlusive dressing for four hours. Animals were then observed for three days. Clinical observation were made 1, 24, 48 and 72 hours after patch removal. Irritation was scored by the method of Draize's Criteria (1959).

No clinical signs, skin irritation reactions or body weight changes were observed in any of the rabbits during the observation period of 72 hours following the removal of patches. The irritation potential of broflanilide towards the skin of rabbits was judged as "Non irritant" under the conditions of this study (Dreher, 2012c).

(c) Ocular irritation

In a primary eye irritation study, 100 mg of powdered broflanilide (purity 99.67%) was instilled into the conjunctival sac of the left eye of three male New Zealand White rabbits (HsdIf:NZW). The eyes were not washed. Clinical observation were made 30 minutes after application, then at one hour and approximately at 24, 48 and 72 hours post treatment. Body weight was recorded the day before dosing and after the last observation.

No clinical signs or effects on body weight were observed. A score of 1 for conjunctival chemosis in 3/3 animals, and redness and discharge in 1/3 animals were observed one hour after application.

From the 24-hour observation onward these scores decreased and eye irritation reactions were no longer observed in any animal by 24 hours after application. No fluorescein staining marks on the cornea were observed in any animal at the 24-hour examination.

The mean eye irritation scores at 24, 48 and 72 hours were 0 for cornea, iris and conjunctival effects. The irritation potential of broflanilide towards the eyes of rabbits was judged as "non irritant" under the condition of this study (Dreher, 2012d).

(d) Dermal sensitization

Study 1

In a first dermal sensitization local lymph node assay (LLNA) study, five female mice (CBA/J) were administered broflanilide (purity 99.67%) in methyl ethyl ketone (MEK) at a limit concentration of 50% (w/v) broflanilide. Two additional groups of five mice were used in this study, one group receiving the vehicle alone and the other receiving the positive control substance α-hexylcinnamaldehyde (HCA) in the vehicle. Each test animal was treated with 25 µL per ear of the test substance preparation, applied to the dorsal surfaces of both ears on three consecutive days. The control group was treated with 25 µL per ear of the vehicle alone. The animals of the positive control group were treated with 25 µL of a 15% preparation of HCA in methyl ethyl ketone.

No mortality or clinical signs of systemic toxicity were observed. There was a slight body weight reduction in animals given broflanilide or the positive control (15% HCA).

A statistically significant increase in sensitization index (SI) of 1.43 for the 50% broflanilide preparation was reported, however this increase was not considered biologically relevant. Broflanilide in its 50% preparation and the 15% HCA positive control caused a statistically significant increase in ear weights, indicative of ear irritation.

It is concluded that based on the findings of this study, Broflanilide does not exhibit skin sensitizing potential (Remmele & Landsiedel, 2012).

Study 2

In a second dermal sensitization LLNA study, groups of four female mice (CBA/CaCrl) received dermal applications of broflanilide (purity 99.67 %) at 10%, 25%, and 50% (maximum suitable concentration) in dimethylformamide (DMF). An additional group of four mice served as control (vehicle only). Each test animal was treated with 25 µL per ear of the test substance preparation, applied to the dorsal surface of both ears for three consecutive days. The control group was treated with 25 µL per ear of the vehicle alone. The sensitivity and reliability of the test system was being checked at least every six months.

There were no deaths related to treatment. One animal in the control group died following intravenous injection and one animal in the 5% group died in restraint prior to injection of tritium-labelled methyl thymidine (^{3}H-TdR). No clinical signs indicative of systemic effects or treatment-related effects on body weight or local irritation were reported for any treatment group. Stimulation index in animals dosed at 10%, 25% and 50% were 0.4, 1.2 and 0.4 respectively.

It was concluded that under the experimental condition of the study broflanilide is not a skin sensitizer (Dreher, 2012e).

Study 3

In a third dermal sensitization study broflanilide (purity 98.67%), female Hartley Guinea pigs (Slc:Hartley) were tested by the maximization test method. The following four groups were prepared: Group A (20 animals), exposed to the test substance both at the induction and challenge; Group B (10 animals), exposed to the test substance only at the challenge; Group C (10 animals), exposed to HCA both at the induction and challenge; and Group D (five animals), exposed to HCA only at the challenge. Based on results of a preliminary range-finding study (Ueda, 2014), each concentration for intradermal induction, topical induction and topical application challenge was 1% (w/v), 50% and 50% (w/w) in liquid paraffin (in Freund's complete adjuvant, FCA, blended with saline) or white petrolatum for the test substance and 1%, 50% and 10% (v/v) in liquid paraffin for HCA, respectively. Skin reaction to the challenge was observed 24 and 48 hours after the patch removal and the sensitization rate was calculated for Groups A and C.

On the observation days after intradermal induction, erythema and scab were found on the induction site treated with FCA in all animals. The scab was observed on the dorsal region of all animals, which was the intradermal injection site, and was seen as a clinical sign continuously from 9–24 days after intradermal induction. These skin reactions were also observed in the unsensitized groups (Groups B and D), therefore it was concluded that these skin reactions were related to the adjuvant treatment rather than the test substance itself, or HCA.

It was concluded that under these experimental conditions broflanilide is not a skin sensitizer (Ueda, 2015).

2.2 Short-term studies of toxicity

The short-term toxicity of broflanilide was evaluated in mice, rats and dogs by oral administration, and in rats by dermal application and inhalation. For all studies, broflanilide purity was greater than 99% unless otherwise specified.

(a) Oral administration

Mouse

In a range-finding study, groups of 12 male and 12 female Crl:CD1 (ICR) mice were fed diets for 28 days containing broflanilide at 0, 200, 700, 2000 or 7000 ppm (equal to 0, 28.2, 107.2, 296.1 and 1019.9 mg/kg bw per day for males, 0, 31.0, 119.4, 315.0 and 1068 mg/kg bw per day for females). Diets were evaluated for their homogeneity and stability. All animals were observed for mortality and clinical signs during the study and their body weight and food consumption were recorded weekly. At termination of treatment all surviving animals were subjected to haematology, clinical chemistry, necropsy and organ weight analysis (adrenal, brain, heart, kidney, liver, spleen, testes and epididymides). Histopathology examinations were conducted on adrenals, any gross lesions, heart, kidney, liver, pancreas, spleen and thymus in all animals of the control and high dose groups.

No treatment-related adverse effects were seen in either sex on survival, clinical signs, body weight, body weight gain, food or test article consumption, clinical pathology (haematology and clinical chemistry), organ weights, gross pathology or histopathology.

Based on the results of this study, the limit dose of 7000 ppm (equal to 1020 mg/kg bw per day for males and 1068.0 mg/kg bw per day for females) was selected as the top dose for the follow-on 90-day study (Heal, 2014).

In a subchronic toxicity study (not compliant with guidelines), groups of 10 males and 10 females Crl:CD1 (ICR) mice were fed diets for 90 days containing broflanilide at concentrations of 0, 200, 1500 or 7000 ppm (equal to 0, 26.3, 199.4 and 955.3 mg/kg bw per day for males, 0, 32.3, 229.8 and 1147.6 mg/kg bw per day for females). An additional four male and four female mice from each group were assessed by LC-MS/MS for the presence in plasma of broflanilide and its primary metabolite DM-8007. Diets were evaluated for their homogeneity and stability. All animals were observed for mortality and clinical signs during the study, and their body weight and food consumption recorded weekly. Ophthalmoscopy investigations were performed pretreatment and in week 12 on all control and high-dose animals. At termination of treatment, all surviving animals were subjected to haematology, necropsy and organ weight analysis (adrenal, brain, heart, kidney, liver, ovaries, spleen, testes and epididymides, thymus and uterus with cervix). Histopathology examinations were conducted on all tissues (except in the femur bone marrow) on all animals of the control and high-dose groups, and on the adrenal, liver, and any gross lesions in all animals at the low and intermediate doses.

No treatment-related mortality or clinical signs of toxicity were observed. A slight suppression of body weight gain (−8%) was observed in males at 7000 ppm. Food consumption was unaltered and there were no treatment related findings from ophthalmoscopy investigations.

Haematology investigation revealed a statistically significant ($p < 0.05$) decrease in haemoglobin distribution width (HDW) in males at 1500 and 7000 ppm, as great as −6% when compared to controls, but with no true dose–response relationship. Mean platelet volume (MPV) also showed a statistically significant reduction in females at 1500 and 7000 ppm (up to −7%) when compared to controls. Considering their magnitude and the absence of changes in other related parameters, these findings

were not considered adverse. The number of large, unstained cells (LUCs) was reduced in females at 7000 ppm by around −60% compared to controls (statistically significant). Since almost all individual values (9/10) fell within the concurrent control range these changes were considered of no toxicological relevance. Females at 200 and 7000 ppm showed a statistically significant decrease in white blood cells (WBCs) compared to controls, but these changes did not show any relationship to dose.

Increased adrenal weights compared to control means were reported in females at 1500 and 7000 ppm (by 18% and 43% respectively), achieving statistical significance at the high dose. At the high dose adrenal weight increases were consistent with an increased incidence of animals with slight cortical vacuolation (zona fasciculata), and so were considered adverse.

Bioanalysis of plasma indicated that broflanilide and its primary metabolite DM-8007 were not present in the plasma of controls. Plasma levels of broflanilide and DM-8007 increased with increasing dose; the increases were less than proportional and were generally similar for males and females. Plasma levels of metabolite DM-8007 exceeded those of parent broflanilide.

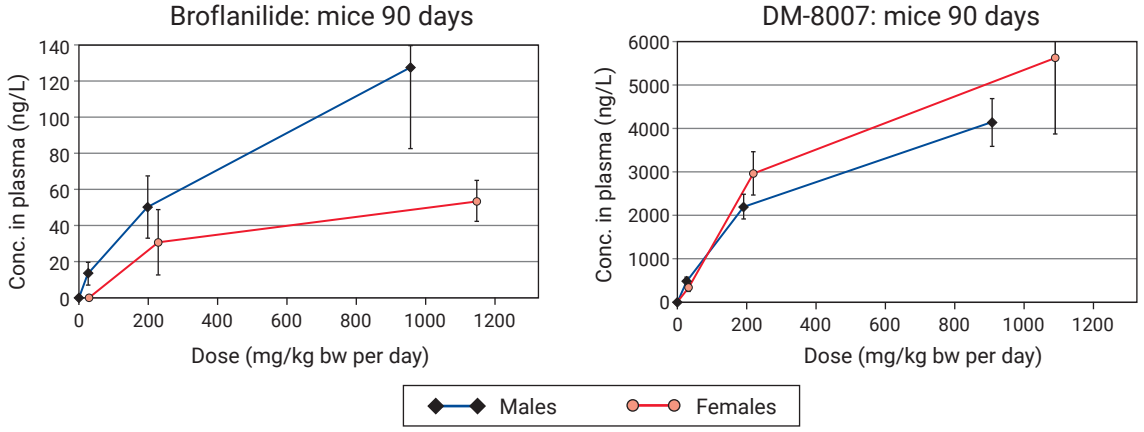
Table 13. Key findings of the 90-day toxicity study with broflanilide in the mouse

	Sex, dietary dose (ppm)							
	Males				Females			
Parameters	**0**	**200**	**1500**	**7000**	**0**	**200**	**1500**	**7000**
Organ weight; adrenal glands								
Absolute organ weight (g)	0.007 ± 0.0019	0.008 ± 0.0032	0.007 ± 0.0016	0.008 ± 0.0023	0.011 ± 0.0022	0.011 ± 0.0022	0.013 ± 0.0027	0.014 ± 0.0034**
% of control	–	114	100	114	–	100	118	127
Relative organ weight (%)	0.0161 ± 0.00472	0.0181 ± 0.00702	0.0164 ± 0.00422	0.0192 ± 0.00601	0.0343 ± 0.00741	0.0374 ± 0.00966	0.0406 ± 0.00839	0.0492 ± 0.01321**
% of control	–	112	102	119	–	109	118	143
Histopathology; adrenal glands								
Number examined	10	10	10	10	10	10	10	10
Cortical vacuolation	0	0	0	0	0	0	0	6
minimal	0	0	0	0	0	0	0	6
Mean plasma level ± standard deviation (day 90)								
Broflanilide (ng/L)	< 10.0 [a]	13.6 ± 6.3	50.3 ± 17.2	127.8 ± 45.1	< 10.0 [a]	< 10.0 [a]	31.0 ± 18.2	53.6 ± 11.4
DM-8007 (ng/L)	< 200 [b]	486 ± 87	2198 ± 289	4137 ± 556	< 200 [b]	336 ± 95	2960 ± 519	5623 ±1752

[a] Limit of quantification for broflanilide = 10.0 ng/mL, [b] for DM-8007 = 200 ng/mL; Source: Heal, 2016a

Statistically significant using ANOVA and Dunnett's test: * $p < 0.05$, ** $p < 0.01$;

Figure 5. Plasma broflanilide and metabolite DM-8007 in male and female mice at 90 days



(Redrawn from Heal, 2016a)

Under the experimental conditions of the study the no-observed-adverse-effect level (NOAEL) was 1500 ppm (equal to 229.8 mg/kg bw per day) based on effects on the adrenal glands (increased adrenal weight and increased incidence of minimal cortical vacuolation) in females at 7000 ppm (equal to 1147.6 mg/kg bw per day) (Heal, 2016a).

Rat

In a subchronic toxicity study, broflanilide (purity 99.58%) was administered in the diet for 90 days to groups of 10 Wistar (Crl:WI(Han) rats of each sex per dose, at concentrations of 0, 500, 1500, 5000 and 15 000 ppm (equal to 0, 35, 104, 345 and 1109 mg/kg bw per day for males, 0, 41, 126, 418, and 1239 mg/kg bw per day for females). Two additional groups of 10 rats/sex per group were administered 0 and 15 000 ppm for 90 days and then maintained for four weeks without access to broflanilide to assess recovery. The highest dose tested was selected based on the result from maximum tolerated dose (MTD) study in which body weight loss had been observed in males and females at 20 000 ppm (Bartlett, 2010). Diets were evaluated for their homogeneity and stability. Food consumption and body weight were determined weekly. The animals were examined for signs of toxicity or mortality at least once a day. Detailed clinical examinations in an open field were conducted prior to the start of the administration period and weekly thereafter. Ophthalmological examinations were performed before the beginning and at the end of the administration period on animals from the control and high-dose groups. A functional observational battery (FOB), as well as measurement of motor activity (MA), were carried out at the end of the administration period. Clinical chemistry and haematological examinations as well as urinalyses were performed towards the end of the administration period on both main and recovery groups. Additional plasma samples were taken from 10 animals/sex per group after 14, 42 and 72 days of test item administration to investigate the content of broflanilide and its main metabolite DM-8007 by tandem utrahigh-performance liquid chromatography/mass spectrometry (UPLC-MS/MS) for which the limit of quantification was (LOQ) was 0.01 mg/L. At termination of treatment, organ weights were recorded for all animals (adrenal glands, kidneys, brain, liver, cervix, spleen, ovaries, testes, epididymides, heart, thymus, thyroid and uterus). At sacrifice all animals were subject to gross pathology, followed by histopathological examinations. Histopathology examination was conducted on all tissues in all animals of the control and high-dose groups, and on the adrenal, spleen, heart (female), liver (female), ovaries and any gross lesions in all animals at the low and intermediate doses.

There were no treatment-related mortalities, clinical signs of toxicity or effects on water or food consumption. Body weight (from day 35 to 91) and body weight gain (from day 7 to 91) were decreased to a statistically significant extent in males at 15 000 ppm with a maximum depression by 8% in body weight and 15% in weight gain when compared to control means. Lower body weights were also observed in females in this dose group, but their differences did not achieve statistical significance. Body weights in both sexes were similar to controls after the recovery period.

No treatment-related findings of toxicological concern were observed in examinations in home cage or open-field environments, nor in tests of sensorimotor/reflexes or quantitative parameters. A statistically significant increase in rearing counts was reported for males at 500 and 15 000 ppm, however with no dose–response relationship, and rearing counts in these animals that were similar to those of the control recovery group, the changes were not considered treatment-related. No treatment-related deviations in motor activity were observed in either sex of the main groups. In recovery group males at 15 000 ppm, at week 13, a significant increase in motor activity relative to controls was reported for single intervals. No significant deviations in the overall motor activity, however, were apparent. Since no treatment-related effects on motor activity were observed in the 90-day neurotoxicity study (Buesen et al., 2015b) up to a dose level of 15 000 ppm, the significant changes during single intervals reported in this study were considered not to be related to treatment.

There were no treatment-related findings from the ophthalmoscopy investigations.

Investigation of haematology parameters revealed a statistically significant increase in relative reticulocyte counts of up to around 50% compared to controls, which was seen in males and females at all doses. No meaningful effects on the other red blood cell or coagulating parameters were observed.

Investigation of clinical biochemistry parameters revealed increases in cholesterol in females by 45%, 62%, 39% and 22% compared to controls at 500, 1500, 5000 and 15 000 ppm, respectively. These achieved statistical significance at 500, 1500 and 5000 ppm. In the recovery groups, cholesterol

was increased in males and females at 15 000 ppm. It was noted that all values were within historical control data (HCD) ranges and that the concurrent control values were on the low end of the historical range. In the absence of treatment-related changes correlating with any physiopathological findings the increase in was considered of no toxicological significance. In the recovery group at 15 000 ppm alanine transaminase (ALT) was decreased in females with statistical significance, however decreases in ALT are not considered adverse. In males at 500 and 1500 ppm potassium levels were increased compared to controls, but as the values recorded were consistent with the controls for the recovery group changes were regarded as not treatment-related.

There was a significant decrease in urine specific gravity and a nonsignificant increase in urine volume in males at 5000 and 15 000 ppm compared with controls.

No treatment-related changes in bone marrow cytology were observed.

A treatment-related, statistically significant ($p < 0.01$) increase in absolute and relative adrenal glands weights was observed at all dose levels in males and females of the main group, with a relatively flat dose response. In the recovery group at 15 000 ppm both males and females showed an increase in absolute and relative adrenal gland weights, achieving statistical significance in all instances except for relative adrenal weight in females.

In females of the main group a statistically significant increase in absolute heart weight was reported at 1500 and 5000 ppm, by up to 17% compared with controls. Females at 15 000 ppm also showed an absolute heart weight increase (by 9%) but this did not achieve statistical significance. In these animals, relative heart weight was increased (with statistical significance) at 1500, 5000 and 15 000 ppm by up to 16%, with a flat dose response. Heart weights from the recovery group were similar to controls for both sexes. Given the magnitude of these changes and the absence of correlating histopathology findings, increase in heart weight was not regarded as toxicologically relevant.

In females of the main group absolute liver weight showed a statistically significant increase at 500, 1500 and 5000 ppm (up to 15%). Relative liver weight showed a statistically significant increase in females at 500, 1500, 5000 and 15 000 ppm (up to 14%) and in males at 1500 and 5000 ppm (up to 8%). Liver weights of animals in the recovery group were similar to controls. Given the magnitude of these changes, and the absence of correlating physiopathological findings, the reported increase in liver weights was considered of no toxicological relevance.

In females of the main group absolute spleen weights were increased at all dose levels (by up to 33%), achieving statistical significance at 500, 1500 and 5000 ppm. No treatment-related effect was observed on the absolute spleen weight of males in the main group or males and females in the recovery group at 15 000 ppm. Relative spleen weight of animals in the main group were also increased at all dose levels (by up to 15% in males and 32% in females), achieving statistical significance in males at 500, 1500 and 5000 ppm and in females at all dose levels. No meaningful effects on relative spleen weight were observed for animals in the recovery group receiving 15 000 ppm.

In females, an increase in absolute and relative ovary weight was noted at 1500, 5000 and 15 000 ppm (by up to 21% in absolute weight and up to 27% in relative weight) achieving statistical significance for the relative weight at 15 000 ppm. No ovary weight changes compared to controls were noted in the recovery group.

In males, a statistically significant increase in relative epididymis weight were reported at 500, 1500 and 15 000 ppm (by up to 15%) and in relative testis weight at 1500 and 15 000 ppm (by up to 19%). No weight changes in these organs were observed in males from the recovery group at 15 000 ppm. As no correlating histopathological findings were observed the increased weights of these organs were considered to be of no toxicological significance.

A statistically significant increase in relative kidney weight was reported in main-group males at 1500 ppm. In the absence of substantial kidney weight changes at the next two higher dose levels the increased relative kidney weight at 1500 ppm was not considered treatment-related. A statistically significant increase in relative brain weight (by 5%) reported in females at 15 000 ppm was ascribable to the slightly reduced body weight in these animals. There were no histopathology alterations induced by treatment.

No treatment-related gross lesions were observed.

Histopathology investigation revealed a statistically significant increase in vacuolation in the cortex of the adrenal gland (zona fasciculata and glomerulosa) in all treated animals from the main group, with grade of severity increasing at higher dose levels. The cytoplasmic vacuolation was characterized by multiple, round, clearly demarcated to coalescing vacuoles of varying size. In treated females, cortical cells were also hypertrophic. This finding was reversible in female animals and partially reversible in males.

A statistically significant increase in vacuolation of ovarian interstitial cells was noted in all treated female animals, with increased severity at higher dose levels. Vacuoles were multiple, round, clearly demarcated to coalescing, and of varying size. This finding was partially reversible as both incidence and severity in the recovery group was lower than for the main group.

An increase in the incidence of extramedullary haemopoiesis was noted in several animals from the main group with a relatively flat dose response in males and no dose response in females, nor did the effect's severity show any clear dose response. Overall, this finding correlates with the increase of reticulocytes and spleen weights and was considered a compensatory effect of a presumed anaemia that could not, itself, be detected in this study.

Table 14a. Key findings of the 90-day dietary toxicity study in rats; results for males

	Males: dietary dose (ppm)				
Parameters	**0**	**500**	**1500**	**5000**	**15 000**
Body weight; mean ± standard deviation (g)					
MG day 0	144.6 ± 8.7	145.7 ± 9.1	145.4 ± 9.4	145.4 ± 8.6	144.2 ± 8.1
MG day 91	394.9 ± 19.4	385.7 ± 33.6	385.7 ± 19.1	392.5 ± 32.2	**365.0*** **± 10.3**
percentage of control	100	98	98	99	**92**
Body weight gain; mean ± standard deviation (g)					
MG day 0–91	250.3	239.9	240.3	247.1	**220.8***
percentage of control	100	95.8	96.0	98.7	**88.2**
Haematology;					
MG reticulocytes (%)	1.5 ± 0.2	**1.8** **± 0.4***	**2.0** **± 0.4****	**2.1** **± 0.4****	1.9 ± 0.3**
percentage of control	100	**120**	**133**	**140**	127
RG reticulocytes (%)	1.6 ± 0.3	-	-	-	**2.0** **± 0.2****
percentage of control	100	-	-	-	**125**
Urine analysis					
Volume; mean ± standard deviation (mL)	3.2 ± 1.4	3.9 ± 1.0	3.6 ± 1.2	4.5 ± 1.0	4.5 ± 1.2
Specific gravity; mean ± standard deviation (g/L)	1.070 ± 25	1.052 ± 15	1.066 ± 24	1.050 ± 15*	1.044 ± 8*
Absolute organ weight					
Terminal body weight ± standard deviation (g)	374.4 ± 17.5	365.2 ± 33.9	362.7 ± 22.2	370.4 ± 33.1	346.1 ± 11.8
MG; adrenal gland ± standard deviation (mg)	61.1 ± 6.86	**79.1** **± 8.90****	**83.5** **± 10.44****	**84.8** **± 14.64****	**82.2** **± 8.97****
percentage of control	100	**129**	**137**	**139**	**135**
RG; adrenal gland ± standard deviation (mg)	53.6 ± 5.52	-	-	-	**69.9** **± 11.1****
percentage of control	100	-	-	-	**130**

	Males: dietary dose (ppm)				
Parameters	**0**	**500**	**1500**	**5000**	**15000**
MG; spleen	0.59	0.64	0.65	0.65	0.60
± standard deviation (g)	± 0.06	± 0.09	± 0.04	± 0.05	± 0.08
percentage of control	100	109	110	110	102
Relative organ weights (percentage of body weight)					
MG; adrenal glands	0.016	**0.022**	**0.023**	**0.023**	**0.024**
± standard deviation	± 0.002	**± 0.003****	**± 0.003****	**± 0.003****	**± 0.002****
percentage of control	100	**133**	**141**	**140**	**145**
RG; adrenal glands	0.013	-	-	-	**0.018**
± standard deviation	± 0.001				**± 0.002****
percentage of control	100	-	-	-	**132**
MG; spleen	0.156	**0.175**	**0.179**	**0.175**	**0.172**
± standard deviation	± 0.012	**± 0.019****	**± 0.019****	**± 0.017***	**± 0.020**
percentage of control	100	**112**	**115**	**112**	**110**
Histopathology					
Number examined	10	10	10	10	10
Adrenal cortex – main group					
Vacuolation, increased	0	**10****	**10****	**10****	**10****
minimal	0	1	1	0	3
slight	0	8	3	3	1
moderate	0	1	3	5	1
severe	0	0	3	2	5
Hypertrophy, diffuse	0	0	0	0	0
minimal	0	0	0	0	0
slight	0	0	0	0	0
moderate	0	0	0	0	0
Adrenal cortex – recovery group					
Vacuolation, increased	0	-	-	-	5*
minimal	0	-	-	-	5
moderate	0	-	-	-	0
Spleen – main group					
Haematopoiesis, extramedullary	2	6	5	7	6
minimal	2	3	2	1	3
slight	0	3	3	6	3
moderate	0	0	0	0	0
Spleen – recovery group					
Haematopoiesis, extramedullary	0	-	-	-	0
Minimal	0	-	-	-	0
Slight	0	-	-	-	0
Plasma bioanalysis[a] (mean of 10 animals ± SD; ng/L)					
Compound intake (mg/kg bw per day)	0	35	104	345	1109
Broflanilide					
day 14	0	43 ± 11	60 ± 19	78 ± 21	116± 43
day 42	0	40 ± 10	43 ± 9	67 ± 14	154 ± 69
day 72	0	31 ± 6	40 ± 9	78 ± 19	116 ± 30

	Males: dietary dose (ppm)				
Parameters	**0**	**500**	**1500**	**5000**	**15000**
Metabolite DM-8007					
day 14	0	6600 ± 1800	11400 ± 3600	13300 ± 4100	15700 ± 3200
day 42	0	13200 ± 2100	7500 ± 1700	9800 ± 2400	11800 ± 5000
day 72	0	7600 ± 2400	12800 ± 4600	19700 ±4900	19300 ±5800

MG: Main group; RG: Recovery group; Sources: Buesen, 2017a; Richter, Taraschewski & Wotske, 2015

[a] Bioanalysis; limit of quantification for broflanilide and DM-8007 was 10.0 ng/mL;

Statistically significant: * $p < 0.05$, ** $p < 0.01$

Table 14b. Key findings of the 90-day dietary toxicity study in rats; results for females

	Females: dietary dose (ppm)				
Parameters	**0**	**500**	**1500**	**5000**	**15000**
Body weight; mean ± standard deviation (g)					
MG day 0	119.5 ± 5.8	118.4 ± 6.4	121.3 ± 6.8	119.1 ± 5.0	120.0 ± 7.2
MG day 91	227.3 ± 19.0	233.7 ± 16.4	229.7 ± 18.4	232.6 ± 7.3	217.6 ± 21.1
percentage of control	100	103	101	102	96
Body weight gain; mean ± standard deviation (g)					
MG day 0–91	107.8	115.3	108.4	113.4	97.7
percentage of control	100	107.0	100.6	105.2	90.6
Haematology					
MG Reticulocytes (%)	2.0 ± 0.3	**2.9 ± 0.5****	**3.1 ± 0.8****	**3.1 ± 0.6****	**3.0 ± 0.6****
percentage of control	100	**145**	**155**	**155**	**150**
RG Reticulocytes (%)	2.2 ± 0.5	-	-	-	**3.3 ± 0.4****
percentage of control	100	-	-	-	**150**
Urine analysis					
Volume; mean ± standard deviation (mL)	2.2 ± 1.2	2.2 ± 1.1	2.6 ± 1.4	2.9 ± 0.9	2.2 ± 1.1
Specific gravity; mean ± standard deviation (g/L)	1.076 ± 28	1.082 ± 38	1.061 ± 20	1.062 ± 24	1.069 ± 20
Absolute organ weight					
Terminal body weight ± standard deviation (g)	214.0 ± 15.9	218.7 ± 16.1	216.3 ± 16.1	218.7 ± 9.0	202.1 ± 18.0
MG; adrenal gland ± standard deviation (mg)	**67.0 ± 9.82**	**104.1 ± 9.33****	**100.5 ± 13.99****	**99.8 ± 9.57****	**112.0 ± 20.58****
percentage of control	**100**	**155**	**150**	**149**	**167**
RG; adrenal gland ± standard deviation (mg)	**71.4 ± 5.76**	–	–	–	**79.2 ± 6.50****
percentage of control	**100**	–	–	–	**111**
MG;ovary ± standard deviation (mg)	100.1 ± 9.93	104.3 ± 9.93	119.0 ± 23.3	114.9 ± 15.2	121.5 ± 30.4
percentage of control	–	104	119	115	121

	Females: dietary dose (ppm)				
Parameters	**0**	**500**	**1500**	**5000**	**15000**
MG; spleen	0.39	**0.48**	**0.51**	**0.47**	**0.44**
± standard deviation (g)	± 0.06	**± 0.07****	**± 0.05****	**± 0.05****	**± 0.06**
percentage of control	100	**125**	**133**	**121**	**113**
Relative organ weight					
MG; adrenal glands	0.031	**0.048**	**0.046**	**0.046**	**0.055**
± standard deviation	± 0.004	**± 0.005****	**± 0.005****	**± 0.004****	**± 0.007****
percentage of control	100	**152**	**148**	**146**	**176**
RG; adrenal glands	0.031	-	-	-	0.034
± standard deviation	± 0.002				± 0.003
percentage of control	100	-	-	-	108
MG; ovaries	0.047	0.048	0.055	0.053	**0.060**
± standard deviation	± 0.01	± 0.01	± 0.01	± 0.01	**± 0.01****
percentage of control	100	102	117	112	**127**
MG; spleen	**0.181**	**0.221**	**0.238**	**0.214**	**0.215**
± standard deviation	**± 0.024**	**± 0.031****	**± 0.015****	**± 0.028****	**± 0.023****
percentage of control	**100**	**122**	**132**	**118**	**119**
Histopathology					
Number examined	10	10	10	10	10
Adrenal cortex – main group					
Vacuolation, increased	0	**10****	**10****	**10****	**10****
minimal	0	2	0	1	0
slight	0	1	1	0	3
moderate	0	6	3	1	0
severe	0	1	6	8	7
Hypertrophy, diffuse	0	**8****	**8****	**10****	**9****
minimal	0	2	4	5	4
slight	0	6	4	5	4
moderate	0	0	0	0	1
Adrenal cortex – recovery group					
Vacuolation, increased	0	-	-	-	1
minimal	0	-	-	-	0
moderate	0	-	-	-	1
Ovary – main group					
Vacuolization, interstitial glands	0	**10****	**10****	**10****	**10****
minimal	0	4	6	6	1
slight	0	5	2	1	1
moderate	0	1	2	3	8
Ovary – recovery group					
Vacuolization, interstitial glands	0	-	-	-	**6***
minimal	0	-	-	-	5
slight	0	-	-	-	1

	Females: dietary dose (ppm)				
Parameters	**0**	**500**	**1500**	**5000**	**15000**
Spleen – main group					
Haematopoiesis, extramedullary	7	6	9	10	5
minimal	5	4	1	4	4
slight	2	2	4	3	1
moderate	0	0	4	3	0
Spleen – recovery group					
Haematopoiesis, extramedullary	0	-	-	-	3
minimal	0	-	-	-	2
slight	0	-	-	-	1
Plasma bioanalysis [a] (mean of 10 animals ± SD; ng/L)					
Compound intake (mg/kg bw per day)	0	41	126	418	1239
Broflanilide					
day 14	0	183 ± 62	232 ± 46	348 ± 116	367 ± 108
day 42	0	212 ± 75	285 ± 89	279 ± 65	483 ± 348
day 72	0	192 ± 58	257 ± 86	353 ± 145	290 ± 59
Metabolite DM-8007					
day 14	0	5700 ± 2500	9000 ± 1900	11700 ± 3700	10700 ± 2900
day 42	0	31200 ± 13800	35900 ± 14200	31600 ± 27200	35700 ±12800
day 72	0	6500 ±1600	11300 ± 6500	20900 ±10900	12100 ±5500

MG: Main group; RG: Recovery group; Source: Buesen et al., 2017a; Richter, Taraschewski & Wotske, 2015

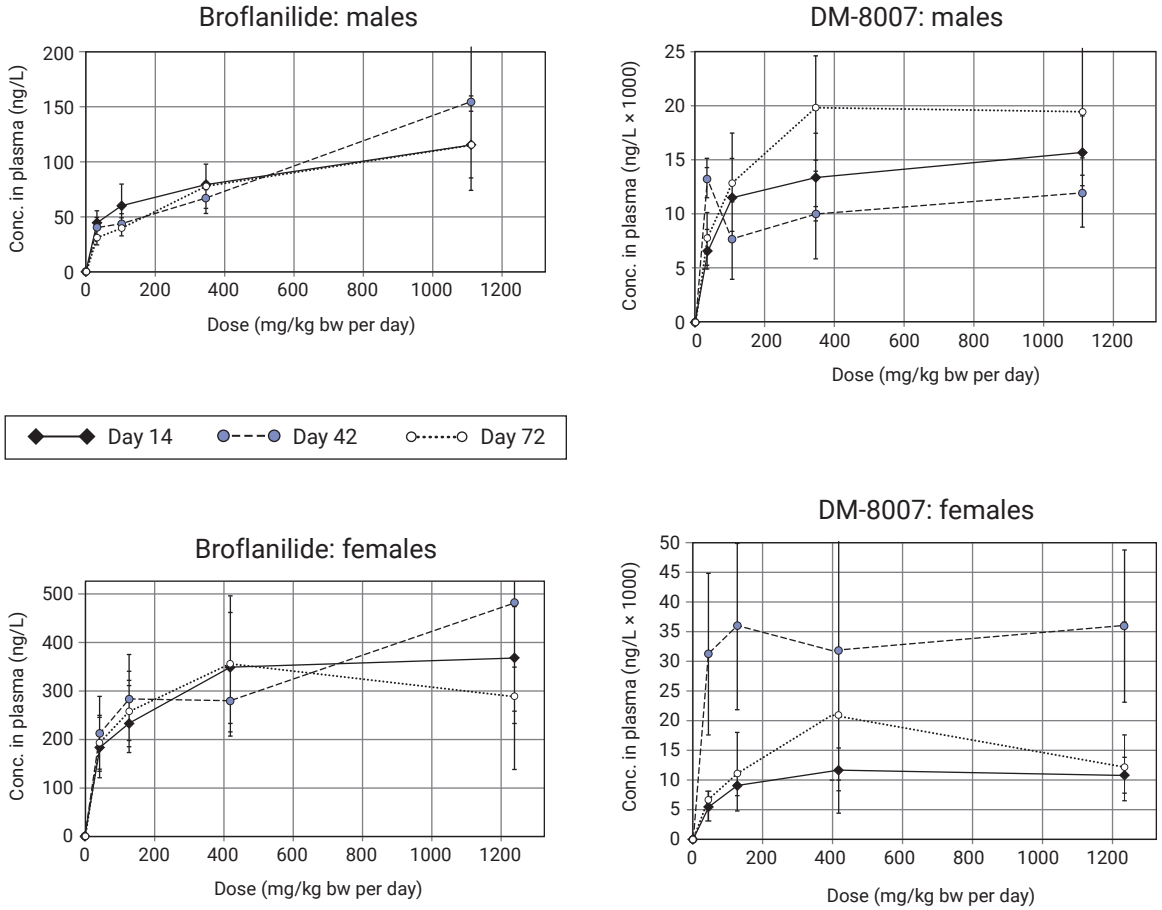
[a] Bioanalysis; limit of quantitation for broflanilide and DM-8007 was 10.0 ng/mL;

Statistically significant: * $p < 0.05$, ** $p < 0.01$

Bioanalysis of plasma samples showed that plasma levels of metabolite DM-8007 were higher than the parent compound in all cases, by approximately 100-fold. The increases in plasma concentration of broflanilide and DM-8007 were less than proportional to intake of broflanilide.

Female rats had higher broflanilide plasma values than males. Except for day 42 females, plasma levels of both broflanilide and DM-8007 varied only slightly over the time period and there was no noticeable difference in magnitude between the sampling time points. On day 42 the plasma levels of metabolite DM-8007 in females were approximately three times higher than those measured in samples from days 14 or 72. On days 14 and 72 plasma level of DM-8007 were higher in males. No parent compound or metabolite was detected in the control samples.

Figure 6. Plasma broflanilide and DM-8007 levels in male and female rats on days 14, 42 and 72



(Drawn from original data in Buesen et al., 2017a and Richter, Taraschewski & Wotske, 2015)

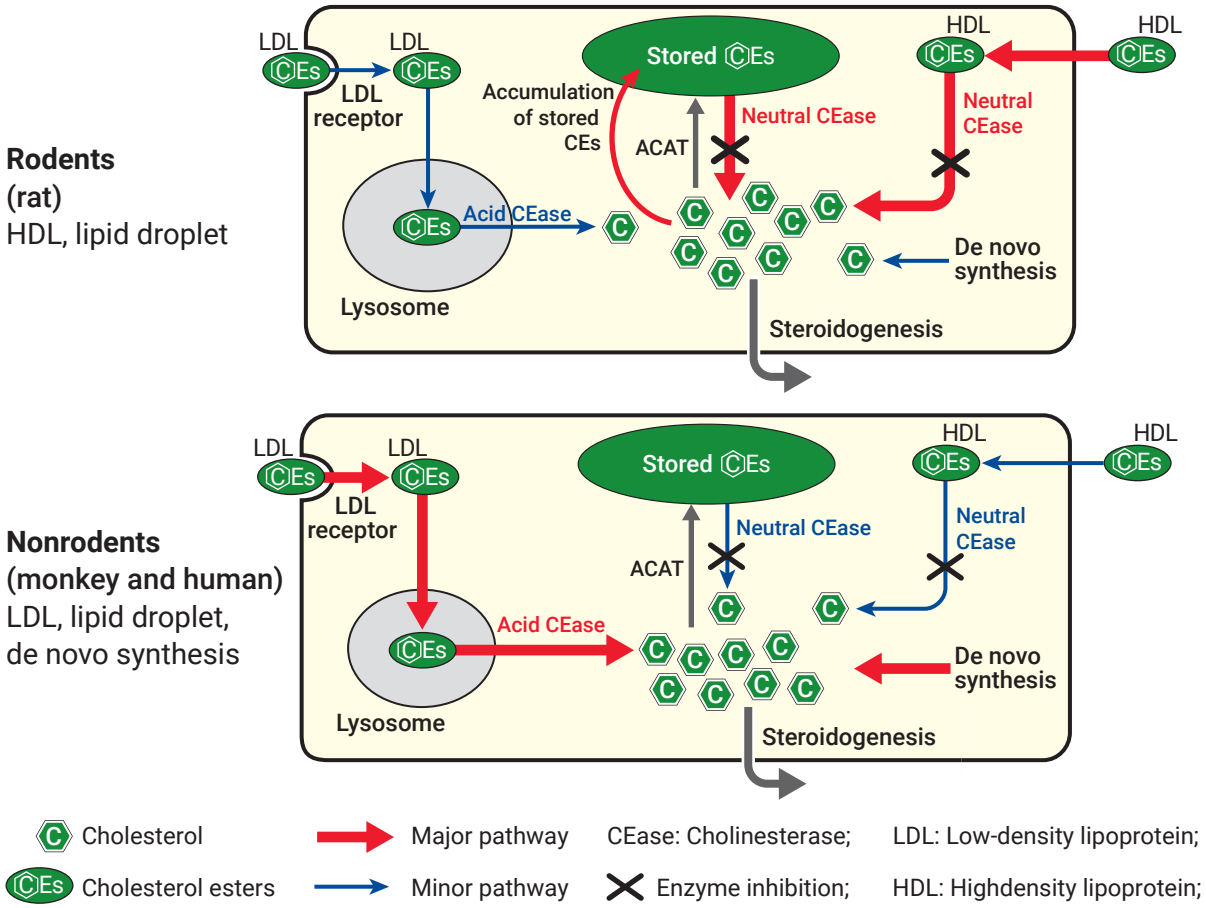
In order to assess the adversity and human relevance of vacuolation in the adrenal gland cortex (zona fasciculata and glomerulosa) and in the ovarian interstitial cells a number of in vitro and in vivo investigative mechanistic studies were conducted.

Results from an in vivo investigative study and a subsequent analysis of adrenal and ovary vacuole contents showed that subchronic treatment of male and female Wistar rats for 90-days via the diet with broflanilide at 500 and 15 000 ppm induced a treatment-related increase in lipid, cholesterol and cholesterol ester content in the adrenal cortex in both sexes and in the ovaries, although the cholesterol levels were lower compared to cholesterol esters levels (Herold, 2020; Lourens, 2017).

Cholesterol esters (CEs) in the adrenal gland and ovary are converted through enzymatic de-esterification by cholesterol esterase (CEase) to cholesterol which is converted in a follow-up enzymic process to pregnenolone catalyzed by mitochondrial cytochrome P450 11A1 (CYP11A1) the rate-limiting enzyme in steroidogenesis, leading ultimately to the biosynthesis of steroid hormones (mineralcorticoids, glucocorticoids as well as estrogens and androgens). Cholesterol esterase occurs in different isoforms: neutral cholesterol esterase (nCEase) and acid cholesterol esterase (aCEase). Evidence from the literature indicate that the main source of cholesterol for steroidogenesis is from hydrolysis of stored CEs or from plasma high density lipoproteins (HDLs) by nCEase, and hydrolysis of plasma CEs, and from plasma low density lipoproteins (LDLs) by aCEase.

In rats cholesterol is mainly supplied by hydrolysis of stored sources of CEs or HDL, meaning a predominant role for the nCEase isoenzyme. In mammals other than rodents (humans and monkeys) cholesterol is mainly supplied by de novo cholesterol synthesis or by hydrolysis of CEs from plasma LDLs by the aCEase isoenzyme (Nishizato et al., 2014; see Fig. 7).

Figure 7. Species differences between rodents and non-rodents with respect to inhibition of steroidogenesis pathways due to the inhibition of neutral CEase (Nishizato et al., 2014)



(Redrawn from Nishizato et al., 2014)

In vitro mechanistic studies showed that broflanilide and its metabolite DM-8007 did not inhibit CYP11A1 activity in mitochondrial-lysosomal fractions from the male rat adrenal gland, nor inhibit aCEase activity in male rat testes mitochondrial lysosomes. Broflanilide and its metabolite DM-8007 did however inhibit nCEase in the cytosolic fraction of the female rat adrenal gland, with an inhibitory potency that was greater for broflanilide than the positive control, and equal to the positive control for its metabolite DM-8007. No inhibition of nCEase was noted in the cytosolic fractions of female rat ovary (Bachelor, 2020a, b, c). The sponsors hypothesized that treatment-related accumulation of lipids/cholesterol and cholesterol esters in steroidogenic-competent organs (such as adrenals and ovary) is due to the ability of broflanilide and its metabolite DM-8007 to interfere with cholesterol supply to the steroid synthesis pathway, and this may consequently reduce steroid hormone biosynthesis in rodents, but is unlikely to do so in humans.

The study of Lourens, (2017) included a functional investigation of adrenal glands following adrenocorticotropic hormone (ACTH) challenge to animals treated with broflanilide at 500 and 15 000 ppm. The results showed that both controls and treated animals responded normally to the challenge, with corticosterone levels higher than prior the ACTH challenge. These results were interpreted by the sponsor as providing evidence of no impairment of glucocorticoids production from the zona fasciculata. However, it was noted that after ACTH activation, a greater increase of corticosterone than in the controls was observed in treated males and females Wistar rats after 90 days, achieving statistical significance at the high dose. In the same study, serum hormone levels of pituitary and steroidogenesis origin were also assessed (without the ACTH challenge) in animals treated with broflanilide. Overall the levels of analyzed hormones showed a high degree of variability, making the data inadequate for any quantitative evaluation. However, notable changes were observed for progesterone, prolactin, luteinizing hormone, testosterone and aldosterone. Plasma progesterone levels were generally depressed in all

females at the high dose. Prolactin plasma levels showed high variability and the direction of change was inconsistent between sexes (increase in males, decrease in females). Plasma luteinizing hormone levels were increased in both sexes, testosterone levels decreased in males, and urine aldosterone concentration increased in both sexes.

These results were considered by the Meeting to partially support the hypothesized MOA, since in rat ovary the same nCEase was not inhibited, and results from the in vitro steroidogenic assay showed a decrease in estradiol in a human adrenal tissue-derived cell line (H295R), suggesting the involvement of other, potentially human-relevant factor(s) interfering with steroidogenesis. It was also noted that the observed plasma/urine hormone changes could not be exclusively correlated with the postulated MOA. Additionally, no evidence was available to support the postulated MOA on fractions obtained from human steroidogenic-competent organs. Finally, the occurrence of vacuolation in the ovary of dogs was considered to be an indication that this toxic effect is not rodent specific, and the effect was therefore was considered to be of human relevance.

Overall, based on a weight of evidence approach, the Meeting concluded that the data do not support the MOA proposed by the sponsor and hence human relevance cannot be excluded.

With the above discussion in mind, a NOAEL could not be identified, due to effects on adrenal glands (increased adrenal weights correlated with increased vacuolation in both sexes and hypertrophy in females in the adrenal cortex), ovaries (increased incidence of vacuolation in interstitial cells) at the lowest dose tested of 500 ppm (equal to 35 mg/kg bw per day for males, 41 mg/kg bw per day for females) (Buesen et al., 2017a; Richter, Taraschewski & Wotske, 2015).

In a non-GLP and non-guideline complementary 90-day study, 10 Wistar (Crl:WI(Han)) rats of each sex per group were administered broflanilide (purity 98.7%) in the diet at dose levels of 0 or 30 ppm (equal to 0 and 2.0 mg/kg bw per day for males, 0 and 2.2 mg/kg bw per day for females). Diets were evaluated for their homogeneity and stability. Food consumption and body weights were determined on a weekly basis and the animals were observed for clinical signs of toxicity and mortality at least once a day. Detailed clinical examinations in an open field were conducted prior to the start of the study and weekly thereafter. At termination of treatment, organ weights were recorded for all animals (adrenal glands, brain, epididymides, heart, kidneys, liver, ovaries, spleen, testes, thymus, thyroid glands, uterus with cervix). At study termination animals were sacrificed and assessed for gross pathology and histopathological examinations made of the adrenal glands and ovaries.

No treatment-related clinical signs of toxicity, mortality or clinical findings were observed.

Body weight was slightly reduced during the course of the study in both sexes (by up to 5% in males and less than 5% in females), however differences were not statistically significant. Similarly, body weight gain was slightly lower than for controls throughout the treatment period in both sexes (by ca 10% in males and less than 15% in females) with females showing a few statistically significant decreases at single time intervals. In both sexes overall body weight gain was suppressed by less than 10% compared with controls.

Evaluation of organ weights revealed a statistically significant decrease in absolute and relative thymus weight in females of 13% and 11% respectively, and an increase in relative thymus weight in males by 22%. Given the opposite direction in males and females of thymus weight changes, and that no effects on this organ was noted in the main 90-day study (Buesen et al., 2017a) up to a dose level of 15 000 ppm, these changes were considered unrelated to treatment.

A statistically significant increase in the relative weight of epididymides by 8% was noted. Since no histopathological findings for this organ were noted in the main 90-day study (Buesen et al., 2017a) up to a dose level of 15 000 ppm, this weight change was considered to be of no toxicological significance.

There were no macroscopic or microscopic findings suggestive of effects on the adrenal glands or ovaries.

This study had a limited experimental protocol focusing the investigations on broflanilide target organs (adrenal glands and ovaries). Under these experimental conditions the NOAEL was 30 ppm (equal to 2.0 mg/kg bw per day) (Buesen et al., 2015a).

Dog

In a non-GLP and non-guideline compliant range-finding study, three male Beagle dogs were administered broflanilide (purity unknown) via capsule at a dose level of 1000 mg/kg per day for 14 days. Initially a single dog was dosed and treatment for the remaining two started on day 4 (so continuing until day 18). Animals were observed daily and body weight and food consumption measured. The dogs were not necropsied at the end of the treatment period.

There were no clinical signs of toxicity or effects on body weight or food consumption.

The male beagle dogs used in this study tolerated well the capsule administration of broflanilide at 1000 mg/kg bw over a period of two weeks (Buesen, 2013).

In a 28-day toxicity study, broflanilide (purity 98.67%) was administered by capsule to groups of four male and four female beagle dogs at dose levels of 0, 100, 300 or 1000 mg/kg bw per day. Food consumption and body weight were determined weekly. The animals were examined for signs of toxicity or mortality at least once a day. Detailed clinical observations were conducted prior to the start of the administration period and weekly thereafter. Clinical chemistry and haematological examinations as well as urinalyses were performed before and towards the end of the administration period. After the administration period all animals were sacrificed and assessed for gross pathology, and organ weights were recorded for all animals (adrenal glands, brain, epididymides, heart, kidneys, liver, ovaries, pituitary gland, prostate, spleen, testes, thymus, thyroid glands and uterus). This was followed by histopathological examination of all tissues in all animals from the control and high-dose groups, and in all animals in all groups in the case of adrenal glands, heart, liver, ovaries, spleen, thyroid glands and any gross lesions. In addition, after 15 days of administration, all high-dose animals (1000 mg/kg bw per day) were subject to plasma analyses for concentration of broflanilide and metabolite DM-8007.

No treatment-related clinical signs of toxicity or mortality were observed. There were no treatment-related effects on body weight or food consumption.

Haematology investigation revealed a statistically significant increase in haemoglobin (Hb) and haematocrit (Ht) in males at 1000 mg/kg bw per day, by 7% when compared to controls. These slight changes were considered to be of no toxicological significance and of doubtful relation to treatment as no effects on red cells parameters were observed in the 90-day or one-year dog studies up to 1000 mg/kg bw per day (Buesen et al., 2016a; Keller et al., 2016).

In males, increased cholesterol concentration was noted at 300 and 1000 mg/kg bw per day, when compared to controls and to pretreatment values. It was noted that the higher mean value at 300 mg/kg bw per day was driven by a one dog with an extremely high cholesterol level. All other individuals had cholesterol values in the range of the concurrent controls. In the absence of treatment-related triglyceride changes the cholesterol increase in males at 1000 mg/kg bw per day was considered of no toxicological significance and of doubtful relation to treatment as no similar effect was observed in the one-year dog studies up to 1000 mg/kg bw per day (Keller et al., 2016). There were no treatment-related effects on urine parameters.

Increased absolute and relative liver weights were reported in males at 1000 mg/kg bw per day. As no treatment-related liver weight changes were observed in the one-year dog studies up to 1000 mg/kg bw per day (Keller et al., 2016), the increased liver weights were considered of doubtful relation to treatment and of no toxicological relevance. Increased absolute adrenal weight was reported in males at 100, 300 and 1000 mg/kg bw per day (by 22%, 17% and 40%, respectively, compared to controls), achieving statistical significance at the high dose. Similarly, relative adrenal weights in males were increased compared to controls by 17%, 17% and 40% at the low, mid and high dose respectively. This result could be explained by the fact that the absolute weights of adrenal glands of male control animals (1.015 g) were below the mean weight of adrenal glands in the laboratory historical control database (absolute HCD mean 1.226 g; range 1.07–1.503 g). In the absence of treatment-related histopathological findings, adrenal weight changes were considered of doubtful toxicological relevance. No effects were observed in the adrenals of females. Absolute and relative weights of the thymus was lower in females at 100, 300 and 1000 mg/kg bw per day (by 29%, 28% and 40% for absolute weight, and by 28%, 23% and 36% for relative weight, respectively) but with no dose–response relationship for relative weight. The decreases in thymus weight were statistically significant for absolute weight at 1000 mg/kg bw per day

and relative weight at 100 and 1000 mg/kg bw per day. No treatment-related histopathological changes were observed in the thymus.

Changes to reproductive organs in males and females were noted and were considered a consequence of the how young the animals were and the different stages of sexual maturity. Only one male in each group reached the age of sexual maturity. This influence is also reflected in the histopathological pattern of testes, epididymides and prostate glands. For females, only one (from the control group) reached its first estrous cycle. There were no treatment-related changes in the weights of these organs nor histopathology findings in the related 90-day and one-year dog studies up to 1000 mg/kg bw per day (Buesen et al., 2016a; Keller et al., 2016).

The results of the bioanalysis showed that broflanilide and its metabolite DM-8007 were quantified in both males and females (see Table 16). Plasma concentrations of DM-8007 were greater than broflanilide by approximately 100-fold in both sexes.

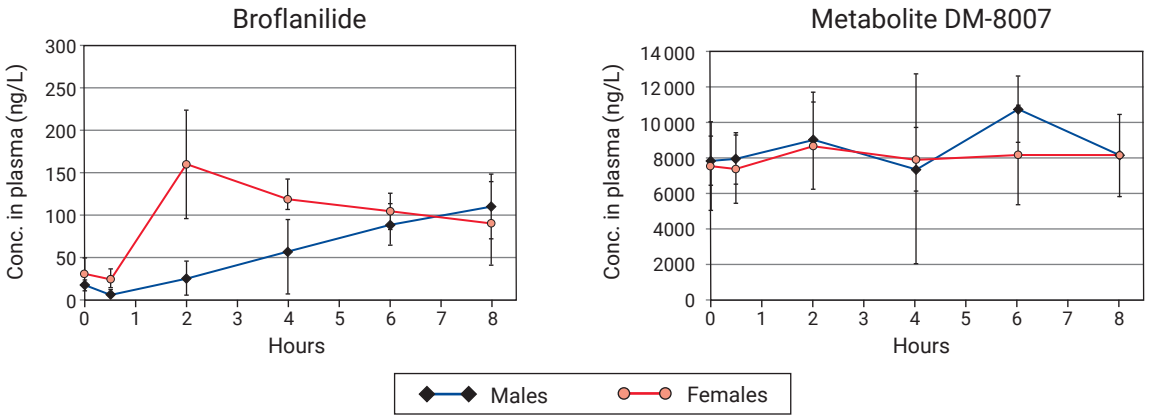
Table 16. Plasma broflanilide and DM-8007 in dogs dosed at 1000 mg/kg bw per day after 15 days

	Hours after administration					
	0	**0.5**	**2**	**4**	**6**	**8**
			Males			
Broflanilide; ± SD (ng/L)	16.54 ±6.24	6.18 ±7.84	25.70 ±19.70	57.17 ±50.06	88.57 ±24.65	109.7 ±38.10
DM-8007; ± SD (ng/L)	7765 ±1378	7920 ±1411	8954 ±2702	7319 ±5346	10713 ±1865	8068 ±2285
			Females			
Broflanilide; ± SD (ng/L)	30.51 ±19.35	24.06 ±12.49	159.7 ±63.71	118.2 ±23.64	104.2 ±21.31	90.08 ±49.88
DM-8007; ± SD (ng/L)	7520 ±2492	7324 ±1933	8637 ±2464	7888 ±1760	8122 ±2798	8068 ±2285

SD: Standard deviation; Source: Buesen et al., 2015c

Bioanalysis: working limit of quantification (wLOQ) was around 5 ng/L for broflanilide and DM-8007

Figure 8. Bioanalysis over time of broflanilide and DM-8007 in beagle dogs at 1000 mg/kg bw/day



(Drawn from data in Buesen et al., 2015c)

The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Buesen et al., 2015c).

In a 90-day dog study broflanilide (purity 98.67%) was administered by capsule to groups of five male and five female beagle dogs at dose levels of 0, 100, 300 or 1000 mg/kg bw per day. Food consumption and body weight were determined at the beginning of administration and weekly thereafter. The animals were examined for signs of toxicity or mortality at least once a day. Detailed clinical observations were conducted prior to the start of the administration period and weekly thereafter.

Ophthalmoscopy was performed before the beginning of the study and at its end. Clinical chemistry and haematological examinations as well as urinalysis were performed before the administration period, at the half-way stage and towards the end of the administration period. In addition, the plasma concentration of broflanilide and its metabolite DM-8007 were determined in samples collected on days 22, 44 and 71. After the administration period all animals were sacrificed and assessed for gross pathology, and organ weights recorded (adrenal glands, brain, epididymides, heart, kidneys, liver, ovaries, pituitary gland, prostate, spleen, testes, thymus, thyroid glands and uterus), following which a histopathological examination was made on all tissues from all animals.

No treatment-related clinical signs of toxicity or mortality were observed, nor any treatment-related effect on body weight. Although body weight gains were depressed in males at 300 and 1000 mg/kg bw per day these changes were not dose-related. Food consumption was lower (by as much as 17%) in males at 1000 mg/kg bw per day for several days during the administration period, when compared to controls. Since there was no concomitant effect on body weight over the entire study period the reduced food consumption was not considered relevant.

Ophthalmoscopic investigations revealed no treatment-related findings.

No treatment-related effects were observed on RBCs or coagulating parameters. A statistically significant decrease in absolute and relative neutrophil counts was reported in females at 100 and 1000 mg/kg bw per day, by 30% and 27%, respectively when compared to controls. In females only, a statistically significant increase in relative lymphocyte counts was reported at 100 mg/kg bw per day. Considering that results for absolute and relative neutrophils and relative lymphocytes for concurrent female controls were outside the HCD range, and that values for these parameters in treated groups fell broadly within the HCD range, the neutrophil changes observed were not considered adverse.

Turning to the biochemistry parameters investigated, an increase in alkaline phosphatase (ALP) activity was noted in males and females at 1000 mg/kg bw per day at all time points, when compared to controls and to pretreatment values; this achieved statistical significance in females at 43 days. A decrease compared to controls, not dose-related but statistically significant, in aspartate transaminase (AST) activity was reported in females at 43 days of treatment at 300 and 1000 mg/kg bw per day. However, the AST activities of these groups were either equal to, or higher than, the values recorded before administration (day −11). For these animals, at the end of the study, AST activities appeared to decrease in a dose-related manner. In the absence of vitamin B6 deficiency or uraemia, AST decrease is not considered toxicologically relevant. A statistically significant (by 84%) in triglycerides when compared to controls was reported in males receiving 1000 mg/kg bw per day, at the end of the study. It was noted that the mean value of triglycerides in these animals was similar to that for the controls at 43 days of treatment, and that no treatment-related effect was noted after one year at 1000 mg/kg bw per day in the study by Keller et al., (2016). Increased cholesterol, compared to control and pretest values, was observed during the study in males receiving 1000 mg/kg bw per day. In males receiving the low dose and mid dose, cholesterol levels were similar to the controls at pretest (day −11). Cholesterol increase in males at 1000 mg/kg bw per day was considered of no toxicological significance and of doubtful relation to treatment as no effect on this parameter was observed in the one-year dog study of Keller et al., (2016) at doses up to 1000 mg/kg bw per day. A similar trend in cholesterol values over time was observed in females at mid- and high-dose levels. The higher values reported in females at the low dose at the end of the study were driven by two individuals which contributed extremely high values. A statistically significant decrease in chloride was reported in males at 300 and 1000 mg/kg bw per day (by 1%) when compared to controls. Given the magnitude of the change and the absence of treatment-related effects after one year up to 1000 mg/kg bw per day, the decrease in chloride was considered unrelated to treatment and of no toxicological significance.

No treatment-related changes in urinalysis parameters were observed.

Treatment-related liver weight changes were noted in males and females. In males, increases in absolute (by 13% and 21%) and relative (by 19% and 23%) liver weights were noted at 300 and 1000 mg/kg bw per day respectively, achieving statistical significance for the relative weights. In females, treatment-related increases in absolute (by 23%) and relative (by 25%) liver weights were noted at 1000 mg/kg bw per day, the change in relative weight achieving statistical significance. Liver weight increases were also noted in females at 100 and 300 mg/kg bw per day, but with no apparent dose–response

relationship. Considering that no treatment-related liver weight changes were observed in the one-year dog study of Keller et al., (2016) up to a dose level of 1000 mg/kg bw per day the liver weight changes observed were considered of no toxicological relevance. The increased ALP level in animals at the high dose was not considered an indication of liver toxicity per se. In the absence of other physiopathological findings indicative of liver toxicity, effects on liver weights were not considered adverse.

An increase in adrenal gland weights from ca 10% to ca 20% was also noted in males and females at 300 and 1000 mg/kg bw per day. The increases in adrenal weight were considered treatment-related, however in the absence of treatment-related histopathological findings these changes were considered of doubtful toxicological relevance.

There were no treatment-related gross or histopathology findings.

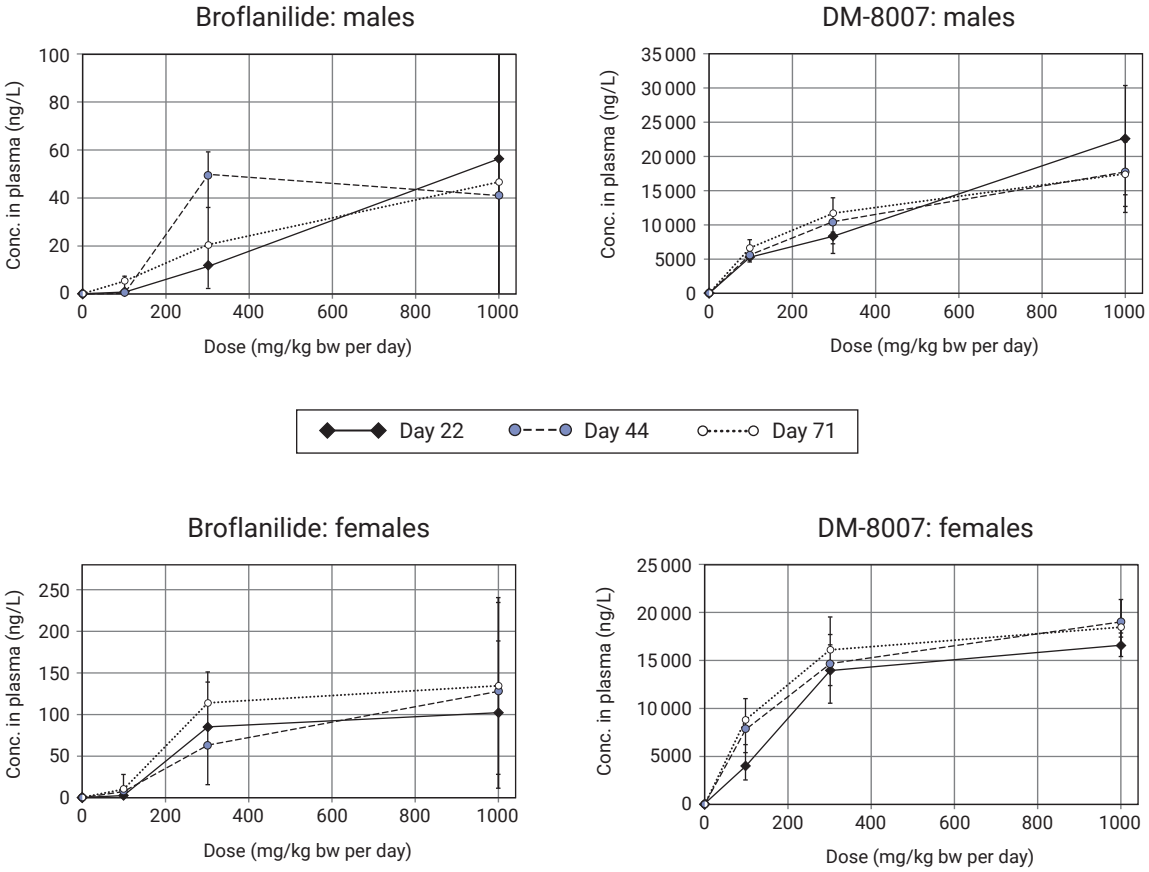
Bioanalysis of plasma showed that broflanilide and metabolite DM-8007 were detected and quantified in both sexes. In some of the control samples small amounts of metabolite DM-8007, but not broflanilide, were detected. This was due to a system carry-over problem that could not be fully circumvented due to the tendency of DM-8007 to remain behind in the apparatus. However, as the amount recovered in control samples was less than 1% of the lowest dose tested (100 mg/kg bw per day), this finding was deemed not to have a significant impact on the interpretation or integrity of the study. The amount of broflanilide was lower than metabolite DM-8007 by approximately 500-fold in males and 150-fold in females. The systemic exposure to broflanilide was supraproportional between the low- and mid-dose levels, and subproportional between the mid- and high-dose levels for both males and females. Systemic exposure to DM-8007 was subproportional between all dose levels for both males and females. Amounts recovered in females were generally higher than in males for both broflanilide and DM-8007.

Table 17. Bioanalysis of broflanilide and DM-8007 in the 90-day toxicity study in beagle dogs

	Sex and dose levels (mg/kg bw per day)							
	Males				**Females**			
	0	**100**	**300**	**1000**	**0**	**100**	**300**	**1000**
Broflanilide; mean ± standard deviation (ng/mL)								
Day 22	< LOQ	< LOQ	11.1 ± 25	55.8 ± 101	< LOQ	< LOQ	83.2 ± 68	99.1 ± 89
Day 44	< LOQ	< LOQ	49.0 ± 46	40.8 ± 88	< LOQ	9.1 ± 13	61.3 ± 77	125.5 ± 114
Day 71	< LOQ	5.2 ± 12	19.4 ± 40	46.0 ± 79	< LOQ	8.6 ± 18	110.9 ± 164	130.5 ± 103
DM-8007; mean ± standard deviation (ng/mL)								
Day 22	< LOQ	5370 ± 885	8050 ± 2196	22 347 ± 7920	4.4	3795 ± 1416	13 947 ± 3595	16 401 ± 995
Day 44	35.6	5551 ± 1037	10 389 ± 3327	17 337 ± 4858	39.1	7569 ± 3296	14 400 ± 2109	18 806 ± 2538
Day 71	26.7	6431 ± 1360	11 416 ± 2659	17 248 ± 5370	31.3	8524 ± 2451	15 884 ± 3584	18 363 ± 632

< LOQ: Less than limit of quantification, ca 5 ng/L; Source: Buesen et al., 2016a

Figure 9. Bioanalysis over time of broflanilide and DM-8007 in beagle dogs at 1000 mg/kg bw/day



(Drawn from data in Buesen, 2016a)

The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Buesen, 2016a).

In a one-year dog study broflanilide (purity 98.67%) was administered by capsule to groups of five male and five female beagle dogs at dose levels of 0, 100, 300 or 1000 mg/kg bw per day. Food consumption and body weight were determined at the beginning of administration and weekly thereafter. The animals were examined for signs of toxicity or mortality at least once a day. Detailed clinical observations were conducted prior to the start of the administration period and weekly thereafter. Ophthalmoscopy was performed before the beginning of the study and at its end. Clinical chemistry and haematological examinations as well as urinalysis were performed before the administration period, at the half-way stage (two time points) and towards the end of the administration period. After the administration period all animals were sacrificed and assessed for gross pathology, their organ weights recorded (adrenal glands, brain, epididymides, heart, kidneys, liver, ovaries, pituitary gland, prostate, spleen, testes, thymus, thyroid glands and uterus), and this was followed by a histopathological examination of all tissues from all animals.

No treatment-related clinical signs of toxicity or mortality were observed. No treatment-related effects on body weights were noted in males, or in food consumption in either sex. Towards the end of the study the mean body weight of females at 1000 mg/kg bw per day showed a reduction (not statistically significant) by 14% compared to the control. In these animals, body weight gain was reduced to a statistically significant extent at several intervals during the study period, with a maximum decrease occurring towards the end of the study, when the weight gain compared to controls was depressed by 47.1%. Reduced body weights compared to controls were also noted in females at the low dose and mid dose but with no clear dose–response relationship. At the end of the study body weights were lowers than for controls by 10% and 8% at 100 and 300 mg/kg bw per day, respectively. Reduced body weight gain was apparent over the whole study period with an overall depression of 35% and 27% compared to controls at 100 and 300 mg/kg bw per day respectively.

Ophthalmoscopic investigations revealed no treatment-related findings.

No treatment-related effects on RBCs or coagulating parameters were observed. After 12 months a dose-related increase in the absolute neutrophil count was reported in females at 100, 300 and 1000 mg/kg bw per day (by 12%, 24% and 38%, respectively) when compared to controls, achieving statistical significance at the high dose. In addition, in females at 1000 mg/kg bw per day white blood cell (WBC) counts increased (not statistically significant) by 24% compared to controls. It was noted that for treated groups, mean values of these parameters were similar to those before treatment (day −12);for this reason WBC changes were considered unrelated to treatment and of no toxicological relevance.

Evaluation of clinical biochemistry showed an expected progressive reduction in plasma ALP activity in control animals when compared to levels seen before commencement of treatment. When compared to controls, a dose-related increase in ALP was observed at all time points in treated males and females. At the twelve month time point the increase in ALP at 100, 300 and 1000 mg/kg bw per day, was by 28%, 49% and 132% for males, and by 41%, 93% and 187% for females, respectively. These changes achieved statistical significance at mid dose in females (days 93, 178 and 361) and males (day 178) and at the high dose in both sexes at all time points. When group means were compared to their pretreatment values, unchanged ALP activities was observed in males and females at 300 mg/kg bw per day, while a slight increase was observed in females at 1000 mg/kg bw per day.

In females after 12 months, a dose-related increases in ALT were reported at 100, 300 and 1000 mg/kg bw per day (21%, 32% and 47%, respectively) when compared to controls. These changes were statistically significant at 100 and 1000 mg/kg bw per day. As the mean ALT values for females at 100 and 300 mg/kg bw per day after 12 months were similar to those before treatment (day −12), these changes were considered to be of doubtful relation to treatment or toxicological relevance. In males after 12 months, an increase in cholesterol levels compared to controls was noted at 100, 300 and 1000 mg/kg bw per day, achieving statistical significance at low and mid doses, but with a relatively flat dose–response relationship. It was noted that the mean cholesterol values for these animals after 12 months were similar to those before treatment (day −12), and for this reason the changes were considered of no toxicological relevance.

Pathology examinations resulted in a statistically significant increase in absolute and relative adrenal glands weights relative to controls in males at all doses, ranging from 30% to ca 40%, with a relative flat dose–response relationship. In females, absolute adrenal gland weight was increased compared to controls at 300 and 1000 mg/kg bw per day by 7% and 15% respectively. Relative adrenal weight in these animals was also increased by 13% and 30% respectively compared to controls. In both sexes, adrenal weight increases correlated with enlargement of the adrenal gland.

Histopathologically, hypertrophy of the cortical cells of the zona fasciculata of the adrenal gland (graded as minimal) was noted in one male from each dosed group. One female at the high dose was graded as slight. This finding was reported in animals with the highest adrenal weights. An increased incidence of minimal macrovesicular vacuolation in the zona fasciculata of the adrenal gland was reported in males at 300 and 1000 mg/kg bw per day. For one male each at the low- and the mid-dose levels, but not at the high dose, slight macrovesicular vacuolation in the zona fasciculata was reported. In females, macrovesicular vacuolation in the zona fasciculata of the adrenal gland was reported in almost all animals at all dose levels; it was graded minimal at 100 and 300 mg/kg bw per day, and from minimal to slight at 1000 mg/kg bw per day.

Table 18. Key results of the one-year study in beagle dogs (weights are shown as mean ± standard deviation)

Parameter	Dose levels (mg/kg bw per day)				Historical control data (mean; range)
	0	100	300	1000	
			Males		
Body weight (kg)					
Week 0	10.5 ± 0.3	10.5 ± 0.8	10.8 ± 0.9	10.5 ± 1.0	-
Week 52	14.5 ± 0.3	14.1 ± 0.6	14.6 ± 1.1	14.3 ± 2.0	-
percentage of control	-	97	101	99	-
Body weight gain (kg)					
Days 0–364	4.0 ± 0.3	3.6 ± 0.9	3.8 ± 0.4	3.8 ± 1.4	-
percentage of control	-	90	95	95	-
Organ weight (absolute organ weights)					
Adrenal glands (g)	1.514 ± 0.113	**1.984 ± 0.476***	**2.038 ± 0.266****	**2.036 ± 0.23****	1.347; 1.164–1.518
percentage of control	-	**131**	**135**	**134**	
Pituitary (g)	69.6 ± 5.128	84.0 ± 13.982	84.6 ± 17.615	74.6 ± 6.95	-
percentage of control	-	121	122	107	-
Organ weight (relative organ weights)					
Adrenal glands (%)	0.01 ± 0.001	**0.014 ± 0.003***	**0.014 ± 0.002****	**0.014 ± 0.002***	0.009; 0.008–0.01
percentage of control	-	**134**	**132**	**138**	
Pituitary (%)	0.0 ± 0.0	**0.001 ± 0.0***	0.001 ± 0.0	0.001 ± 0.0	-
percentage of control	-	**123**	119	109	-
Gross lesions					
Number examined	5	5	5	5	-
Enlarged adrenal glands	0	2	2	3	-
Histopathology					
Number examined	5	5	5	5	-
Adrenal glands					
Hypertrophy: cortical. diffuse	0	1	1	1	-
minimal	0	1	1	1	-
slight	0	0	0	0	-
Vacuolation macrovesicular zona fasciculata	2	2	3	3	-
minimal	2	1	2	3	-
slight	0	1	1	0	-

Parameter	Dose levels (mg/kg bw per day)				Historical control data (mean; range)
	0	100	300	1000	
			Females		
Body weight (kg)					
Week 0	9.4 ± 0.6	9.5 ± 0.7	9.4 ± 0.7	9.5 ± 1.0	-
Week 52	13.4 ± 1.8	12.0 ± 0.8	12.3 ± 0.6	11.6 ± 1.6	-
percentage of control	-	90	92	87	-
Body weight gain (kg)					
day 0–364	4.0 ± 1.4	2.6 ± 0.7	2.9 ± 1.0	**2.2* ± 0.9**	-
percentage of control	-	65	73	**55**	-
Organ weight (absolute organ weights)					
Adrenal glands (g)	1.814 ± 0.342	1.662 ± 0.336	1.948 ± 0.377	2.092 ± 0.647	1.552; 1.330-1.820
percentage of control	-	92	107	115	
Pituitary (g)	83.4 ± 19.819	71.6 ± 10.407	78.2 ± 8.729	82.6 ± 16.165	-
percentage of control	-	86	94	99	-
Organ weight (relative organ weights)					
Adrenal glands (%)	0.014 ± 0.003	0.014 ± 0.002	0.016 ± 0.002	0.018 ± 0.004	0.012; 0.009-0.014
Percentage of control	-	101	113	130	
Pituitary (%)	0.001 ± 0.0	0.001 ± 0.0	0.001 ± 0.0	0.001 ± 0.0	-
Percentage of control	-	97	101	115	-
Gross lesions					
Number examined	5	5	5	5	-
Enlarged adrenal glands	0	0	1	2	-
Histopathology					
Number examined	5	5	5	5	-
Adrenal glands					
Hypertrophy: cortical. diffuse	0	0	0	1	-
minimal	0	0	0	0	-
slight	0	0	0	1	-
Vacuolation macrovesicular zona fasciculata	2	5	4	5	-
minimal	2	5	4	3	-
slight	0	0	0	2	-

Statistically significant: * $p \leq 0.05$, ** $p \leq 0.01$; Source: Keller et al., 2016

In this study no NOAEL could be identified due to reduced body weight in females, increased adrenal weight and enlargement in males and cortical hypertrophy in males, and vacuolation of the zona fasciculata of the adrenal gland in females, all at 100 mg/kg bw per day, the lowest dose tested (Keller et al., 2016).

(b) Dermal application

In a 28-day repeated dermal toxicity study broflanilide (purity 98.7%) was applied as a suspension in drinking water containing 1% CMC to the clipped dorsal skin (at least 10% of body surface) on groups of 10 male and 10 female Wistar rats at dose levels of 0, 100, 300 or 1000 mg/kg bw per day for six hours per day on five days a week. Control animals received the vehicle only; 1% CMC in drinking water. Food consumption and body weights were determined weekly. The animals were examined for signs of toxicity or mortality at least once a day and a detailed examination was performed weekly. Ophthalmological examinations were performed before treatment began and towards the end of the application period. Clinical chemistry and haematological examinations were also performed towards the end of the application period. After the application period, all animals were sacrificed and assessed for gross pathology. The following organs were weighed: adrenal glands, brain, epididymides, heart, kidney, liver, ovaries, spleen, testes, thymus, thyroid and uterus. Histopathological examinations were conducted on gross lesions in all groups, and on all tissues from the control and the high-dose groups.

No treatment-related effects were noted in clinical observations, mortality and body weights. Females at 1000 mg/kg bw per day showed a decrease in body weight gain (by 46%) at the end of the first week of treatment compared to controls; this was not statistically significant and values progressively returned to control levels thereafter.

Haematological investigation revealed a statistically significant increase (c 20%) in total WBC and absolute lymphocyte counts in females receiving 300 or 1000 mg/kg bw per day. Given the magnitude of these changes and that their mean values were within ranges (WBC: $3.10–5.07 \times 10^9$/L; absolute lymphocyte counts: $2.54−4.33 \times 10^9$/L) for historical control data, they were considered of no biological relevance and of doubtful relation to treatment.

Ophthalmoscopic investigations produced no treatment-related findings.

In females at 300 and 1000 mg/kg bw per day, γ-glutamyl transpeptidase (GGTP) activity was increased, with statistical significance, but no obvious dose–response relationship. The mean values were within historical control ranges for GGTP (0–14 nkat/L). In the absence of correlating physiopathological findings this GGTP findings were considered of doubtful relation to treatment and of no toxicological relevance.

Mean absolute brain weight for females receiving 1000 mg/kg bw per day was significantly increased (103%) compared to controls. Mean relative heart weight for males at 1000 mg/kg bw per day was also significantly increased (106%) compared to controls. These small changes in the absence of histopathology correlates were regarded as not treatment-related and of no toxicological significance.

There was no treatment-related gross of histopathology findings.

The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Buesen, 2015d).

(c) Exposure by inhalation

Study 1

In a non-GLP, five-day range-finding inhalation toxicity study, broflanilide (purity 98.67%) was administered to groups of five male and five female Wistar(Crl:WI(Han)) rats by inhalation (described in the study report as "head-nose"). The targeted exposure concentrations were 100, 300 and 1000 mg per cubic metre, administered as a dust aerosol for six hours per day on five consecutive days. A concurrent control group of five male and five female rats was exposed under the same experimental conditions to fresh air. Clinical observations, body weight determinations and haematological examinations were performed on all animals. After sacrifice all animals underwent gross pathological examination, organ weight assessment and histological examination of the respiratory tract, adrenal glands, liver, ovaries and spleen.

Particle size distribution, measured by cascade impactor, demonstrated mass median aerodynamic diameters (MMADs) within the respirable range, with over 70% of particles of MMAD 3 µm or less with a geometric standard deviation (GSD) between 2.0 and 2.4.

No clinical signs of toxicity were observed and all animals survived until scheduled necropsy.

A slightly reduced body weight gain was noted in males and females in the 300 and 1000 mg per cubic metre dose groups. No effect on mean body weight was detectable.

In males at 300 and 1000 mg per cubic metre, absolute and relative eosinophil counts were elevated. This alteration was not considered biologically relevant because this was the only one of several differential blood cell types which showed any effects.

Absolute and relative adrenal weights were increased compared to controls in females at 300 and 1000 mg per cubic metre, achieving statistical significance at the high dose. Increases were by 15% and 13% for absolute and relative weights respectively at 300 mg per cubic metre, and of 25% and 29% for absolute and relative weights respectively at 1000 mg per cubic metre.

A statistically significant increase of absolute (up to 22%) and relative (up to 23%) heart weight was reported in females at all dose levels. In females at 1000 mg per cubic metre absolute and relative thymus weights were decreased by 23% and 20% respectively, achieving statistical significance for the absolute weight. The toxicological relevance of weight changes in thymus and heart could not be definitively determined as no histopathological examination was performed on these organs, however no treatment-related histopathological effects were noted in the 28-day inhalation study relating to these changes in organ weight (Ma-Hock et al., 2017).

Statistically significant increases in relative brain and kidney weights (both by 7%) were noted in females receiving 1000 mg per cubic metre. Changes in brain and kidney weight were considered a consequence of a slight overall body weight reduction in these animals.

Histopathological examination showed an increased incidence of minimal or slight epithelial alterations (very few) in the larynx, minimal to mild inflammatory cell infiltrates in the bronchio-alveolar transition region, and mild hypertrophy/hyperplasia of terminal bronchioles in the lungs in both sexes at 1000 mg per cubic metre. No other treatment-related histopathological findings were noted. The epithelial alteration of the larynx was considered to be treatment-related but not adverse because it was not associated with any dysfunction of the larynx; this is as recommended by International ESTP Expert Workshop (Kaufmann et al., 2009).

Under the non-GLP experimental conditions used the NOAEC was 300 mg per cubic metre based on increased incidence of inflammatory cell infiltrates and hypertrophy/hyperplasia in the lungs (Ma-Hock, 2015).

Study 2

In a 28-day inhalation toxicity study, broflanilide (purity 98.67%) was administered to groups of 10 male and 10 female Wistar (Crl:WI(Han)) rats by nose-only inhalation. The targeted exposure concentrations were 30, 200 and 1000 mg per cubic metre (equal to 31, 193 and 940 mg per cubic metre for males and females) as a dust aerosol for six hours per day, on five consecutive days per week. A concurrent control group of ten male and ten female rats was exposed to fresh air. In addition, recovery groups of five males and five females were exposed simultaneously to the main group animals to the high concentration and control conditions and observed for further four weeks. Clinical observations, body weight determinations and haematological and clinical chemistry examinations were performed on all animals. After sacrifice, all animals underwent gross pathological evaluation, organ weight assessment (adrenal glands, brain, epididymides, heart, kidneys, liver, lungs ovaries, spleen, testes, thymus, thyroid glands and uterus). Histological examination was conducted on all animals for gross lesions, adrenal glands, epididymides, larynx, liver, lung, ovaries, spleen and testes. Additionally all other organs from animals in the control and high-dose groups were examined histologically.

All measurements of particle size resulted in MMADs between 1.3 and 2.0 µm with GSDs from 2.3 to 2.5. The calculated mass fractions of with MMAD below 3 µm ranged between 67.7% and 83.3%.

No treatment-related clinical signs of toxicity or mortalities were observed. Ophthalmologic examinations revealed no treatment-related effects. No changes in body weight compared to controls were noted. Reduced body weight gain was observed in males at all concentrations and in females at the high concentration, achieving statistical significance only in males at 1000 mg per cubic metre. Decreases in body weight gain were not considered of biological relevance. No substance-related changes in food consumption were observed.

Haematological investigations revealed a statistically significant increase in relative reticulocyte counts in both sexes at 200 and 1000 mg per cubic metre, accompanied in females by a slight decrease in haemoglobin (Hb) and increase in mean corpuscular volume (MCV). In males at 200 and 1000 mg per cubic metre relative neutrophil counts were significantly increased and relative lymphocyte counts significantly decreased compared to controls. As no significant changes were observed in counts of WBCs, absolute neutrophils or lymphocytes the reported changes were considered of no biological relevance.

Among clinical chemistry parameters, cholesterol was increased in both sexes at 200 and 1000 mg per cubic metre, by 15% in males and 25% in females at the mid concentration and by 28% in males and 47% in females at the high concentration, achieving statistical significance at the highest concentration tested. The mean cholesterol values for females were outside of the historical control range. In females at 1000 mg per cubic metre, GGTP activity and total bilirubin were significantly increased compared to controls. However, one female rat was noted to have spontaneous small fibrotic liver and bile duct hyperplasia. Excluding the value from this animal, all other animals had GGTP activities within the range of HCD controls. In males at 1000 mg per cubic metre inorganic phosphate was significantly decreased and in females at 200 and 1000 mg per cubic metre creatinine levels were significantly decreased. Both parameters were within HCD ranges, therefore the observed changes were considered of no biological relevance. There were no treatment-related changes in histopathology parameters that might be correlated.

All changed parameters recovered after a four week period, except creatinine in highest-dose females, which remained lower than for the main group, but only slightly so by the end of recovery.

Organ weight assessment revealed a dose-related and statistically significant increase in absolute and relative adrenal weights in both sexes at all concentrations, absolute and relative heart weights in females at 200 and 1000 mg per cubic metre, relative liver and ovary weights in females at 200 and 1000 mg per cubic metre, relative lung weight in both sexes at 1000 mg per cubic metre. Absolute ovary weights in females at 200 and 1000 mg per cubic metre were increased but did not achieve statistical significance. All organ weight changes were reversed by the end of the 28-day recovery period.

Histopathology showed statistically significant treatment-related increases in microvesicular vacuolation of all zones of the adrenal cortex in both sexes at 200 and 1000 mg per cubic metre.

Minimal to slight epithelial alterations in the larynx were increased in all animals at all concentrations, with no clear dose–response relationship in males and relatively flat response in females. Incidences were statistically significant in males at mid and high doses, and in females at all doses. This alteration was considered treatment-related but not adverse, as it was not associated with any dysfunction of the larynx (as recommended by International ESTP Expert Workshop, Kaufmann et al., 2009).

In the lungs, treatment-related minimal to slight regenerative hyperplasia of the bronchial epithelium characterized by basophilic epithelium with focal loss of cilia and a minimal infiltration of inflammatory cells and cellular debris within bronchial lumina was observed in both sexes at 200 and 1000 mg per cubic metre. The increased relative weight of the lungs in high dose animals was considered to be related to these findings. An increased incidence of alveolar histiocytosis occurred in both sexes at 1000 mg per cubic metre.

With respect to the spleen, there was a treatment-related increase (not statistically significant) in pigment storage with brown flaky appearance (plausibly haemosiderin) in rats receiving 200 and 1000 mg per cubic metre, but this showed no statistically significant differences from controls. Extramedullary haematopoiesis was observed in rats of from groups including the controls, with an increased incidence and severity in males and females at 200 and 1000 mg per cubic metre, reaching statistical significance in females at 200 mg per cubic metre and in both sexes at 1000 mg per cubic metre.

In the ovaries, a significant treatment-related increase in microvesicular vacuolation of interstitial cells (minimal to moderate) was noted at 200 and 1000 mg per cubic metre.

In the testes, multifocal tubular degeneration occurred in several control (minimal to severe) and treated (minimal to moderate) male animals, but without any relationship to the concentrations which resulted in some of these animals having debris in the epididymides. Multifocal tubular degeneration has been observed frequently in nose-only exposed control animals (Wistar rats) in this laboratory; HCD mean 20%, ranging from 0–100%, with grading from minimal (minimal) to extreme (grade 5).

In the epididymides, an increase in minimal cribriform changes (not statistically significant) was observed at 1000 mg per cubic metre.

It was noted that none of these alterations were observed in the testes or epididymides of animals in the 90-day oral toxicity study (Buesen et al., 2015b).

After the four-week recovery period, treatment-related findings were no longer observed in the larynx, lungs or spleen of either sex, or the adrenal glands of females. In the adrenal glands of males and ovaries in females, treatment-related findings were reduced in incidence and severity. The findings in the testes and epididymides of males related to the mode of exposure were reduced in severity after the recovery period.

Table 19. Key findings of the 28-day inhalation toxicity study in Wistar rats (weights are shown as mean ± standard deviation)

Parameter	Dose levels (mg per cubic metre)				Historical control data (mean; range)
	0	**30**	**200**	**1000**	
			Males		
Haematology (day 28)					
Haemoglobin (mmol/L)	8.9 ± 0.3	8.9 ± 0.2	8.9 ± 0.2	8.7 ± 0.3	-
percentage of control	-	100	100	98	-
Mean corpuscular volume (fL)	50.3 ± 1.3	50.2 ± 1.4	49.2 ± 1.4	49.6 ± 1.4	-
percentage of control	-	100	98	99	-
Reticulocytes (%)	1.7 ± 0.3	1.7 ± 0.3	**2.1 ± 0.4***	**2.4 ± 0.5****	1.9; 1.5–2.6
percentage of control	-	100	**124**	**141**	
Clinical chemistry (day 28)					
Total bilirubin (µmol/L)	1.91 ± 0.32	1.80 ± 0.35	1.97 ± 0.40	2.01 ± 0.33	-
percentage of control	-	94	103	105	-
Cholesterol (mmol/L)	1.88 ± 0.38	1.93 ± 0.30	2.16 ± 0.34	**2.40 ± 0.30****	1.79; 1.40–2.17
percentage of control	-	103	115	**128**	
Organ weight (absolute organ weights)					
Terminal body weight (g)	273.68 ± 19.80	265.66 ± 18.46	265.25 ± 20.97	261.31 ± 13.36	-
percentage of control	-	97	97	95	-
Adrenal glands (mg)	57.9 ± 4.58	**65.5 ± 7.52***	**77.7 ± 7.07****	**95.0 ± 10.3****	-
percentage of control	-	**113**	**134**	**164**	-
Ovaries (mg)	-	-	-	-	-
percentage of control	-	-	-	-	-
Organ weight (relative organ weights)					
Adrenal glands (%)	0.021 ± 0.003	**0.025 ± 0.003***	**0.029 ± 0.004****	**0.036 ± 0.004****	-
percentage of control	-	**116**	**138**	**171**	-
Ovaries (%)	-	-	-	-	-
percentage of control	-	-	-	-	-

Parameter	Dose levels (mg per cubic metre)				Historical control data (mean; range)
	0	30	200	1000	
Histopathology					
Number examined	10	10	10	10	-
Adrenal glands					
Vacuolation, increased	0	0	**7****	**10****	-
minimal	0	0	7	4	-
slight	0	0	0	2	-
moderate	0	0	0	3	-
severe	0	0	0	1	-
Lungs					
Regenerative hyperplasia, bronchiolar	0	0	2	**8****	-
minimal	0	0	2	8	-
slight	0	0	0	0	-
Histiocytosis, alveolar	2	2	2	6	-
minimal	2	1	2	5	-
slight	0	1	0	1	-
Debris, bronchial	0	0	1	2	-
minimal	0	0	1	2	-
spleen					
Pigment storage	10	10	10	10	-
minimal	10	10	10	4	-
slight	0	0	0	5	-
moderate	0	0	0	1	-
Haematopoiesis extramedullary	3	3	5	**8***	-
minimal	3	3	3	3	-
slight	0	0	2	4	-
moderate	0	0	0	1	-
Ovaries					
Increased vacuolation	-	-	-	-	-
minimal	-	-	-	-	-
slight	-	-	-	-	-
moderate	-	-	-	-	-
Testes					
Degeneration (multifocal), tubular	5	5	6	7	
minimal	3	2	3	2	
slight	1	3	3	4	
moderate	0	0	0	1	
severe	1	0	0	0	

Parameter	Dose levels (mg per cubic metre) 0	30	200	1000	Historical control data (mean; range)
Epididymides					
Cribriform change (focal)	1	1	0	4	
minimal	0	1	0	4	
slight	1	0	0	0	
Debris	5	4	5	5	
minimal	4	2	5	3	
slight	1	1	0	2	
moderate	0	1	0	0	
			Females		
Haematology (day 28)					
Haemoglobin (mmol/L)	8.8 ± 0.3	8.8 ± 0.3	**8.5 ± 0.3***	**8.4 ± 0.5****	8.5; 8.1–9.3
percentage of control	-	100	**97**	**95**	
Mean corpuscular volume (fL)	51.3 ± 1.5	51.1 ± 0.8	**52.4 ± 1.5***	52.1 ± 1.3	52.2; 50.6–54.6
percentage of control	-	100	**102**	102	
Reticulocytes (%)	1.9 ± 0.5	2.2 ± 0.5	**2.8 ± 0.4****	**3.3 ± 0.9****	2.1; 1.4–3.0
percentage of control	-	116	**147**	**174**	
Clinical chemistry (day 28)					
Total bilirubin (µmol/L)	2.03 ± 0.38	2.08 ± 0.36	2.13 ± 0.52	**3.11 ± 1.41****	1.78; 0.95–2.60
percentage of control	-	102	105	**153**	
Cholesterol (mmol/L)	1.40 ± 0.25	1.47 ± 0.32	1.75 ± 0.49	**2.06 ± 0.42****	1.29; 1.04–1.65
percentage of control	-	105	125	**147**	
Organ weight (absolute organ weights)					
Terminal body weight (g)	171.83 ± 7.57	172.11 ± **7.**30	169.37 ± **5.9**1	168.94 ± **9.67**	-
percentage of control	-	100	99	98	-
Adrenal glands (mg)	68.7 ± 8.00	**82.2 ± 9.95****	**89.8 ± 11.69****	**99.6 ± 18.72****	-
percentage of control	-	**120**	**131**	**145**	-
Ovaries (mg)	85.0 ± 7.572	94.2 ± 13.307	95.9 ± 13.828	98.7 ± 25.647	-
percentage of control	-	111	113	116	-
Organ weight (relative organ weights)					
Adrenal glands (%)	0.040 ± 0.004	**0.048 ± 0.006****	**0.053 ± 0.006****	**0.059 ± 0.009****	-
percentage of control	-	**120**	**133**	**147**	-
Ovaries (%)	0.049 ± 0.004	0.055 ± 0.007	**0.057 ± 0.008***	**0.058 ± 0.015***	-
percentage of control	-	110	**114**	**118**	-

Parameter	Dose levels (mg per cubic metre)				Historical control data (mean; range)
	0	30	200	1000	
Histopathology					
Number examined	10	10	10	10	-
Adrenal glands					
Vacuolation, increased	0	0	**7****	**10****	-
minimal	0	0	5	2	-
slight	0	0	2	1	-
moderate	0	0	0	7	-
severe	0	0	0	0	-
Lungs					
Regenerative hyperplasia, bronchiolar	0	0	2	**10****	-
minimal	0	0	2	6	-
slight	0	0	0	4	-
Histiocytosis, alveolar	3	2	1	**9****	-
minimal	3	2	1	9	-
slight	0	0	0	0	-
Debris, bronchial	0	0	1	**4***	-
minimal	0	0	1	4	-
Spleen					
Pigment storage	10	10	10	10	-
minimal	8	5	0	3	-
slight	2	5	8	5	-
moderate	0	0	2	2	-
Haematopoiesis extramedullary	4	2	**9***	**9***	-
minimal	4	2	7	2	-
slight	0	0	1	1	-
moderate	0	0	1	6	-
Ovaries					
Increased vacuolation	0	0	**7****	**9****	-
minimal	0	0	6	0	-
slight	0	0	1	6	-
moderate	0	0	0	3	-

Statistically significant: * $p \leq 0.05$, ** $p \leq 0.01$; Source: Ma-Hock et al., 2017

The NOAEC was 30 mg per cubic metre (equal to 31 mg per cubic metre) based on increased adrenal gland weight, increased incidence of adrenal vacuolation in both sexes, increased incidence of ovary vacuolation, regenerative hyperplasia of the bronchial epithelium and cellular debris in the bronchial lumina of the lungs, histological findings in spleen and correlated changes in haematology parameters in both sexes at 200 mg per cubic metre (equal to 193 mg per cubic metre) (Ma-Hock et al., 2017).

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

In a carcinogenicity study, groups of 51 male and 51 female Crl:CD-1(ICR) mice were fed diets containing broflanilide (purity 98.67%) for 78 weeks at 0, 200, 1500 or 7000 ppm (equal to 0, 21, 157 and 745 mg/kg bw per day for males, 0, 22, 172 and 820 mg/kg per day for females). Additionally, five male and five female mice from each group were assessed by LC-MS for broflanilide and its primary metabolite DM-8007 in their plasma at weeks 4, 24, 52 and 78. All animals were observed for mortality and clinical signs twice a day during the study and their body weight and food consumption recorded once weekly from week 1 to week 16 and then at least once in every four weeks thereafter. At termination of treatment all surviving animals were euthanized and subjected to haematology, necropsy, their organs weighed (adrenal gland, brain, kidney, liver, ovary, testes and epididymides) and histopathological examination conducted on all tissues.

There were no statistically significant differences in mortality rates between treated and control groups of either sex during the treatment period. In macroscopic clinical observations, pale lower teeth and abnormal teeth were noted in both sexes at 7000 ppm. Repetitive circling (often associated with excessive activity) was observed in a number of animals, predominantly treated females. The occurrence of this isolated finding, not associated with any treatment-related mortalities or increased frequency of the observation, was considered by the Meeting to be a chance finding. It was also noted that no treatment-related auditory effects were seen across the database. Several animals were noted as having raised hair (graded minimal), or being hunched throughout the study. A veterinary review of mice throughout the study determined that these findings were unrelated to treatment.

Body weights and food consumption of males and females in all treated groups were comparable with those of controls throughout the treatment period.

As a result of haematological investigations a statistically significant increase in relative mean eosinophil count was reported in males at 7000 ppm (two-fold relative to control). A review of the individual animal data indicated that this higher value was primarily seen in two animals, both of which had no other associated findings.

For females receiving 200 ppm, statistically significantly higher mean absolute (three-fold relative to control) and relative lymphocyte counts were reported. Evaluation of individual data showed that one female in this group had a WBC count of 81.8×10^9 cells/L, and an absolute lymphocyte count of 79.36×10^9 cells/L. White blood cell and lymphocyte counts were found to be higher (but not statistically significant) in females at 1500 ppm, however, this was ascribable to a single female that had a WBC count of 412.7×10^9 cells/L, a neutrophil count of 66.0×10^9 cells/L and a lymphocyte count of 346.66×10^9 cells/L. Review of the individual animal pathology data indicated that both animals had haemolymphoreticular tumours which correlated with their high white blood cell counts.

In the organ weight assessment, a statistically significant increase in adrenal weight (absolute and relative to body and brain weight) was reported in females at 7000 ppm, increased by up to 27% relative to control). An increase in adrenal weight of up to 15% relative to controls was also reported in males at the same dose level, however, this did not achieve statistical significance. An increase in absolute and relative ovary weights was noted in animals at 1500 and 7000 ppm, but this was not dose-related. The increase relative to control was by 374% and 90% for absolute weight, and by 379% and 122% for relative weight at 1500 and 7000 ppm respectively. Evaluation of individual data showed that the ovary weight increase was due to three animals at 1500 and two animals at 7000 ppm. In these animals the absolute ovary weight was from between four- and 14-fold (at 1500 ppm) and three- and five-fold (at 7000 ppm) higher than the control upper value. All other individual ovary weight in these groups fell within the concurrent control values.

In females, microscopic analysis revealed a slightly increased incidence of accessory lobe, minimal to slight haemopoiesis, minimal to slight cortical vacuolation, minimal corticomedullary vacuolation, and minimal to slight inflammatory cell foci in the adrenals of females at 7000 ppm. In the ovary of females at 7000 ppm a slight increase in occurrence of cysts was observed both macroscopically and microscopically.

In males at 7000 ppm, a slightly increased incidence in cystic tubules in the kidney (graded minimal) was observed.

There were no treatment-related neoplastic findings.

Bioanalysis showed that neither broflanilide nor its metabolite DM-8007 were detected in control samples. The amount of DM-8007 detected was 30- to 50-fold, 45- to 80-fold, and 35- to 70-fold higher than that of broflanilide at 200, 1500 and 7000 ppm respectively. Plasma levels of broflanilide and DM-8007 were broadly sub-proportional when compare to external dose levels. With respect to the intake of broflanilide, the change (expressed as a fold-wise increase) at 1500 ppm and 7000 ppm was eight times and 35 times higher, respectively, when compared to the external dose at 200 ppm. For broflanilide, the corresponding increase in plasma concentration was between roughly two-and nine-fold, and between seven- and 15-fold at 1500 ppm and 7000 ppm, respectively, when compared to the value for 200 ppm. For metabolite DM-8007, the corresponding increase in plasma concentration was between roughly five-fold and eight-fold, and between 11-fold and 23-fold at 1500 ppm and 7000 ppm, respectively, when compared to the value at 200 ppm. In general no sex differences in plasma levels were seen for either compound.

Table 20. Key results of the long-term toxicity and carcinogenicity study in mice

	Sex and dietary dose (ppm)							
	Males				**Females**			
Parameter	**0**	**200**	**1500**	**7000**	**0**	**200**	**1500**	**7000**
Organ weights								
Adrenal glands; weight								
Absolute (g) SD	0.008 ± 0.004	0.008 ± 0.004	0.008 ± 0.002	0.009 ± 0.003	0.012 ± 0.004	0.012 ± 0.004	0.013 ± 0.003	**0.015** **± 0.004****
% of control	-	95	97	112	-	101	109	**120**
Relative to bw (%) SD	0.0144 ± 0.0067	0.0138 ± 0.0061	0.0143 ± 0.0047	0.0167 ± 0.0055	0.0269 ± 0.0092	0.0264 ± 0.0085	0.0301 ± 0.0099	**0.0341** **± 0.0122****
% of control	-	95	99	115	-	98	112	**127**
Ovary; weight								
Absolute (g) SD	-	-	-	-	0.158 ± 0.25	0.147 ± 0.32	0.749 ± 2.59	0.30 ± 0.83
% of control	-	-	-	-	-	93	474	190
Relative to bw (%) SD	-	-	-	-	0.32 ± 0.47	0.35 ± 0.87	1.51 ± 5.00	0.70 ± 1.84
% of control	-	-	-	-	-	111	479	222
Macroscopic observations								
Examined	51	51	51	51	51	51	51	51
Adrenal: large	0	0	0	0	0	0	0	4[#]
Ovary: cyst/s	-	-	-	-	26	28	26	34
Microscopic observations								
Adrenal; examined	50	50	49	50	51	51	51	51
Accessory lobe	0	0	0	0	0	0	0	4[#]
Haemopoiesis	0	0	0	0	1	1	2	3
Cortical vacuolation	0	0	0	0	0	0	1	3[#]
Corticomedullary vacuolation	0	0	0	0	1	1	2	3
Inflammatory cell foci	0	1	0	1	1	1	0	4[#]

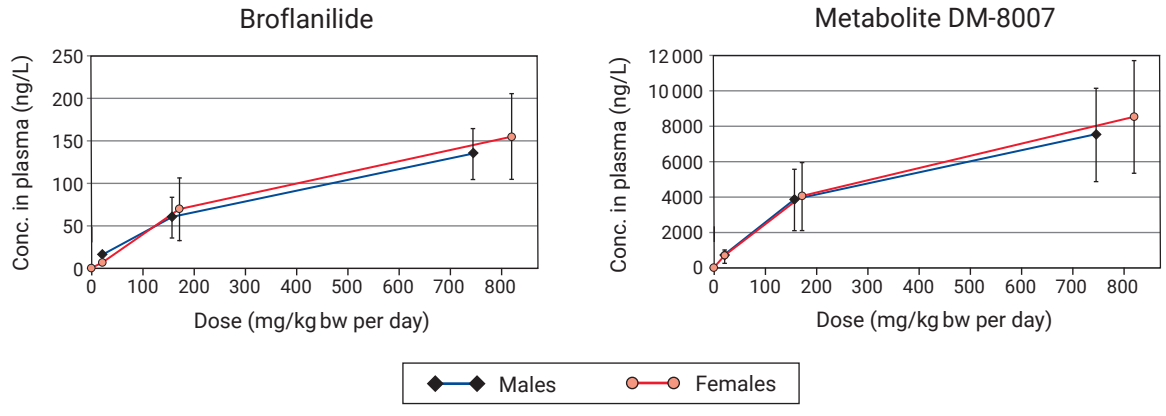
	Sex and dietary dose (ppm)							
	Males				Females			
Parameter	**0**	**200**	**1500**	**7000**	**0**	**200**	**1500**	**7000**
Ovary; examined	-	-	-	-	51	51	51	51
Cyst	-	-	-	-	30	33	29	38
Kidney; examined	51	51	51	51	51	51	51	51
Cystic tubules	1	0	0	6[#]	1	0	1	2
Broflanilide; plasma bioanalysis (mean ± SD for 5 individuals; ng/mL)								
Week 4	< 10.0[a]	18.9 ± 5.9	92.0 ± 39.6	150.8 ± 51.9	< 10.0[a]	14.3 ± 4.2	87.4 ± 44.3	199.6 ± 90.9
Week 24	< 10.0[a]	16.2 ± 7.5	39.6 ± 16.9	105.6 ± 31.3	< 10.0[a]	< 10.0	44.7 ± 22.1	93.2 ± 48.5
Week 52	< 10.0[a]	16.1 ± 6.9	65.5 ± 26.5	169.3 ± 61.5	< 10.0[a]	12.5 ± 0.8	112.5 ± 39.7	190.8 ± 142.2
Week 78	< 10.0[a]	10.4	42.4 ± 30.5	113.4 ± 40.8	< 10.0[a]	< 10.0	33.5 ± 11.9	135.3 ± 114.2
Weeks 4–78	< 10.0[a]	15.0 ± 4.0	60.0 ± 24.0	135.0 ± 30.0	< 10.0[a]	7.0 ± 8.0	70.0 ± 37.0	155.0 ± 50.0
Metabolite DM–8007; plasma bioanalysis (mean ± SD for 5 individuals; ng/mL)								
Week 4	< 200[b]	948 ± 130	5956 ± 784	10 204 ± 2102	< 200[b]	1152 ± 225	6374 ± 1441	13 040 ± 2294
Week 24	< 200[b]	551 ± 92	3140 ± 800	7406 ± 1205	< 200[b]	505 ± 84	3504 ± 801	7490 ± 2602
Week 52	< 200[b]	605 ± 115	4274 ± 1054	8456 ± 1728	< 200[b]	533 ± 136	4418 ± 1526	7794 ± 3548
Week 78	< 200[b]	285 ± 44	1900 ± 438	3982 ± 909	< 200[b]	244 + 34	1792 ± 841	5693 ± 2400
Weeks 4–78	< 200[b]	597 ± 272	3818 ± 1724	7512 ± 2621	< 200[b]	609 ± 385	4022 ± 1909	8504 ± 3163

SD: Standard deviation; bw: Body weight; Source: Heal, 2016b

[a] Limit of quantitation for broflanilide was 10.0 ng/mL; [b] Limit of quantitation for DM=8007 was 200.0 ng/mL;

Statistically significant: * $p \leq 0.05$, ** $p \leq 0.01$; [#] Statistically significant using positive trend test (Cochran–Armitage)

Figure 10. Bioanalysis of broflanilide and DM-8007 in the 78-week study in mice; mean plasma concentrations for weeks 4–78 against dose level



(Drawn from data in Heal, 2016b)

The NOAEL was 1500 ppm (equal to 172 mg/kg bw per day) based on pale and abnormal teeth in both sexes, increased adrenal weights (absolute and relative), enlarged adrenals, a marginally increased incidence of haematopoiesis, cortical vacuolation, corticomedullary vacuolation and inflammatory cell foci in the adrenal glands of females and slight increase in ovary cysts, and a slight increase in cystic tubules in kidneys of males, all at 7000 ppm (equal to 820 mg/kg bw per day) (Heal, 2016b).

Rat

In a combined chronic toxicity/carcinogenicity study in rats broflanilide (purity 98.67%) was administered in the feed for 24 months to Wistar Crl:WI(Han) rats (50/sex per dose level) at doses of 0, 100, 300, 1500 or 15 000 ppm (equivalent to 0, 4.5, 14, 70 and 709 mg/kg bw per day for males, 0, 5.9, 19, 95 and 953 mg/kg bw per day for females). Satellite animals (10/sex per dose level) were given broflanilide for 12 months at doses of 0, 30, 100, 300, 1500 or 15 000 ppm (equivalent to 0, 1.7, 5.7, 16, 84 and 822 mg/kg bw per day for males, 0, 2.1, 7.2, 20, 104 and 1128 mg/kg bw per day for females). Animals in the main and satellite groups were subject to cage-side observations and checked for mortality at least once a day. Animal in the main group were subjected to detailed clinical observations prior to treatment and then once weekly during the treatment period. Body weight and food consumption were determined on a weekly basis until week 13 of treatment and at four-week intervals thereafter. Blood samples were collected from the satellite group at several intervals for determination of haematological and clinical chemistry parameters, and plasma concentrations of broflanilide and its metabolite DM-8007. Urinalysis and ophthalmoscopic examination were conducted on the satellite group. At necropsy, organ weights (brain, thyroids, adrenals, heart, liver, spleen, kidney, testes, epididymides, ovaries and uterus) were determined and histopathology examinations were conducted on liver, testes, ovaries, adrenals, uterus (including cervix), lungs (including bronchi) and any gross lesions in all groups, and on all tissues of all animals in the control and top-dose groups.

No treatment-related clinical signs of toxicity or mortalities were noted in the satellite or main groups. One female from the satellite group at 15 000 ppm was sacrificed moribund during the treatment period. This death was not considered related to treatment.

No treatment-related effects were observed on food consumption or body weights in males or females from the satellite group. on occasions, a statistically significant increase in body weight and body weight gain were reported in females at 100, 300 or 1500 ppm, however these events were not dose related. In the main group, food consumption was increased by approximately 20% relative to controls during several periods in males fed 300, 1500 or 15 000 ppm and in females at 15 000 ppm. Among males of the main group at 15 000 ppm, body weight and body weight gain were slightly reduced (by around 6% and 8% respectively) when compared to controls. Body weights in comparable females were similar to control values.

There were no treatment-related findings from ophthalmoscopic examinations.

No treatment-related effects were observed in males for any haematological parameter in measurements taken at 3, 6 or 12 months. In females, a slight decrease in RBC counts was reported after three and six months at 300, 1500 and 15 000 ppm, but with no clear dose–response relationship. A slight decrease in Hb was noted after three months at 100, 300, 1500 and 15 000 ppm and at all dose levels after six months. After 12 months, lower RBC counts (by 6%) and lower Hb concentrations (by 5%), persisted in females at 15 000 ppm, but with no progression compared to earlier intervals. A slight decrease in haematocrit was reported at 100, 300, 1500 and 15 000 ppm after six months. Relative reticulocyte counts were significantly increased at 100, 300, 1500 and 15 000 ppm after three months, and at 300, 1500 and 15 000 ppm after six months. No significant treatment-related changes in relative reticulocyte count was apparent in females after 12 months.

Clinical chemistry analysis showed an increase in cholesterol (up to 18%) in male rats at 1500 and 15 000 ppm after 6 and 12 months, not statistically significant at the high dose. In females cholesterol was increased after three and six months at 300, 1500 and 15 000 ppm, by as much as 50% relative to controls. After 12 months cholesterol level in females was increased (by up to 37%) at 1500 and 15 000 ppm, achieving statistical significance at the high dose. A slight increase in cholesterol levels (by 9%) was also noted in females at 300 ppm, however as almost all individual values were within the concurrent control range this was not considered toxicologically relevant. Decreased creatinine (by 11%) was observed in females at 15 000 ppm after 12 months.

Bioanalysis results indicated that the parent compound broflanilide could not be quantified properly because concentrations were around the limit of quantitation (LOQ) of 5 ng/mL. Trace amounts of DM-8007 were detected below the LOQ in a single control sample (3.55 ng/mL), but compared to the high concentrations of DM-8007 in treated animals this was not considered relevant. Broflanilide was detected at an amount above the LOQ in a single control sample (491.6 ng/mL). The reason for these observations appeared to be a slight system carry-over phenomenon that could not be fully circumvented due to the tendency of the compounds to remain behind in the apparatus. Compared to the high concentrations of DM-800 in the rat plasma of treated animals, the carry-over problem was regarded as non-relevant. Metabolite DM-8007 could be detected and quantified in plasma samples from all treated rats at concentrations that increased with increasing intake of the test substance in the feed, but not in a dose proportional manner.

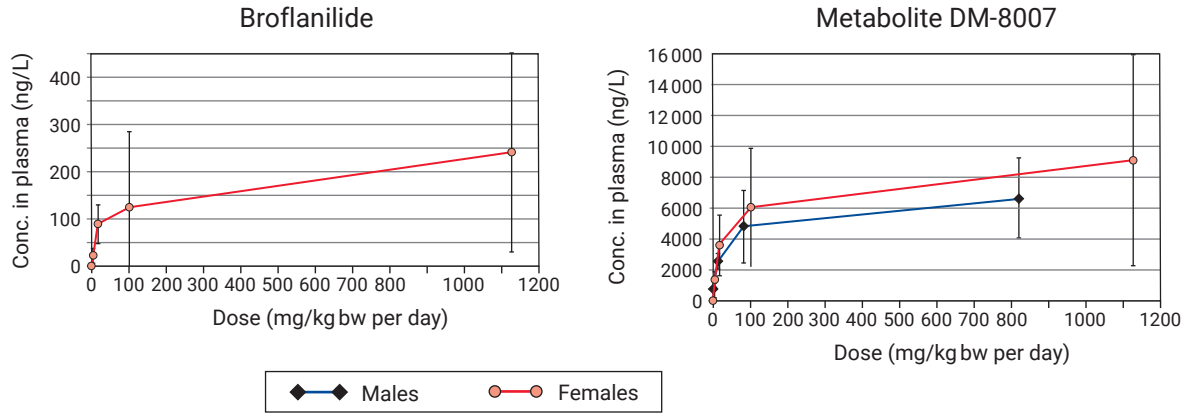
In males the external dose (compound intake) was (fold-wise) 3-, 9-, 49- and 484-times higher at 100, 300, 1500 and 15 000 ppm respectively compared to the lowest dose of 30 ppm. The fold-wise change in plasma concentration of DM-8007 was 5-, 13-, 25- and 35-times at 100, 300, 1500 and 15 000 ppm when compared to the value for the group fed at 30 ppm. In females the external dose (compound intake) was (fold-wise) was 3-, 10-, 50- and 537-time higher at 100, 300, 1500 and 15 000 ppm respectively compared to the lowest dose of 30 ppm. The fold-wise change in plasma concentration of DM-8007 was 3-, 16-, 27- and 41-times at 100, 300, 1500 and 15 000 ppm when compared to the value for the group fed at 30 ppm. Overall, plasma levels of DM-8007 in both sexes increased in a supra-proportional manner at 100 and 300 ppm and in a sub-proportional manner at 1500 and 15000 ppm.

Table 21a. Key results of the combined chronic toxicity and carcinogenicity study in rats; plasma bioanalysis for parent and DM-8007 (mean ± SD shown for 10 individuals in ng/mL)

	Sex and dietary dose (ppm)											
	Males						Females					
	0	**30**	**100**	**300**	**1500**	**15 000**	**0**	**30**	**100**	**300**	**1500**	**15 000**
Broflanilide												
Day 8	-	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	-	< LOQ	4.7 ± 14.9	93.2 ± 66.3	23.4 ± 74.1	22.4 ± 70.9
Days 81–82	-	< LOQ	< LOQ	< LOQ	140.8 ± 149.1	< LOQ	-	< LOQ	43.6 ± 70.9	154.1 ± 90.8	376.6 ± 125.2	519.0 ± 263.2
Days 172–173	-	< LOQ	< LOQ	< LOQ	24.6 ± 77.7	< LOQ	-	< LOQ	11.4 ± 24.3	75.4 ± 68.9	187.8 ± 169.8	326.0 ± 152.0
Days 263–264	-	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	-	< LOQ	17.6 ± 28.9	54.7 ± 31.7	< LOQ	33.3 ± 105.2
Days 354–358	-	< LOQ	< LOQ	< LOQ	165.1 ± 178.6	< LOQ	-	21.8 ± 68.9	31.1 ± 53.0	60.6 ± 46.8	30.1 ± 95.1	302.1 ± 235.0
Days 8–358	-	< LOQ	< LOQ	< LOQ	66.0 ± 80.0	< LOQ	-	4.0 ± 10.0	22.0 ± 16.0	88.0 ± 40.0	124.0 ± 160.0	241.0 ± 212.0
Metabolite DM–8007												
Day 8	-	169.1 ± 117.3	1112 ± 521.4	3319 ± 1415	4285 ± 2873	7620 ± 2350	-	184.5 ± 77.78	576.7 ± 334.4	1948 ± 1078	3198 ± 1453	2223 ± 1849
Days 81–82	-	216.2 ± 25.64	846.7 ± 120.0	2606 ± 595.2	4714 ± 2030	4977 ± 2901	-	417.2 ± 118.2	3067 ± 3460	6949 ± 2070	12411 ± 3789	19861 ± 8778
Days 172–173	-	227.5 ± 44.62	915.4 ± 196.0	2496 ± 615.9	8442 ± 5224	10385 ± 6538	-	187.4 ± 34.72	914.1 ± 212.1	3246 ± 397.3	6013 ± 1527	8807 ± 1569
Days 263–264	-	183.1 ± 24.70	794.3 ± 126.3	2442 ± 492.2	4737 ± 659.6	6784 ± 1867	-	130.4 ± 75.07	753.3 ± 315.1	2256 ± 1205	2769 ± 1843	4415 ± 2751
Days 354–358	-	154.7 ± 64.26	657.4 ± 288.9	1855 ± 928.9	1845 ± 2181	3491 ± 2820	-	191.2 ± 40.55	811.7 ± 178.6	3414 ± 345.3	5738 ± 1723	10100 ± 5295
Days 8–358	-	190.0 ± 31.0	865.0 ± 167.0	2544.0 ± 522.0	4805.0 ± 2360.0	6651.0 ± 2631.0	-	222.0 ± 112.0	1225.0 ± 1037.0	3563.0 ± 1994.0	6026.0 ± 3855.0	9081.0 ± 6821.0

SD: Standard deviation; < LOQ: Below the level of quantitation; Source: Buesen et al., 2017b

Figure 11. Bioanalysis at 12 months of broflanilide and DM-8007 in the two-year rat study; mean plasma concentrations for weeks 4–78 against dose level



(Drawn from data in Buesen et al., 2017b)

Organ weights

Organ weight assessment showed a dose-related, statistically significant increase in absolute and relative adrenal weights in females at 100 ppm, and in both sexes at 300, 1500 and 15 000 ppm for animals in the satellite and main groups. Adrenal weight increase was dose-related and time-related.

In the satellite group a statistically significant increase in absolute and/or relative heart weight was reported in females at 100, 300, 1500 and 15 000 ppm, by up to 28% relative to control at the high dose. It was noted that the heart weights of females at 100 and 300 ppm were within the range of the background values. In the main group, females showed a statistically significant increase in absolute and relative heart weights at 1500 and 15 000 ppm (by up to around 20%) and of relative weights at 300 ppm. The mean value at 300 ppm was within the range of the background values. In males from the main group a statistically significant increase in relative heart weights was reported at 1500 and 15 000 ppm, this by up to 6% relative to controls. There were no histopathology findings for the heart, thus these weight changes were considered of low toxicological relevance.

In males of the satellite and main groups, increased relative liver weights (statistically significant) were noted at 1500 and 15 000 ppm, reaching increases by up to 9% relative to controls at the high dose. In females of the satellite and main groups, absolute and/or relative liver weights were significantly increased at 1500 and 15 000 ppm, by up to 15% relative to controls, but with no clear dose–response relationship in animals of the main group. A significant increase in relative liver weights was also reported in females of the main group at 300 ppm, however this change was marginal in size (by 2%), and it was considered of doubtful relation to treatment. Treatment-related histopathological liver findings (multinucleated hepatocytes) were observed in the main groups at 1500 and 15 000 ppm.

Statistically significant changes were report in the spleen weights of females from the satellite and main groups. Absolute weights at 100, 1500 and 15 000 ppm and relative spleen weights at 1500 and 15 000 ppm were increased in the satellite groups. In the main groups, absolute and relative spleen weights were decreased relative to controls at all dose levels, achieving statistical significance in absolute weights for the 1500 and 15 000 ppm groups, and in relative weight for the 300, 1500 and 15 000 ppm groups. These changes were opposite in direction at 12 months and 25 months, and there were no correlating histopathological findings; they were therefore not considered treatment-related.

In the satellite group, increased absolute and relative uterus weights were noted at 15 000 ppm, achieving statistical significance in the case of relative weight. This increase was not considered treatment-related as it was mainly due to histopathologically observable estrus dilation. In the main group, uterus weights at 300, 1500 and 15 000 ppm were reduced compared to controls, achieving statistical significance at 300 ppm for absolute weight. It was noted that two individuals among the concurrent controls had extremely high uterus weights compared to the rest of the group. Almost all treated animals had uterus weights similar to those of the majority of the concurrent controls.

Statistically significant changes in absolute and/or relative brain weight were noted in the satellite and main groups. These changes were slight when compared to controls, they were in opposite directions at 12 and 25 months, and no dose–response relationship was apparent, they were therefore considered unrelated to treatment.

Statistically significant increases in relative kidney weight were noted in both sexes in the main 1500 and 15 000 ppm groups, by up to 10% and 11% in males and females respectively. Given the small increase, the absence of relevant changes in absolute weight or any treatment-related histopathological findings, these changes were considered of doubtful relation to treatment and of no toxicological significance.

Gross pathology and histopathology

After 12 months, no treatment-related gross findings were noted in either sex. Histopathological examination of these animals showed an increased incidence and severity of vacuolation in the adrenal cortex in males fed 100 ppm and above, and in females fed 300 ppm and above. Increased incidence of diffuse hypertrophy was noted in the adrenal cortex of females at 1500 and 15 000 ppm. In addition, the incidence and severity of vacuolation in the interstitial glands of the ovary was increased at 300 ppm and above. In males, a minimal to slight increase in extramedullary haematopoiesis (mainly reticulopoiesis) in the spleen was observed at all dose levels, with a relatively flat dose–response relationship. After 24 months the incidence of extramedullary haematopoiesis in the spleen of treated animals was similar to that in the controls.

After 24 months, gross pathology examinations showed treatment-related increases in discolouration of the adrenal cortex in males at 15 000 ppm and in females at 1500 and 15 000 ppm. In females, enlarged adrenal and adrenal masses were observed at 1500 and 15 000 ppm. In both sexes at 15 000 ppm, foci in the lung were observed. In males, discolouration of the testes, foci and masses were observed at 15 000 ppm.

Histopathology examination after 24 months showed treatment-related effects in the adrenal glands of both sexes, consisting of increased incidence and severity (minimal to moderate) of vacuolation in the adrenals of both sexes at 300 ppm and above, increased incidence and severity of cystic degeneration (minimal to slight) in males at 1500 ppm and increased incidence of fatty changes in females at 1500 ppm and in both sexes at 15 000 ppm. In lungs the occurrence of very small, very few cholesterol clefts (with associated granulomatous responses) was significantly increased in male and female animals at 1500 ppm and 15 000 ppm. In males, alveolar histiocytosis (very few to a moderate number of sites) was noted with a statistically significant increase in incidence and severity at 15 000 ppm.

In the liver, increased incidence and severity (slight to moderate) of multinucleated hepatocytes were observed in females at 15 000 ppm. A slight increase in multinucleated hepatocytes (moderate) was noted in females at 1500 ppm. In the ovary, an increased incidence and severity of vacuolation of the interstitial glands (minimal to severe) was noted at all doses tested. A statistically significant increase in the incidence of follicular cysts was observed in the ovary of animals at 15 000 ppm. A significant increase in the occurrence of this finding was also reported at 100 ppm and 1500 ppm, but with no dose–response relationship. In the brain, an increase in the incidence of compression by the pituitary was noted in females at all dose levels, achieving statistical significance at 15 000 ppm compared to controls. As this did not reflect the true incidence of pituitary adenomas or carcinomas or the size of tumours, the Meeting concluded that brain compression by pituitary tumours in females was unrelated to treatment.

The Meeting noted the following findings occurred only in females at the highest dose and only in survivals. In the extra-orbital lacrimal gland, a statistically significant increase in degeneration was reported in females at 15 000 ppm. Considering that these changes were of lesser grade in treated animals than in controls and were unilateral, they were considered a chance findings. In the kidney, a statistically significant increase in cysts and pelvic dilatation was reported in females at 15 000 ppm. In the absence of treatment-related chronic progressive nephropathy, obstructive change in the urinary system in the 15 000 ppm groups or renal toxicity in other rat studies, these findings were not considered to be treatment-related. In the stomach a statistically significant increase in erosion/ulcer formation was reported in females at 15 000 ppm; this finding correlated with the macroscopic stomach finding "focus". However, the Meeting noted that no local effects were observed across the database, so this stomach effect was considered a chance finding.

Treatment-related neoplastic and/or preneoplastic lesions were observed in the testes of males and in the ovaries, uterus and adrenals of female rats.

In males, there was a statistically significant, treatment-related increase in Leydig cell adenomas at 15 000 ppm, Leydig cell multifocal hyperplasia at 1500 and 15 000 ppm, and an increase (not statistically significant) in Leydig cell diffuse hyperplasia at 15 000 ppm. The incidence of diffuse hyperplasia at the high dose exceeded the mean and upper limit of the historical control data. The majority of these findings were present in survivors. The incidence of adenomas in the highest dose (28%) group clearly exceeded the mean and upper limit of both the older (1999–2011) and the more recent (2009–2015) laboratory historical control data. At 300 ppm, the increase in Leydig cell adenoma incidence (10%) was above the mean and the upper limit of both sets of HCD, however the increase in incidence was not greater at the next highest dose of 1500 ppm (8%). The incidence of Leydig cell adenomas at 1500 ppm was above the mean of both sets of HCD, and exceeded the upper limit of the most recent dataset. Base on the assumption of tumour genesis characterized by a continuum of events from hypertrophy to adenomas the Meeting identified a NOAEL for Leydig cell adenomas at 1500 ppm.

In the ovaries, a number of benign tumours of sex cord stromal origin were observed in treated animals. Benign luteomas and sex cord tumours at 100 ppm and 15 000 ppm were increased, but not to a statistically significant extent. These tumours did not occur in the concurrent or historical controls or in animals at 300 or 1500 ppm. An increased incidence of benign granulosa cell tumours was observed in animals at 300, 1500 ppm and 15 000 ppm with no clear dose–response relationship, and achieving statistical significance only at 1500 ppm. The incidence of granulosa cell tumours at 300 ppm (6%), 1500 ppm (22%) and at 15 000 ppm (12%) were above the upper limit of HCD from 1999–2011 (2%). Benign thecoma were reported at 15 000 ppm an incidence equal to the concurrent controls. Combining all subtypes of tumour of sex cord stromal origin, including thecoma benign tumours, results in an increased incidence in animals at 1500 and 15 000 ppm.

In the uterus, an increased incidence of adenocarcinomas was reported in animals at 1500 ppm (22%) and 15 000 ppm (28%) compared to controls (10%), achieving statistical significance at the highest dose. These incidences exceeded the control means of both HCD sets, were within the range of both HCD sets at 1500 ppm, and exceeded the upper limit of the most recent HCD set (2009–2015) at 15 000 ppm. Given their dose–response pattern the relationship to treatment could not be disregarded. An increase in glandular hyperplasia, a precursor to uterine adenocarcinoma, was observed in animals at 300, 1500 and 15 000 ppm with a relatively flat dose–response relationship, achieving statistical significance at 300 and 15 000 ppm. Hyperplasia was also noted in animals at 100 ppm, but was considered comparable to effects seen in the controls. Incidences of glandular hyperplasia in treated animals were all above the upper limit of both HCD datasets (2009–2015). It worth noting that historical datasets for this particular finding contained only four and seven studies. The concurrent control incidence for this finding was within one of the two historical datasets.

In females at 15 000 ppm adrenal cortical carcinomas (4%) were noted when compared to the control incidence (0%). The incidence of adrenal cortex carcinomas was above the upper limit of the HCD range (1999–2015).

In females there was an increase in the total number of malignant and metastasized neoplasms at 1500 ppm and 15 000 ppm. The percentage of malignant and metastasizing neoplasms tended to be higher in decedents.

Assessment of potential MOA and the human relevance of vacuolation in the adrenal gland cortex and ovary is outlined and discussed in more detail in the 90-days rat study summary (Buesen et al., 2017a; Richter, Taraschewski & Wotske, 2015). Overall, based on a weight of evidence approach the Meeting concluded that the data do not support the proposed MOA, hence human relevance cannot be excluded. As the same MOA was also hypothesized for Leydig cell adenomas and sex cord stromal tumours in the ovaries of rats the same conclusions apply for the rat tumours.

In addition, assays (Brock, 2018; Chen, 2019) were performed to investigate dopamine agonistic potential as a plausible MOA, that would not be relevant to humans, for the formation of Leydig cell adenomas. These showed that broflanilide and DM-8007 had little capability as orthosteric agonists or antagonists towards the radioligand receptors tested (D1-receptor, D2-receptor, dopamine transporter).

The results of these studies do not support the dopamine agonist alternative mode of action (non-human relevant) for Leydig cells adenomas observed in rats.

Table 21b. Key results of the combined chronic toxicity and carcinogenicity study in rats; body and organ weights (mean ± standard deviation shown)

	Dietary dose (ppm)					
	0	**30**	**100**	**300**	**1500**	**15000**
			Males			
Weights at week 52 – males						
Terminal body weight (g)	493.2	482.9	480.8	485.9	476.6	495.8
standard deviation	± 52.5	± 48.0	± 63.8	± 53.9	± 35.5	± 42.7
percentage of control	-	98	97	99	97	101
Adrenal						
Absolute weight (mg)	57.7	56.9	54.2	**69.2****	**72.5****	**81.6****
standard deviation	± 7.6	± 7.5	± 7.5	**± 10.7**	**± 7.9**	**± 11.2**
percentage of control	–	99	94	**120**	**126**	**141**
Relative weight (%)	0.012	0.012	0.011	**0.014***	**0.015****	**0.017****
standard deviation	± 0.002	± 0.001	± 0.001	**± 0.003**	**± 0.002**	**± 0.002**
percentage of control	-	100	96	**122**	**129**	**140**
Liver						
Absolute weight [a] (g)	10.0	9.8	9.9	9.9	10.3	11.0
standard deviation	± 1.0	± 0.9	± 1.1	± 1.2	± 0.8	± 1.5
percentage of control	-	98	99	99	103	110
Relative weight [b] (%)	2.03	2.03	2.07	2.03	**2.17****	**2.22****
standard deviation	± 0.07	± 0.10	± 0.14	± 0.14	**± 0.09**	**± 0.21**
percentage of control	-	100	102	100	**107**	**109**
Weights at week 104 – males						
Terminal body weight (g)	559.465	-	563.618	539.183	553.303	530.107
standard deviation	± 50.022		± 67.162	± 71.766	± 63.224	± 65.185
percentage of control	-	-	101	96	99	95
Adrenal						
Absolute weight (mg)	60.62	-	61.07	**67.34****	**76.26****	**92.00****
standard deviation	± 14.9		± 9.24	**± 13.32**	**± 16.14**	**± 42.96**
percentage of control	-	-	101	**111**	**126**	**152**
Relative weight (%)	0.011	-	0.011	**0.013****	**0.014****	**0.018****
standard deviation	± 0.003		± 0.002	**± 0.003**	**± 0.003**	**± 0.010**
percentage of control	-	-	101	**116**	**127**	**163**
Liver						
Absolute weight [c] (g)	11.575	-	12.073	11.256	11.992	11.958
standard deviation	± 1.346		± 1.887	± 1.750	± 1.688	± 1.890
percentage of control	-	-	104	97	104	103
Relative weight [d] (%)	2.071	-	2.141	2.091	**2.166***	**2.251****
standard deviation	± 0.174		± 0.227	± 0.201	**± 0.145**	**± 0.175**
percentage of control	-	-	103	101	**105**	**109**

	Dietary dose (ppm)					
	0	**30**	**100**	**300**	**1500**	**15 000**
			Females			
Weights at week 52 – females						
Terminal body weight (g)	240.7	240.7	**264.4***	**261.4***	**262.3***	241.2
standard deviation	± 16.5	± 16.9	**± 26.2**	**± 16.8**	**± 24.2**	± 19.5
percentage of control	-	100	**110**	**109**	**109**	100
Adrenal						
Absolute weight (mg)	62.0	64.1	**73.5****	**84.5****	**105.1****	**116.1****
standard deviation	± 6.5	± 9.0	**± 7.6**	**± 10.7**	**± 17.9**	**± 19.3**
percentage of control	-	103	**119**	**136**	**170**	**187**
Relative weight (%)	0.026	0.027	0.028	**0.032****	**0.040****	**0.048****
standard deviation	± 0.003	± 0.004	± 0.005	**± 0.005**	**± 0.006**	**± 0.008**
percentage of control	-	103	109	**125**	**155**	**186**
Liver						
Absolute weight [a] (g)	5.4	5.2	5.8	5.8	**6.2***	**6.2****
standard deviation	± 0.7	± 0.4	± 0.7	± 0.7	**± 0.9**	**± 0.5**
percentage of control	-	96	108	108	**115**	**115**
Relative weight [b] (%)	2.24	2.16	2.20	2.22	2.36	**2.58***
standard deviation	± 0.25	± 0.16	± 0.21	± 0.26	± 0.26	**± 0.23**
percentage of control	-	96	98	99	106	**115**
Weights at week 104 – females						
Terminal body weight (g)	313.129	-	320.661	301.758	296.096	307.125
standard deviation	± 39.646		± 48.413	± 41.246	± 41.947	± 45.622
percentage of control	-	-	102	96	95	98
Adrenal						
Absolute weight (mg)	69.50	-	**80.33****	**95.53****	**118.79****	**207.15****
standard deviation	± 11.45	-	**± 17.34**	**± 21.55**	**± 51.48**	**± 454.49**
percentage of control	-	-	**116**	**137**	**171**	**298**
Relative weight (%)	0.022	-	0.026	**0.032****	**0.041****	**0.070****
standard deviation	± 0.005	-	± 0.010	**± 0.009**	**± 0.018**	**± 0.167**
percentage of control	-	-	116	**143**	**182**	**312**
Liver						
Absolute weight [c] (g)	7.13	-	7.069	7.096	**7.844****	**8.029****
standard deviation	± 2.223		± 1.071	± 1.233	**± 1.793**	**± 1.305**
percentage of control	-	-	99	100	**110**	**113**
Relative weight [d] (%)	2.327	-	2.234	**2.366****	**2.673****	**2.637****
standard deviation	± 1.046		± 0.423	**± 0.348**	**± 0.574**	**± 0.412**
percentage of control	-	-	96	**102**	**115**	**113**

Statistically significant: * $p \leq 0.05$, ** $p \leq 0.01$; Source: Buesen et al., 2017b

[a] HCD for absolute liver weight in males given as 10.146 (range 9.290–11.352)

[b] HCD for relative liver weight in males given as 2.091 (range 1.950–2.305)

[c] HCD for absolute liver weight in females given as 7.232 (range 6.674–7.835)

[d] HCD for relative liver weight in females given as 2.243 (range 2.094–2.386)

Note: [a] and [b] are for males at weeks 52 and 104; [c] and [d] are for females at weeks 52 and 104.

Table 21c. Key results of the combined chronic toxicity and carcinogenicity study in rats; gross pathological findings at week 104; numbers in [square brackets] indicate survivors

	Sex, dietary dose (ppm)											
	Males						Females					
Parameter	**0**	**30**	**100**	**300**	**1500**	**15000**	**0**	**30**	**100**	**300**	**1500**	**15000**
Number examined [Number of survivors]	50 [34]	-	50 [44]	50 [42]	50 [42]	50 [43]	50 [42]	-	50 [36]	50 [38]	50 [28]	50 [40]
Adrenal cortex												
Discolouration (*N*)	0	-	0	2 [0]	0	**6* [5]**	0	-	0	1 [1]	**8** [2]**	**6* [2]**
percentage	0	-	0	4 [0]	0	**12 [12]**	0	-	0	2 [3]	**16 [7]**	**12 [5]**
Enlarged (*N*)	0	-	0	0	0	2 [2]	1 [1]	-	0	2 [2]	**11** [4]**	**10** [10]**
percentage	0	-	0	0	0	4 [5]	2 [2]	-	0	4 [5]	**22 [14]**	**20 [25]**
Mass (*N*)	0	-	0	0	0	0	0	-	0	0	2 [1]	2 [2]
percentage	0	-	0	0	0	0	0	-	0	0	4 [4]	4 [5]
Lung												
Focus (*N*)	10 [6]	-	11 [11]	18 [18]	18 [16]	**27** [25]**	15 [12]	-	6 [5]	5 [4]	15 [7]	23 [16]
percentage	20 [18]	-	22 [25]	36 [43]	36 [38]	**54 [58]**	30 [29]	-	12 [14]	10 [11]	30 [25]	46 [40]
Testes												
Discolouration (*N*)	0	-	1 [1]	1 [1]	1 [1]	3 [2]	-	-	-	-	-	-
percentage	0	-	2 [2]	2 [2]	2 [2]	6 [5]	-	-	-	-	-	-
Focus	6 [6]	-	2 [2]	6 [6]	8 [7]	11 [10]	-	-	-	-	-	-
percentage	12 [18]	-	4 [5]	12 [14]	16 [17]	22 [23]	-	-	-	-	-	-
Mass	0	-	1 [1]	1 [1]	0	3 [3]	-	-	-	-	-	-
percentage	0	-	2 [2]	2 [2]	0	6 [7]	-	-	-	-	-	-
Ovary												
Cyst	-	-	-	-	-	-	1 [1]	-	4 [4]	5 [4]	3 [3]	**8* [7]**
percentage	-	-	-	-	-	-	2 [2]	-	8 [11]	10 [11]	6 [11]	**16 [18]**

N: Number of individuals examined

Source: Buesen et al., 2017b

Statistically significant: * $p \leq 0.05$, ** $p \leq 0.01$

Table 21d. Key results of the combined chronic toxicity and carcinogenicity study in rats; histopathology at weeks 52 and 104; numbers in [*square brackets*] *indicate survivors*

	Sex, dietary dose (ppm)											
	Males						Females					
Parameter	**0**	**30**	**100**	**300**	**1500**	**15000**	**0**	**30**	**100**	**300**	**1500**	**15000**
Histopathology at week 52												
Number examined	10	10	10	10	10	10	10	10	10	10	10	10
Adrenal cortex												
Vacuolization increased	0	0	**7****	**9****	**10****	**10****	1	0	1	**10****	**9****	**10****
minimal	0	0	3	2	0	1	1	0	1	6	3	2
slight	0	0	4	4	4	5	0	0	0	2	3	5
moderate	0	0	0	3	5	3	0	0	0	2	3	3
severe	0	0	0	0	1	1	0	0	0	0	0	0
Hypertrophy, diffuse	0	0	0	0	0	0	0	0	0	0	**6****	**10****
minimal	0	0	0	0	0	0	0	0	0	0	6	0
moderate	0	0	0	0	0	0	0	0	0	0	0	10
Spleen												
Haematopoiesis	3	6	**8***	**9****	7	**9****	8	6	-	-	-	10
minimal	1	3	2	2	2	6	5	3	-	-	-	4
slight	2	3	6	7	5	3	2	3	-	-	-	4
moderate	0	0	0	0	0	0	0	0	-	-	-	2
severe	0	0	0	0	0	0	1	0	-	-	-	0
Ovary												
Vacuolation of interstitial glands	-	-	-	-	-	-	3	2	2	7	**9****	**8***
minimal	-	-	-	-	-	-	2	0	0	1	0	2
slight	-	-	-	-	-	-	1	2	2	2	3	1
moderate	-	-	-	-	-	-	0	0	0	4	5	2
severe	-	-	-	-	-	-	0	0	0	0	1	3
Histopathology at week 104; non-neoplastic findings												
Number examined [survivors]	50 [34]	-	50 [44]	50 [42]	50 [42]	50 [43]	50 [42]		50 [36]	50 [38]	50 [28]	50 [40]
Adrenal cortex												
Vacuolation increased	1 [0]	-	2 [1]	**18**** **[15]**	**23**** **[18]**	**33**** **[28]**	1 [0]	-	0	6 [5]	**24**** **[11]**	**15**** **[8]**
as a percentage	2 [0]	-	4 [2]	**36** **[36]**	**46** **[43]**	**66** **[65]**	2 [0]	-	0	12 [13]	**48** **[39]**	**30** **[20]**
minimal	1	-	2	12	18	14	1	-	0	6	19	6
slight	0	-	0	4	3	16	0	-	0	0	4	5
moderate	0	-	0	1	2	3	0	-	0	0	1	4
extreme	0	-	0	1	0	0	0	-	0	0	0	0
Degeneration, cystic	3 [3]	-	2 [2]	3 [3]	4 [3]	**15**** **[13]**	0	-	0	0	0	0
as a percentage	6 [9]	-	4 [5]	6 [7]	8 [7]	**30** **[30]**	0	-	0	0	0	0
minimal	3	-	2	3	4	10	0	-	0	0	0	0
slight	0	-	0	0	0	5	0	-	0	0	0	0

	Sex, dietary dose (ppm)											
	Males						Females					
Parameter	**0**	**30**	**100**	**300**	**1500**	**15000**	**0**	**30**	**100**	**300**	**1500**	**15000**
Fatty change, multifocal, present	16 [14]	-	13 [11]	6 [5]	13 [8]	**30** [25]**	6 [5]	-	2 [2]	9 [8]	**15* [9]**	**34** [25]**
as a percentage	32 [41]	-	26 [25]	12 [12]	26 [19]	**60 [58]**	12 [12]	-	4 [6]	18 [21]	**30 [32]**	**68 [63]**
Ovary												
Cysts, present	-	-	-	-	-	-	2 [2]	-	**8* [7]**	4 [3]	**9* [5]**	**17** [14]**
as a percentage	-	-	-	-	-	-	4 [5]	-	**16 [19]**	8 [8]	**18 [18]**	**34 [35]**
Vacuolation of interstitial glands	-	-	-	-	-	-	9 [7]	-	**19* [14]**	**23** [19]**	**28** [14]**	**26** [19]**
as a percentage	-	-	-	-	-	-	18 [17]	-	**38 [39]**	**46 [50]**	**56 [50]**	**52 [48]**
minimal	-	-	-	-	-	-	8	-	11	18	13	12
slight	-	-	-	-	-	-	1	-	6	4	10	7
moderate	-	-	-	-	-	-	0	-	2	1	5	6
severe	-	-	-	-	-	-	0	-	0	0	0	1
Brain												
Number examined	50	-	8	10	9	50	50	-	50	49	50	50
Compressed by pituitary tumour	6	-	3	4	1	4	5	-	12	6	14	13*
Lung												
Clefts	1 [1]		1 [1]	5 [5]	**14** [14]**	**13** [12]**	0		1 [1]	3 [3]	**10** [8]**	**10** [8]**
as a percentage	2 [3]		2 [2]	10 [5]	**28 [33]**	**26 [28]**	0		2 [3]	6 [8]	**20 [29]**	**20 [20]**
minimal	1		1	5	10	13	0		1	3	7	8
slight	0		0	0	4	0	0		0	0	3	2
Histiocytosis	27 [19]		32 [30]	34 [29]	32 [29]	**39** [36]**	0		0	0	0	0
as a percentage	54 [59]		64 [68]	68 [69]	64 [69]	**78 [84]**	0		0	0	0	0
minimal	20		27	29	24	16	0		0	0	0	0
slight	7		4	3	6	14	0		0	0	0	0
moderate	0		1	2	2	9	0		0	0	0	0
Liver												
Hepatocytes, multinucleated	6 [6]		0	1 [0]	0	1 [1]	21 [19]		9 [8]	11 [9]	20 [18]	**34** [29]**
as a percentage	12 [18]		0	2 [0]	0	2 [2]	42 [45]		18 [22]	22 [24]	40 [64]	**68 [73]**
minimal	-		-	-	-	-	14		7	11	14	15
slight	-		-	-	-	-	7		1	0	4	17
moderate	-		-	-	-	-	0		1	0	2	2

	Sex, dietary dose (ppm)											
	Males						Females					
Parameter	**0**	**30**	**100**	**300**	**1500**	**15 000**	**0**	**30**	**100**	**300**	**1500**	**15 000**
Histopathology at week 104; pre/neoplastic findings												
Testes												
Adenoma, Leydig cell [a]	1 [1]	-	2 [2]	5 [5]	4 [4]	**14** [14]**	-	-	-	-	-	-
as a percentage	2 [3]	-	4 [5]	10 [12]	8 [10]	**28 [33]**	-	-	-	-	-	-
unilateral	0	-	2	5	3	12	-	-	-	-	-	-
bilateral	1	-	0	0	1	2	-	-	-	-	-	-
Hyperplasia, Leydig cell [b], multifocal	8 [6]	-	4 [4]	8 [8]	**17* [17]**	**31** [30]**	-	-	-	-	-	-
as a percentage	16 [18]	-	8 [9]	16 [19]	**34 [40]**	**62 [70]**	-	-	-	-	-	-
unilateral	6	-	4	5	10	15	-	-	-	-	-	-
bilateral	2	-	0	3	7	16	-	-	-	-	-	-
Hyperplasia, Leydig cell, diffuse	2 [1]	-	2 [2]	1 [0]	2 [1]	7 [6]	-	-	-	-	-	-
as a percentage	4 [3]	-	4 [5]	2 [0]	4 [2]	14 [14]	-	-	-	-	-	-
Ovary												
Luteoma, benign [c]	-	-	-	-	-	-	0	-	1, [1]	0	0	3, [2]
as a percentage	-	-	-	-	-	-	0	-	2, [3]	0	0	6, [5]
Thecoma, benign [c]	-	-	-	-	-	-	2, [2]	-	0	0	0	2, [2]
as a percentage	-	-	-	-	-	-	4, [5]	-	0	0	0	4, [5]
Tumour, granulosa cell, benign [d]	-	-	-	-	-	-	1 [1]	-	1 [1]	3 [3]	**11** [7]**	6 [6]
as a percentage	-	-	-	-	-	-	2 [2]	-	2 [3]	6 [8]	**22 [25]**	12 [15]
Tumour, sex cord stromal, benign	-	-	-	-	-	-	0	-	2 [1]	0	0	3 [3]
as a percentage	-	-	-	-	-	-	0	-	4, [3]	0	0	6, [8]
Tumours with sex cord stromal origin (*N*)		-	-	-	-	-	3 [3]	-	4 [3]	3 [3]	11 [7]	14 [13]
Animals with tumours of sex cord stromal origin (*N*)		-	-	-	-	-	3	-	3	3	11	12
Uterus												
Adenocarcinomas [e]	-	-	-	-	-	-	6 [4]	-	4 [2]	6 [4]	11 [6]	**14* [10]**
as a percentage	-	-	-	-	-	-	12 [10]	-	8 [6]	12 [11]	22 [21]	**28 [25]**
Hyperplasia, glandular [f]		-	-	-	-	-	9 [9]	-	13 [12]	18* [14]	17 [12]	**18* [15]**
as a percentage	-	-	-	-	-	-	18 [21]	-	26 [33]	36 [37]	34 [43]	**36 [38]**
Adrenal cortex												
Carcinoma [g]	-	-	-	-	-	-	0	-	0	0	0	2 [2]
as a percentage	-	-	-	-	-	-	0	-	0	0	0	4 [3]

	Sex, dietary dose (ppm)											
	Males						Females					
Parameter	**0**	**30**	**100**	**300**	**1500**	**15000**	**0**	**30**	**100**	**300**	**1500**	**15000**
Adenoma [g]	-	-	-	-	-	-	6 [6]	-	1 [1]	2 [1]	0 [0]	3 [3]
as a percentage	-	-	-	-	-	-	12 [14]	-	-	2 [3]	4 [3]	0 [0]
Hyperplasia	-	-	-	-	-	-	35 [32]	-	-	36 [27]	38 [32]	28 [20]
as a percentage	-	-	-	-	-	-	70 [76]	-	-	72 [75]	76 [84]	56 [71]
Incidence of tumour bearing												
Number of animals, total [survivors]	50 [34]		50 [44]	50 [42]	50 [42]	50 [43]	-	50 [42]		50 [36]	50 [38]	50 [28]
Malignant	21 [10]		7 [6]	22 [16]	11 [6]	12 [7]	-	19 [12]		10 [5]	13 [7]	35 [14]
as a percentage	42 [29]		14 [14]	44 [38]	22 [14]	24 [16]	-	38 [29]		20 [14]	26 [18]	70 [50]
Metastasizing	6 [1]		1 [1]	2 [1]	2 [0]	2 [1]	-	4 [1]		2 [2]	5 [3]	8 [1]
as a percentage	12 [3]		2 [2]	4 [2]	4 [0]	4 [2]	-	8 [2]		4 [6]	10 [8]	16 [4]

N: Number of individuals examined Source: Buesen et al., 2017b

Historical control data: Severity grades: 1 minimal; 2 slight; 3 moderate; 4 severe; 5 extreme;

HCD for pre/neoplastic findings [mean%, min%-max%]:

[a] Adenoma, Leydig cell:
first time range 1999–2011 [4.4%, 0–8%]; eleven 24-month Wistar rat studies (50 rats per study, total animals 550);
second set time range 2009–2015 [2.9%, 1.7–6.0%] (seven studies).

[b] Leydig cell hyperplasia: second time range 2009–2015 [3.9%, 1.7–10.0%] (seven studies).

[c] Ovary sex cord stromal benign tumour [1.3%, 0–6%] / thecoma [0.4%, 0–2%] time range 1999–2011;
eleven 24-month Wistar rat studies (50 or 48 animals per study, total animals 548).

[d] Granulosa benign cell tumour: first time range, 1999–2011 [0.2%, 0–2%]; eleven 24-month Wistar rat studies
(50 animals per study, total animals 550).

[e] Uterus adenocarcinoma:
first time range, 1999–2011 [16.5%, 2–30%]; eleven 24-month Wistar rat studies (50 animals per study, total animals 550);
second time range, 2009–2015 [9.2%, 2–22%]; seven studies.

[f] Uterus glandular hyperplasia:
first time range, 2009–2015 [16%, 12–24%]; four 24-month Wistar rat studies (50 animals per study, total animals 200);
second time range, 2009–2015 [4.6; 0-12]; seven studies.

[g] Adrenal cortex carcinoma [0.2%, 0–2%]/adenoma [1.6%, 0–10%] time range 1999–2011; eleven24-month Wistar rat studies (50 animals per study, total animals 550).

Statistically significant: * $p \leq 0.05$, ** $p \leq 0.01$

The LOAEL for chronic toxicity in males was 300 ppm (equal to 14 mg/kg bw per day) based on increased adrenal weight, and incidence of adrenal vacuolization, whereas in females the LOAEL was the 100 ppm (equal to 5.9 mg/kg bw per day), the lowest dose tested, based on ovarian vacuolization after 24 months of treatment, therefore overall no NOAEL for chronic toxicity could be identified in this study.

The NOAEL for carcinogenicity was 300 ppm (equal to 19 mg/kg bw per day) based on increased incidence of ovarian tumours of sex cord stromal origin and uterus adenocarcinomas in females at 1500 ppm (equal to 95 mg/kg bw per day).

2.4 Genotoxicity

Table 22. Results of genotoxicity studies performed with broflanilide

Type of study	Organism/cells	Concentration	Purity	Result	Reference
		In vitro			
Ames test	*Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100; *Escherichia coli* strain WP2 *uvrA*	33–10 000 µg/plate (± S9)	99.67%	Negative	Ruelker, 2020
Chromosomal aberration	Chinese hamster lung cells	6 h: 72–5000 µg/mL (–S9) 6 h: 72–1080 µg/mL (+S9) 24 h: 43.8–700 µg/mL (–S9)	99.67%	Negative	Kasamoto, 2019
Gene mutation	Chinese hamster ovary (CHO) cells (HPRT locus assay)	10–5000 µg/mL (± S9)	99.67%	Negative	Schulz & Landsiedel, 2014
		In vivo			
Mouse micronucleus	Male NMRI mouse bone marrow	0, 500, 1000 and 2000 mg/kg bw	99.67%	Negative	Schulz & Landsiedel, 2013
		Systemic exposures of 500 and 2000 mg/kg bw were confirmed			Fabian & Landsiedel, (2017)

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g* ;

HPRT: Hypoxanthine–guanine phosphoribosyl transferase

(a) In vitro studies

In a reverse gene mutation assay in bacteria, strains TA1535, TA1537, TA98 and TA100 of *Salmonella typhimurium* and WP2 uvrA of *Escherichia coli* were exposed to broflanilide (purity 99.67%) in dimethyl sulfoxide (DMSO) at concentrations ranging from 0 to 10 000 µg/plate in the presence and in the absence of induced rat liver metabolic activation (S9 mix) using the standard plate and pre-incubation procedures. The test was performed in two independent experiments, each with triplicate plates for each concentration. Precipitation of the test substance was seen at 1000 µg/plate and above with and without S9 mix. Cytotoxicity was occasionally observed, depending on the strain and test conditions, from at concentrations of about 1000 µg/plate and above. Positive controls showed a marked increase in the number of revertant colonies. Broflanilide was not mutagenic under the test conditions used (Ruelker, 2020).

Broflanilide (purity 99.67%) was evaluated for its clastogenic potential in a chromosome aberration test using a Chinese hamster lung fibroblast cell line (CHL/IU). The test item was dissolved in DMSO. Cells were exposed for six hours at concentrations from 0 to 3000 µg/mL with S9 metabolic activation, and at concentrations from 0 to 648 µg/mL without S9 activation. An additional test without S9 was conducted for 24 hours at concentrations of 0 to 350 µg/mL. In a preliminary cytotoxicity test, precipitation was observed at 320 µg/mL and above with or without metabolic activation using both short-term and continuous exposures. Two hundred metaphases per concentration were examined for each experimental condition. For the short-term exposure assays the highest concentration analyzed was the concentration that showed the lowest relative cell growth (84.4%) and the lowest concentration was the highest that showed no precipitation. In the long-term exposure assay, the highest concentration analyzed was the lowest that elicited precipitation, with three consecutive concentrations at which precipitation did not occur. No induction of structural chromosome aberrations was noted at any of the broflanilide concentrations in any assay during this study. The incidences of polyploid cells in all treatment groups were comparable to those in the negative control groups in all assays. Positive control groups were produced the required results, demonstrating the validity of the study. Broflanilide did not induce chromosome aberrations in cultured mammalian cells under the conditions of this study (Kasamoto, 2019).

Broflanilide (purity 98.67%), was assessed for its potential to induce gene mutations at a hypoxanthine–guanine phosphoribosyl transferase (HPRT) locus in Chinese hamster ovary (CHO) cells. Two independent experiments were performed, both with and without metabolic activation (± S9) and both with an exposure period of four hours. Based on results of a preliminary range-finding study, concentrations from 39.1 to 5000 µg/mL were tested in the first experiment and from 10 to 1280 µg/mL in the second experiment, with and without metabolic activation. The study included vehicle control (DMSO alone) and positive controls. Duplicate cultures were used for all groups.

In both the absence and presence of metabolic activation no cytotoxicity (indicated by cloning efficiencies of 10%–20% of control) was observed at soluble test substance concentrations. Precipitates were evident at concentrations of about 78.1 µg/mL and above, with and without metabolic activation. A statistically significant, dose-dependent increase in mutant colonies was detected in the second experiment in the presence of metabolic activation. However, all mutant rates were well within the range of the historical negative control data, and therefore this finding was regarded as biologically irrelevant. The positive controls gave the expected increase in the frequencies of mutations. It was concluded that broflanilide is not mutagenic under these experimental conditions (Schulz & Landsiedel, 2014).

(b) In vivo studies

In a bone marrow micronucleus test, four groups of five male Crl:NMRI mice were administered a single oral gavage dose of broflanilide (purity 98.67%) at 0, 500, 1000 or 2000 mg/kg bw in a volume of 10 mL/kg bw. The use of males was chosen on the basis of a preliminary acute oral toxicity study in which no acute signs of toxicity were observed in males or females dosed at 2000 mg/kg bw. Two positive control groups were included; one dosed with cyclophosphamide at 20 mg/kg bw (in 10 mL/kg bw of deionized water), and one dosed with vincristine sulfate at 0.15 mg/kg bw (in 10 mL/kg bw of deionized water). As a vehicle control, a mixture of DMSO and corn oil was administered. Bone marrow smears were obtained from all groups 24 hours after dosing and in addition from the controls and the 2000 mg/kg bw group after 48 hours. After staining, 2000 polychromatic erythrocytes (PCEs) were evaluated per animal and investigated for micronuclei. The numbers of normocytes with and without micronuclei occurring per 2000 PCEs were also recorded. Samples taken from each dose level and the control were frozen and subsequently analyzed by HPLC to determine test material concentration.

The administration of the test substance did not lead to any clinical signs of toxicity.

No statistically significant or biologically relevant differences were reported in the frequency of erythrocytes containing micronuclei, either between the vehicle control groups and the three dose groups, or between groups from the two sacrifice intervals (24 and 48 hours) . The number of normochromatic and polychromatic erythrocytes containing small micronuclei or large micronuclei did not deviate from the vehicle control values at any of the sacrifice intervals and was within the historical vehicle control data ranges. Broflanilide exerted no relevant influence on erythropoiesis at any administered dose level. Both positive control substances induced a statistically significant increase in the number of PCEs containing small and/or large micronuclei within the range of the historical positive control data or above. It was concluded that broflanilide does not induce the formation of micronuclei in mouse polychromatic erythrocytes under these experimental conditions (Schulz & Landsiedel, 2013).

To provide evidence of bone marrow exposure a repeated dose toxicokinetic study was conducted in mice to evaluate whether broflanilide reaches the circulatory system. Two groups of four male mice (Crl:NMRI) were orally (gavage) administered [*B-ring*-^{14}C]broflanilide (purity 98.7%) twice at an interval of 24 hours. The initial target dose level was 500 mg/kg bw, then at 25 hours the target dose level for the second administration was 2000 mg/kg bw. Animals were sacrificed by decapitation under isoflurane anaesthesia two hours after the second dose, and blood samples collected. Blood samples were separated into plasma and blood cells and the radioactivity measured by LSC.

No mortality occurred during the study period and no clinical signs were observed. No abnormalities in body weight were observed.

The mean total (blood cells plus plasma) radioactivity residue values were 35.0 ± 4.1 µg equiv./g and 40.5 ± 10.3 µg equiv./g in animals given broflanilide at 500 and 2000 mg/kg bw, respectively. Radioactivity residue in plasma was three-fold higher than in blood cells, at both dose levels.

It was concluded that in male mice, after two oral administrations of [*B-ring*-^{14}C]broflanilide at target dose levels of 500 and 2000 mg/kg bw, radioactive residues of the test substance and/or its metabolites are present in the systemic circulation (Fabian & Landsiedel, 2017).

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a combined repeat-dose toxicity study (not guideline compliant) with a reproduction/developmental toxicity screening test in Crl:WI(Han) rats, broflanilide (purity 99.7%) was administered in the diet to 10 animals/sex per group at concentrations of 0, 5000, 10 000 or 15 000 ppm (equal to 0, 299, 644 and 983 mg/kg per day for males, 0, 360, 711 and 1067 mg/kg per day for females). Broflanilide was administered from pairing (two weeks), during the pairing period and until postpartum day (PPD) 22 for females, and until the day before necropsy in week 6 for the males. Additional groups of six female rats (toxicity group) were given broflanilide in the diet at dose levels of 0, 5000, 10 000 or 15 000 ppm for 37 days, until the day before necropsy. The following parameters were evaluated: mortality, clinical signs of toxicity, body weight, body weight gain, food consumption, test article intake, estrous cycle and mating, litter data, functional observations (littering and toxicity group animals), functional tests (toxicity group animals and pups at postnatal day 22) and motor activity (toxicity group animals). Haematology, clinical chemistry and organ weights (adrenals, brain, heart, kidney, liver, mandibular lymph nodes, spleen, testes and epididymides) were determined for toxicity group animals. Adults from the toxicity experiment, culled pups and pups killed at postnatal day (PND) 22 were subject to macroscopic examination. Microscopic pathology was conducted on animals from the toxicity experiment on all tissue from the control and high-dose groups, and on adrenal and ovary tissue from the low- and mid-dose groups.

No treatment-related mortality or clinical observations were recorded during the study. In males, a transient decrease in body weight gain, that showed a significant dose–response relationship, was seen during weeks 1 and 2 when compared to controls. In females, effects on body weight gain were noted at 10 000 and 15 000 ppm during the lactation period, consisting of an approximately 25% suppression of weight gain over lactation days (LDs) 4–7, an approximately 60% increase over LDs 7–14 (significant for dose–response), and a body weight loss over LDs 14–21. No treatment-related effects were observed in food consumption.

There were no effects on mating data or estrous cyclicity.

One female at 15 000 ppm suffered a total litter loss. There was no effect on length of gestation, number of implantation sites, pup numbers or survival. Pups weights at 10 000 and 15 000 ppm were lower than for controls at PND 1 by 8% and 10%, and at PND 7 by 13% and 12%, respectively; they showed a significant dose–response relationship. The start of physical development (pinna unfolding, incisor eruption) was slightly delayed in these animals. No treatment-related effects on pups were observed from functional tests. Macroscopic examination of the weanling offspring at necropsy produced no findings indicative of an adverse effect due to treatment.

In adult animals, there were no treatment-related effects on functional or behavioural development. No adverse effect due to treatment was revealed from motor activity data. Minor motor activity variations between the groups (including some that reached statistical significance) were within the background range seen at the test facility and were considered to represent normal variation for animals of this strain and age.

There was a dose-related increase compared with controls in absolute and relative reticulocyte counts in males (by 26%) and females (by 60%), reaching statistical significance in males (absolute) and in females (absolute and relative) at 15 000 ppm, and in females at all doses (relative only). Additionally, females showed a statistically significant and dose-related reduction in RBCs (by up to 7%) compared with controls, with a lower packed cell volume, which was statistically significant at 10 000 and 15 000 ppm and an increased (by 36%) red cell distribution width at 15 000 ppm. In females at 15 000 ppm mean platelet volume was significantly increased (by 6%), with a greater (by 7%) platelet distribution width. In females at 10 000 and 15 000 ppm there was a slight, decrease (by up to 31%) in white blood cell count, showing a significant dose–response relationship.

Clinical chemistry analysis revealed a significant decrease in potassium concentration in males (by 11%) at 15 000 ppm and in females at 10 000 (by 12%) and 15 000 ppm (18%). In females at 15 000 ppm there was a slight, dose-related and significant decrease in calcium and inorganic phosphorous concentration, as well as a significant increase in glucose concentration, but this last showing no dose–response relationship.

In males and females, there was an increase (by up to 41% in females) in adrenal weights (absolute and relative) at all dose levels, with no clear dose–response relationship, achieving statistically significance in females at 5000 and 15 000 ppm for relative adrenal weight. There was also a dose-related increase in liver weights (absolute and relative) in females (by up to 14% for absolute, and up to 9% for relative weight), which achieved statistical significance at 10 000 and 15 000 ppm for relative weight. No significant effects in liver weight were observed in the treated males. In females at 10 000 and 15 000 ppm an increase in heart weights was observed, achieving statistical significance for the relative weight.

Macroscopic examination of adults revealed enlarged adrenal glands in most treated (toxicity group) females (1/6 controls and 5/6 in each of the treated groups). This showed some correlation with cortical hypertrophy seen microscopically in the group given 15 000 ppm.

Microscopic examination showed an increased incidence in minimal haematopoiesis in the spleen of males at 10 000 and 15 000 ppm, and in females at 5000, 10 000 or 15 000 ppm. Adrenal cortical hypertrophy (eosinophilic or finely vacuolated cytoplasm in zona fasciculata) was present in females at 15 000 ppm. This finding correlated with the gross observation of enlarged adrenals and the increased organ weight in this sex.

The study did not follow current test guidelines. Limitations included: lower number of females for littering, limited reproductive and developmental parameters (for example, anogenital distance), and lack of investigation into haematology and clinical chemistry of littering females.

Under these experimental conditions the NOAEL for parental toxicity could not be identified due to increased adrenal weights in both sex, increased reticulocytes and decreased RBC counts, enlarged adrenal and increase spleen haematopoiesis in females at 5000 ppm (equal to 299 mg/kg bw per day), the lowest dose tested.

The NOAEL for reproductive toxicity was 15 000 ppm (equal to 1067 mg/kg bw per day), the highest dose tested.

The NOAEL for offspring toxicity was 5000 ppm (equal to 369 mg/kg bw per day) based on reduced pup weight on PND 1 and PND 7 at 10 000 ppm (equal to 774 mg/kg bw per day) (Perks, 2014).

In a two-generation reproductive toxicity study broflanilide, (purity 98.7%) was administered in the diet to groups of 25 male and 25 female healthy young Wistar Crl:WI(Han) rats (the F0 parental generation) at constant, homogeneous concentrations of 0, 30, 100, 300, 1500 or 15 000 ppm (equal to 0, 2.3, 7.5, 22.6, 112.3 and 1146.7 mg/kg bw per day for males, 0, 2.3, 7.5, 22.8, 111.3 and 1152.5 mg/kg bw per day for females). At least 73 days after the beginning of treatment, F0 animals were mated to produce a litter (the F1 generation). Groups of 25 animals/sex per group were selected from F1 pups to become the F1 parental generation. After weaning these were administered the test item as a constant, homogeneous addition to the diet at concentrations of 0, 30, 100, 300, 1500 or 15 000 ppm. This breeding program was repeated to produce an F2 litter. In addition, during gestation the diet concentration was adjusted due to the dams' increased food intake. The study was terminated by the terminal sacrifice of the F2 weanlings and F1 parental animals. The health status of the parents and pups was checked daily, and parental animals were examined for their mating and reproductive performances. Food consumption of parents (F0 and F1) was determined once weekly, also during gestation (GDs 0–7, 7–14, 14–20) and lactation periods on lactation days (LDs) 1–4, 4–7, 7–14 and 14–21. Body weights of parents (F0 and F1) were determined once weekly. However, during gestation and lactation F0/F1 females were weighed on gestation days (GDs) 0, 7, 14 and 20 and on PND 1, 4, 7, 14 and 21. Estrous cycle data were evaluated for F0 and F1 generation females over a three-week period prior to mating until evidence of mating occurred, and the estrous stage of each female was determined on the day of scheduled sacrifice. The F1 and F2 pups were sexed on the day of birth (PND 0) and were weighed on the first day after

birth (PND 1) as well as on PNDs 4, 7, 14 and 21. Their viability was also recorded. At necropsy on PND 21, all pups were examined macroscopically and for one pup of each sex per litter the brain, spleen, uterus and thymus were weighed. Blood samples for clinical pathological investigations were withdrawn from 12 selected F0 and F1 parental animals per sex and group shortly before scheduled sacrifice. Date of sexual maturation, (day of vaginal opening in females or balanopreputial separation in males) was recorded for all F1 pups selected to become the F1 parental generation. Sperm parameters (motility, sperm head count, morphology) were assessed in the F0 and F1 generation males at scheduled sacrifice after appropriate staining. All F0 and F1 parental animals underwent a gross pathology assessment (including weight determinations of several organs) and were subjected to an extensive histopathological examination, special attention being paid to the organs of the reproductive system. A quantitative assessment of primordial and growing follicles in the ovaries was performed for all control and high-dose F1 parental females.

Results for F0 parental animals

There were no treatment-related mortalities, clinical signs or changes in general behaviour in F0 adult males and females during the course of the study. No treatment-related effects on food consumption were observed. Males at 15 000 ppm had a lower body weight than controls at the end of the premating period (by 5%) although the difference was not statistically significant. Body weight gain for males at 1500 and 15 000 ppm was depressed (statistically significant) at the beginning (days 0–7; by about 9% and 13% respectively) and the end (days 63–70; by about 32% and 42% respectively) of premating. At 15 000 ppm the overall body weight gain for the premating treatment period was 8% below that for controls. In males during postmating a statistically significant decrease of body weight gain that was not dose-related, was reported at all dose levels. Body weights in these animals were similar to controls. These body weight changes were not considered treatment-related and therefore of no biological relevance.

In females, at 1500 and 15 000 ppm statistically significantly mean body weight decreases (by about 6–7% compared with controls) were reported during lactation (PNDs 14–21) and body weight gain was depressed by 44%–53% during PNDs 7–14. Overall body weight gains during lactation (PNDs 1–21) decreased at 1500 and 15 000 ppm by about 11% and 22% respectively, although these changes were not statistically different from control. The body weight gain of the parental females in all test groups was comparable to their concurrent control group during the entire premating and gestation period.

There were no treatment-related effects on estrus cycle, sperm parameters, male or female fertility, conception, gestation, parturition or lactation.

Haematological analysis revealed a statistically significant decrease in RBC counts (by 4%–10%), haemoglobin concentration (by 5%–10%) and haematocrit (by 5%–10%) in females at 300, 1500 and 15 000 ppm. In these animals a statistically significant increase in relative reticulocyte counts (by 57%–257%) was also observed. It was noted that RBC counts, Hb and Ht values in females at 300 ppm were either within, or marginally below, those from laboratory background data. It was also noted that relative reticulocyte counts of the concurrent control were below those from laboratory background data. In females at 15 000 ppm a marginally (but statistically significant) lower (by 1%) mean corpuscular haemoglobin concentration (MCHC) was observed. In males, relative reticulocyte counts were higher at 300, 1500 and 15 000 ppm than those of controls, with no obvious dose–response relationship, and achieving statistical significance at 300 and 15 000 ppm. All other statistically significant haematology changes in dosed males were not dose-dependent.

Results of clinical chemistry analysis showed a dose-related increase in cholesterol in females at 300 ppm and in males and females at 1500 and 15 000 ppm (by up to 21% and 80% in males and females respectively) achieving statistical significance only in dosed females. Cholesterol levels in females were outside the laboratory background range. A significant increase of total bilirubin (by 23%–32%) was observed in females at 1500 and 15 000 ppm. In females at 15 000 ppm GGTP activity was higher than for controls to a statistically significant extent, but with the mean that was within the HCD range.

Absolute and relative adrenal weights increased in a statistically significant manner in females at 100 ppm and in males and females at 300, 1500 and 15 000 ppm; increases compared to controls

were by 40%–46% in males and 53%–61% in females. A statistically significant increase in relative adrenal weight (by 9%) was also reported in males at 100 ppm. In males at 15 000 ppm, a slight increase (statistically significant) in relative liver weight (6%) was reported. In females, relative liver weight was increased at doses of 100 ppm and above (by up to 16%; statistically significant), and absolute liver weight was increased at 300 ppm, 1500 and 15 000 ppm (by up to 11% compared to controls), with no obvious dose response and achieving statistical significance at 300 and 15 000 ppm. However all mean liver weight values for males and females fell within the laboratory background range. Absolute and relative spleen weights were significantly increased by 28–34% in females at 300, 1500 and 15 000 ppm. Spleen weights in males were similar to control values. A statistically significant increase in relative pituitary weight was reported in males at 15 000 ppm (by 9% compared to controls). Relative and absolute ovary weights were increased at 300, 1500 and 15 000 ppm, by up to 15% for absolute and up to 20% for relative weight, with no obvious dose–response relationship. These ovary weight differences were statistically significant at 300 ppm and above for the relative weight, and at 300 and 15 000 ppm for the absolute weight. All mean values fell outside the laboratory background ranges.

Gross pathology examination of F0 parents showed an increased incidence of enlarged adrenal cortex in females at doses of 300 ppm and above. Histopathology examination revealed an increased incidence and severity (minimal to marked) of vacuolation in the adrenal cortex of males and females at 100, 300, 1500 and 15 000 ppm, and of hypertrophy (minimal to slight) in females at 300 ppm, and males and females at 1500 and 15 000 ppm. In females receiving 100, 300, 1500 and 15 000 ppm an increased incidence and severity of vacuolated interstitial ovary glands was observed. There were no treatment-related histopathology findings in the liver, spleen or pituitary.

Results for F1 pups

No treatment-related clinical signs were noted in F1 pups. An increased number of pups died between PND 1 and PND 4 at 1500 and 15 000 ppm. At 1500 ppm these deaths were due to a cluster of deaths due to cannibalization noted in a single litter (No. 318): at 15 000 ppm they were mainly due to cannibalization more or less evenly distributed over several litters. It was noted that the number of cannibalized F1 pups at the high dose was similar to that observed in the F2 pups at the lowest dose (100 ppm), therefore this finding was not regarded as treatment-related. No association to treatment was observed on viability index during lactation (PNDs 4–21). The sex distribution and sex ratios of live F1 pups were not affected by treatment.

Body weights of F1 male and female pups at 1500 and 15 000 ppm were lower than controls (statistically significant) on PNDs 21 (by 5%–7%). For these pups a significantly depressed body weight gain (by 8% and 13% in males, and 7% and 14% in females at 1500 and 15 000 ppm, respectively) was observed during the period PND 14–21, leading as well to an overall reduction of weight gain during lactation (PNDs 1–21, by 7–8%). There was no treatment-related effect on sexual maturation of F1 pups. In male pups, a statistically significant delay in balanopreputial opening of 1.5 days compared to controls was reported at 1500 ppm, however as this later onset of puberty was within the laboratory historical control range the finding was considered unrelated to treatment. In females, a statistically significant decrease in body weight at vaginal opening was reported at 1500 ppm. In the absence of an effect on body weight at the next highest dose this finding was considered unrelated to treatment.

When organs of F1 pups were weighed a statistically significant decrease of absolute brain weight (by 3%) and increase in relative brain weight (by 5%–6%) was reported in male pups at 1500 and 15 000 ppm. Similar changes in brain weight were noted in female pups at 15 000 ppm, however these achieved statistical significance only at the high dose and for absolute weight. Absolute and relative thymus weights were decreased in male pups (by up to 16% for absolute and 9% for relative weights) and female pups (by up to 16% for absolute and up to 9% for relative weights) at 1500 and 15 000 ppm. Absolute thymus weight changes were significantly different from controls in male pups at both dose levels. In female pups the absolute thymus weight was significantly different from controls at both dose levels and the relative weight at the high dose only. Brain weight changes at 1500 and 15 000 ppm and the decreased thymus weights of F1 pups at 1500 and 15 000 ppm were considered to be secondary to the lower pup body weights in these groups. Necropsy observations of

F1 pups revealed no treatment-related effects.

Results for F1 generation parental animals

There were no treatment-related mortalities, clinical signs or changes in general behaviour in F1 adult males or females during the course of the study. Food consumption of the F1 parental females at 30, 100, 1500 and 15 000 ppm was lower than control values (by 10%–13%; statistically significant) on several occasions during premating. These decreases did, however neither show a consistent pattern nor were they dose-dependent, thus they are considered spurious findings. Food consumptions for all dosed males and female F1 rats during the entire gestation and lactation period were comparable to those of controls. Males at 1500 and 15 000 ppm had lower body weight than controls (by about 5%) at the end of premating period, although the difference was not statistically significant. Males at 300, 1500 and 15 000 ppm showed depressed body weight gains compared with controls during the last week of premating (days 63–70) by 33%, 23% and 25%, respectively. When calculated for the entire premating period a suppression of body weight gain of about 6% was apparent, although the difference is not statistically significant. Body weight gains in F1 parental females were generally similar to those of controls throughout the study period. Statistically significant suppressions of body weight gain were reported during premating days 21–28 at 15 000 ppm (of about 27%) and GD 0–7 at 100, 1500 and 15 000 ppm (of about 15%, 23% and 23% respectively).

There were no treatment-related effects on estrous cycle, sperm parameters, male or female fertility, conception, gestation, parturition or lactation. The slightly (but statistically significant) lower number of estrous cycles at 300 ppm was considered unrelated to treatment. A statistically significant decrease in sperm head count was reported at 15 000 ppm, however the mean value was similar to that for the F0 generation control. Moreover, there was no concomitant histopathological finding in the testis. Therefore, the lower sperm head counts in the testis were not regarded as treatment-related.

Haematological analysis of F1 parental animals revealed a slight but statistically significant decrease in RBCs in males (by 3% compared with controls) and females (by 5%) at 15 000 ppm, and in Hb in females at 300, 1500 and 15 000 ppm (by up to 3%) and in males at 15 000 ppm (by 3%). A significant reduction in Hb (by 3%) was also reported in females at 30 ppm, but this was not toxicologically relevant; it was noted that no change at the next higher dose was observed. In males at 15 000 ppm, absolute monocyte counts were significantly decreased compared with controls (by about 31%). It was noted that the mean of absolute monocyte counts at the high dose was equal to the laboratory historical control mean.

Results of clinical chemistry analyses showed a dose-related increase in cholesterol for females at 300 ppm, and for males and females at 1500 and 15 000 ppm (by up to 30% and 51% in males and females, respectively).

Absolute and relative adrenal weights were increased (statistically significant) in males at 100 ppm and in males and females at 300, 1500 and 15 000 ppm. Increases compared to controls were by 8%–50% for absolute adrenal weight and by 9%–55% for relative adrenal weight in males; for females increases were by 15%–43% for absolute weights and by 18%–48% for relative weights. Adrenal weights of males at 100 and 300 ppm were within the historical control range. A marginal, but statistically significant, decrease (by 2%) in absolute brain weight and a non-significant increase (by 2%) in relative brain weight were observed in both sexes at 15 000 ppm. The decreased absolute and slightly increased relative weight of the brain was considered related to slightly decreased terminal body weights (not statistically significant at 4% below controls) and was within historical control values. In males at 15 000 ppm liver weights increased by 6% and 10% for absolute and relative values, respectively, achieving statistical significance for the relative weight. In females, liver weights were increased with a relative flat dose response at 300, 1500 and 15 000 ppm, (by 8%, 3% and 8% for the absolute and by 10%, 8% and 12% for the relative weights, respectively). Spleen weights were increased compared to controls in males and females at 15 000 ppm, by 7%–11% in males and 8%–13% in females, achieving statistical significance for relative weight in males. Absolute spleen weight in males was within the laboratory historical control range, while the male mean relative spleen weight was outside the historical control range. There were no treatment-related histopathology findings in the liver or spleen. In males at 300, 1500 and 15 000 ppm, statistically significant increases in the weight of the caudal epididymides were by up to 17% for the absolute weights, and up to 21% for the relative weights. The increases in

caudal epididymides weights exceeded the historical control ranges. In the same dose groups, absolute epididymis weight was significantly increased (by up to 13%) as was relative epididymis weight (by up to 17%). The increase in relative epididymides weight exceeded the historical control range. Additionally, in these dosed groups testis weights were significantly increased compared to controls by up to 19% for the absolute weight and by up to 24% for the relative weight. Testis weights were also increased at 100 ppm, by 5% in the case of absolute weight and 7% for relative weight. Reported significant testis weights changes were all outside the historical control ranges. In males at 15 000 ppm, seminal vesicle weights increased by 8% in the case of absolute weight and 12% for relative weight, achieving statistical significance for the relative weights. In the absence of treatment-related histopathological findings in the male reproductive organs, the reported weight changes were considered of low toxicological relevance.

In females at 300, 1500 and 15 000 ppm ovary weights were significantly increased but with no clear dose–response relationship, however in the presence of correlating histopathology findings (vacuolation of interstitial glands) these weights changes were considered to be treatment-related.

Gross pathology examinations of F1 parents showed an increased incidence of enlarged adrenal cortex in females at 300 ppm and above. Histopathological examination revealed a treatment-related increase in the incidence and severity (minimal to moderate) of vacuolation in the adrenal cortex of males and females at 100, 300, 1500 and 15 000 ppm, and hypertrophy (minimal to slight) in males at 300 ppm and males and females at 1500 and 15 000 ppm. In females at 300, 1500 and 15 000 ppm, increased incidence and severity (minimal to moderate) of vacuolated interstitial ovary glands was observed. Histopathology, including differential ovarian follicle counts, uncovered no other treatment-related findings in F1 parental animals.

Results for F2 pups

No treatment-related clinical signs or mortalities were noted for F2 pups. Mean body weights of the F2 male and female pups at 1500 and 15 000 ppm were decreased compared to controls (statistically significant) during the period PND 7–21, by up to 10% in males and up to 9% in females. The body weight gains of male and female pups at 1500 and 15 000 ppm were lower compared with controls (statistically significant) during major parts of the lactation period (by up to 13% in males and up to 15% in females). Sex distribution and sex ratios of live F2 pups were not affected by treatment.

Organ weight determination of F2 pups revealed a statistically significant decrease compared with controls in absolute brain weight in male and female pups at 1500 and 15 000 ppm, by about 3% and up to 5% in males and females respectively. Changes in brain weight in F2 pups were considered related to decreased body weights. Absolute and relative thymus weights were significantly decreased in male pups (by up to 23% for absolute and by 19% for relative weights) and female pups (by up to 21% for absolute and by up to 13% for the relative weights) at 1500 and 15 000 ppm. The decreased absolute brain weights, as well as thymus weights, of the F2 pups at 1500 and 15 000 ppm were considered to be secondary to the lower pup body weights seen in these groups. At necropsy no treatment-related effects were noted in F2 pups.

Table 23a. Key findings for males in the two-generation reproductive toxicity study

	Sex and dietary doses (ppm)						
	Males						
Dietary dose; (ppm)	**0**	**30**	**100**	**300**	**1500**	**15 000**	**HCD [a]**
Dose; lactation (ppm)	**0**	**15**	**50**	**150**	**750**	**7 500**	
Parameter							
	F0 parental animals						
Dose (mg/kg bw per day)							
Premating	0	2.3	7.5	22.6	112.3	1146.7	-
Gestation	-	-	-	-	-	-	

	Sex and dietary doses (ppm)						
	Males						
Dietary dose; (ppm)	**0**	**30**	**100**	**300**	**1500**	**15 000**	**HCD [a]**
Dose; lactation (ppm)	**0**	**15**	**50**	**150**	**750**	**7 500**	
Parameter							
Lactation	-	-	-	-	-	-	
Body weight (g); mean ± SD							
Premating day 0	150.9 ± 9.8	149.7 ± 10.2	149.7 ± 10.4	150.7 ± 10.8	150.2 ± 10.1	148.8 ± 10.2	-
Premating day 70	387.8 ± 30.6	380.2 ± 36.0	372.4 ± 27.1	384.5 ± 39.8	380.1 ± 40.8	366.8 ± 35.2	-
Postmating day 10	413.6 ± 33.4	406.5 ± 37.3	395.2 ± 28.3	409.3 ± 43.2	407.7 ± 45.4	396.9 ± 38.0	-
Body weight gain (g); mean ± SD							
Pre-mating days 0–7	44.9 ± 4.1	45.0 ± 4.9	43.3 ± 3.9	42.3 ± 5.1	**40.9 ± 4.6****	**39.2 ± 4.4****	-
percentage of control						**87%**	
Premating days 63–70	12.0 ± 3.5	11.8 ± 3.6	12.1 ± 2.8	11.0 ± 4.4	**8.2 ± 4.0****	**7.0 ± 7.8****	-
Premating days 0–70	236.9 ± 25.8	230.5 ± 29.7	222.8 ± 21.2	233.8 ± 33.0	230.0 ± 37.1	218.1 ± 28.7	-
Postmating days 3–10	8.2 ± 4.2	**4.9 ± 3.8****	**3.0 ± 5.3****	**2.5 ± 3.4****	**3.4 ± 4.3****	**4.8 ± 3.9****	-
Haematology; male, day 104; mean ± SD							
Red blood cells (10^9/L)	8.38 ± 0.24	8.67 ± 0.43*	8.67 ± 0.27*	8.46 ± 0.30	8.49 ± 0.23	8.35 ± 0.29	8.70; 8.32–9.23
percentage of control	-	103	103	101	101	100	
Haemoglobin (mmol/L)	9.0 ± 0.3	8.9 ± 0.3	9.0 ± 0.2	8.8 ± 0.3	8.8 ± 0.2	8.8 ± 0.3	9.0; 8.7–9.5
percentage of control	-	99	100	98	98	98	
Haematocrit (L/L)	0.421 ± 0.016	0.423 ± 0.016	0.426 ± 0.012	0.418 ± 0.014	0.414 ± 0.011	0.416 ± 0.014	-
percentage of control	-	100	101	99	98	99	
Mean corpuscular haemoglobin, MCH (fmol)	1.07 ± 0.03	**1.03 ± 0.04****	**1.04 ± 0.03***	**1.04 ± 0.03***	**1.04 ± 0.03***	1.06 ± 0.02	-
percentage of control	-	**96**	**97**	**97**	**97**	99	-
MCHC (mmol/L)	21.34 ± 0.37	21.07 ± 0.31	21.14 ± 0.45	21.12 ± 0.36	21.23 ± 0.46	21.23 ± 0.42	-
percentage of control	-	99	99	99	99	99	-
Reticulocytes (%)	1.4 ± 0.2	1.5 ± 0.4	1.5 ± 0.3	**1.7 ± 0.3***	1.6 ± 0.3	**1.8 ± 0.3****	1.7; 1.4–2.0
percentage of control	-	107	107	**121**	114	**129**	
Clinical chemistry; male, day 104; mean ± SD							
Cholesterol (mmol/L)	2.11 ± 0.40	2.23 ± 0.47	2.13 ± 0.44	2.35 ± 0.45	2.49 ± 0.31	2.55 ± 0.48	-
percentage of control	-	106	101	111	118	121	-
Total bilirubin, by colorimetry (µmol/L)	1.35 ± 0.31	1.37 ± 0.23	1.45 ± 0.22	1.54 ± 0.22	1.48 ± 0.27	1.45 ± 0.15	-
percentage of control	-	101	107	114	110	107	-

	Sex and dietary doses (ppm)						
	Males						
Dietary dose; (ppm)	**0**	**30**	**100**	**300**	**1500**	**15000**	**HCD [a]**
Dose; lactation (ppm)	**0**	**15**	**50**	**150**	**750**	**7500**	
Parameter							
γ-glutamyl transpeptidase, GGTP (nkat/L)	0 ± 0	0 ± 0	0 ± 0	2 ± 5	0 ± 0	0 ± 0	-
Organ weights; mean ± SD							
Terminal body weight (g)	401.408 ± 32.998	397.048 ± 35.996	385.904 ± 30.009	400.472 ± 44.956	398.524 ± 46.932	387.224 ± 36.473	-
Absolute adrenal weight (mg)	60.20 ± 8.00	59.88 ± 8.92	62.72 ± 7.96	**70.92 ± 8.69****	**81.44 ± 12.99****	**84.40 ± 8.99****	64.245; 60.200–71.400
percentage of control	-	99	104	**118**	**135**	**140**	
Relative adrenal weight (%)	0.015 ± 0.002	0.015 ± 0.001	0.016 ± 0.002*	**0.018 ± 0.002****	**0.020 ± 0.002****	**0.022 ± 0.003****	0.017; 0.014–0.020
percentage of control	-	100	109	**119**	**136**	**146**	
Gross pathology							
Number examined	25	25	25	25	25	25	-
Adrenal cortex: enlarged	0	0	0	0	0	0	-
Histopathology							
Adrenal cortex							
Number examined	25	25	25	25	25	25	-
Vacuolation increased	0	0	6	18	21	14	-
minimal	0	0	2	3	4	3	-
slight	0	0	3	10	12	10	-
moderate	0	0	1	5	4	1	-
severe	0	0	0	0	1	0	-
Hypertrophy, diffuse	0	0	0	0	2	2	-
minimal	0	0	0	0	1	2	-
slight	0	0	0	0	1	0	-
F1 pup data							
Body weight (g); mean ± SD							
Lactation day 1	6.8 ± 0.4	6.8 ± 0.6	6.9 ± 0.7	6.8 ± 0.6	6.7 ± 0.9	6.8 ± 0.7	-
Lactation day 4	10.2 ± 1.0	10.3 ± 1.0	10.4 ± 1.4	10.1 ± 1.2	10.0 ± 1.4	10.2 ± 1.2	-
Lactation day 7	16.6 ± 1.3	16.6 ± 1.5	16.9 ± 1.9	16.4 ± 1.4	16.0 ± 1.6	16.3 ± 1.6	-
Lactation day 14	34.6 ± 2.4	34.2 ± 2.6	34.5 ± 3.2	34.3 ± 2.1	33.2 ± 2.2	33.4 ± 2.8	-
Lactation day 21	55.6 ± 3.2	54.5 ± 3.5	55.1 ± 4.9	55.0 ± 3.7	52.2 ± 3.2*	51.5 ± 3.7**	-
percentage of control	-	98	99	99	94	93	-
Body weight gain (g); mean ± SD							

	Sex and dietary doses (ppm)						
	Males						
Dietary dose; (ppm)	**0**	**30**	**100**	**300**	**1500**	**15 000**	**HCD [a]**
Dose; lactation (ppm)	**0**	**15**	**50**	**150**	**750**	**7 500**	
Parameter							
Lactation days 14–21	20.7 ± 1.4	20.3 ± 1.7	20.6 ± 1.9	20.7 ± 1.9	**19.0 ± 1.6****	**18.1 ± 1.4****	
percentage of control	-	98	100	100	**92**	**87**	
Lactation days 1–21	48.8 ± 2.9	47.6 ± 3.3	48.2 ± 4.3	48.2 ± 3.6	**45.5 ± 2.9****	**44.7 ± 3.2****	-
percentage of control	-	98	99	99	**93**	**92**	-
	F1 parental animals						
Dose (mg/kg per day)							
Premating days 0–70	0	2.6	8.6	25.6	127.8	1294.7	-
Gestation	NR	NR	NR	NR	NR	NR	-
Lactation	NR	NR	NR	NR	NR	NR	-
Body weight gain (g)							
Pre-mating (Day 0–70)	283.6 ± 24.5	290.8 ± 34.9	273.6 ± 23.5	272.8 ± 34.5	269.4 ± 19.8	266.7 ± 22.6	-
Gestation				NR			
Haematology; male, day 102; mean ± SD							
Red blood cells (10^9/L)	8.72 ± 0.23	8.90 ± 0.32	8.84 ± 0.36	8.69 ± 0.37	8.57 ± 0.21	8.47 ± 0.24*	-
percentage of control	-	102	101	100	98	97	-
Haemoglobin (mmol/L)	9.2 ± 0.3	9.3 ± 0.2	9.2 ± 0.2	9.1 ± 0.2	9.0 ± 0.2	8.9 ± 0.1**	-
percentage of control	-	101	100	99	98	97	-
Clinical chemistry; male, day 102; mean ± SD							
Cholesterol (mmol/L)	2.06 ± 0.45	2.13 ± 0.41	2.07 ± 0.48	2.40 ± 0.32	2.64 ± 0.36**	2.67 ± 0.42**	-
percentage of control	-	103	100	117	128	130	-
Organ weight; mean ± SD							
Terminal body weight (g)	387.7 ± 27.495	394.276 ± 39.322	382.552 ± 30.617	377.528 ± 43.083	372.748 ± 24.992	373.684 ± 27.397	-
Absolute adrenal weight (mg)	61.2 ± 8.19	62.6 ± 7.077	**65.96 ± 7.657***	**73.04 ± 11.234****	**83.40 ± 12.583****	**91.52 ± 11.344****	66.553; 61.320–73.360
percentage of control	-	102	**108**	**119**	**136**	**150**	
Relative adrenal weight (%)	0.016 ± 0.002	0.016 ± 0.002	**0.017 ± 0.002***	**0.019 ± 0.003****	**0.022 ± 0.003****	**0.025 ± 0.004****	0.017; 0.015–0.019
percentage of control	-	101	**109**	**123**	**141**	**155**	

	Sex and dietary doses (ppm)						
	Males						
Dietary dose; (ppm)	**0**	**30**	**100**	**300**	**1500**	**15000**	**HCD [a]**
Dose; lactation (ppm)	**0**	**15**	**50**	**150**	**750**	**7500**	
Parameter							
Gross pathology							
Number of animals	25	25	25	25	25	25	-
Adrenal cortex: enlarged	0	0	0	0	0	0	
Histopathology							
Number of animals	25	25	25	25	25	25	-
Adrenal glands							
Vacuolation increased	7	9	15	23	25	25	-
minimal	7	8	7	11	2	1	-
slight	0	1	5	11	10	11	-
moderate	0	0	3	1	13	13	-
Hypertrophy, diffuse	0	0	0	3	6	16	-
minimal	0	0	0	3	6	16	-
slight	0	0	0	0	0	0	-
F2 pup data							
Body weight (g); mean ± SD							
Lactation day 1	6.8 ± 0.8	6.7 ± 0.6	6.8 ± 0.8	6.5 ± 0.5	6.6 ± 0.6	6.6 ± 0.7	-
Lactation day 4	10.3 ± 1.6	10.4 ± 1.1	10.2 ± 1.3	9.7 ± 1.0	9.4 ± 1.1	9.6 ± 1.4	-
Lactation day 7	16.6 ± 1.9	16.6 ± 1.4	16.4 ± 1.8	15.9 ± 1.1	15.0 ± 1.7**	15.3 ± 1.8*	-
percentage of control	-	100	99	96	90	92	-
Lactation day 14	33.5 ± 3.0	33.4 ± 2.3	33.0 ± 2.9	32.4 ± 2.0	30.8 ± 2.6**	31.4 ± 3.1*	-
percentage of control	-	100	99	97	92	94	-
Lactation day 21	52.4 ± 4.8	52.2 ± 3.3	52.2 ± 4.2	51.1 ± 2.8	**48.3 ± 3.9****	**47.8 ± 4.5****	-
percentage of control	–	100	100	98	**92**	**91**	-
Body weight gain (g); mean ± SD							
Lactation days 1–4	3.5 ± 0.8	3.6 ± 0.6	3.4 ± 0.7	3.2 ± 0.6	**2.9 ± 0.6***	3.0 ± 0.7	-
percentage of control	-	103	97	91	**83**	86	-
Lactation days 4–7	6.3 ± 0.5	6.3 ± 0.6	6.2 ± 0.8	6.2 ± 0.4	**5.6 ± 0.7****	**5.7 ± 0.7****	-
percentage of control	-	100	98	98	**89**	**90**	-
Lactation days 1–21	45.5 ± 4.2	45.5 ± 3.1	45.5 ± 3.7	44.6 ± 2.6	**41.9 ± 3.5****	**41.2 ± 4.0****	-
percentage of control	-	100	100	98	**92**	**91**	-

(See Table 23b for footnotes that apply to this table also.) Source: Schneider et al., 2017

Table 23b. Key findings for females in the two-generation reproductive toxicity study

	Sex and dietary doses (ppm)						
	Females						
Dietary dose; (ppm)	**0**	**30**	**100**	**300**	**1500**	**15000**	**HCD [a]**
Dose; lactation (ppm)	**0**	**15**	**50**	**150**	**750**	**7500**	
Parameter							
	F0 parental animals						
Dose (mg/kg bw per day)							
Premating	0	2.5	8.3	26.7	126.3	1260.2	-
Gestation	0	2.3	7.5	22.8	111.3	1152.5	-
Lactation	0	2.9	9.6	29.3	139.9	1461.6	-
Body weight (g); mean ± SD							
Premating day 0	125.8 ± 7.1	124.2 ± 7.1	125.0 ± 7.2	125.4 ± 7.2	124.4 ± 8.0	123.9 ± 6.7	-
Premating day 70	231.4 ± 15.6	229.8 ± 14.3	231.4 ± 18.5	226.3 ± 17.8	224.1 ± 14.8	225.1 ± 14.0	-
Postmating day 10	-	-	-	-	-	-	-
Gestation day 20	342.1 ± 21.4	333.4 ± 26.0	337.2 ± 28.9	338.6 ± 29.4	329.8 ± 25.9	330.2 ± 24.4	-
Lactation day 14	305.2 ± 15.6	293.4 ± 20.4	301.7 ± 21.3	297.1 ± 25.1	**283.2 ± 16.4****	**285.6 ± 17.7****	-
percentage of control	-	96	99	97	**93**	**94**	
Lactation day 21	296.1 ± 12.7	286.3 ± 19.5	294.9 ± 25.8	291.2 ± 21.0	**276.8 ± 16.4****	**277.5 ± 14.8****	-
percentage of control	-	97	100	98	**93**	**94**	-
Body weight gain (g); mean ± SD							
Premating day 0–7)	21.0 ± 4.6	21.1 ± 6.2	20.8 ± 4.5	18.9 ± 4.0	20.7 ± 5.6	19.6 ± 5.4	
Premating days 63–70	4.5 ± 5.3	5.6 ± 6.7	6.3 ± 5.6	5.2 ± 4.8	4.5 ± 4.5	6.5 ± 5.4	
Premating days 0–70	105.6 ± 12.2	105.6 ± 9.9	106.4 ± 14.2	100.9 ± 14.5	99.7 ± 11.0	101.2 ± 12.1	-
Postmating days 3–10	-	-	-	-	-	-	-
Gestation days 0–7	23.1 ± 5.9	20.5 ± 3.3	22.4 ± 3.8	23.1 ± 5.3	22.3 ± 4.5	20.8 ± 3.7	-
Gestation days 7–14	22.6 ± 4.9	22.1 ± 5.3	22.9 ± 4.0	23.0 ± 4.4	22.5 ± 5.0	22.5 ± 4.0	-
Gestation days 0–20	106.6 ± 16.0	101.3 ± 15.7	103.2 ± 19.0	109.2 ± 15.2	104.9 ± 17.1	104.1 ± 14.1	-
percentage of control	-	95	97	102	98	98	-
Lactation days 1–4	18.5 ± 8.0	16.7 ± 9.9	17.5 ± 8.5	17.4 ± 9.1	22.4 ± 15.7	19.0 ± 12.4	-
Lactation days 7–14	22.7 ± 11.9	18.3 ± 6.9	18.3 ± 7.6	18.3 ± 8.7	**12.7 ± 9.4****	**10.6 ± 6.7****	-
percentage of control	-	81	81	81	**56**	**47**	-
Lactation days 1–21	39.7 ± 13.3	37.9 ± 10.5	42.0 ± 15.4	41.9 ± 12.9	35.2 ± 21.6	30.8 ± 10.5	-
percentage of control	-	95	106	106	89	78	-

	Sex and dietary doses (ppm)						
	Females						
Dietary dose; (ppm)	**0**	**30**	**100**	**300**	**1500**	**15000**	**HCD [a]**
Dose; lactation (ppm)	**0**	**15**	**50**	**150**	**750**	**7500**	
Parameter							
Haematology; females, day 137; mean ± SD							
Red blood cells (10^9/L)	8.07 ± 0.27	7.90 ± 0.33	7.97 ± 0.33	**7.72 ± 0.24****	**7.32 ± 0.38****	**7.24 ± 0.49****	7.81; 7.51–8.24
percentage of control	-	98	99	**96**	**91**	**90**	
Haemoglobin (mmol/L)	8.8 ± 0.3	8.8 ± 0.3	8.7 ± 0.3	**8.4 ± 0.3****	**8.1 ± 0.3****	**7.9 ± 0.4****	8.6; 8.2–9.1
percentage of control	-	100	99	**95**	**92**	**90**	-
Haematocrit (L/L)	0.413 ± 0.015	0.405 ± 0.017	0.405 ± 0.014	**0.391 ± 0.012****	**0.376 ± 0.016****	**0.373 ± 0.022****	0.406; 0.392–0.428
percentage of control	-	98	98	**95**	**91**	**90**	
Mean corpuscular haemoglobin, MCH (fmol)	1.09 ± 0.04	1.11 ± 0.04	1.09 ± 0.03	1.09 ± 0.04	1.10 ± 0.03	1.09 ± 0.03	-
percentage of control	-	102	100	100	101	100	-
MCHC (mmol/L)	21.35 ± 0.28	21.61 ± 0.32	21.38 ± 0.26	21.41 ± 0.40	21.44 ± 0.36	**21.11 ± 0.22***	21.13; 20.42–22.06
percentage of control	-	101	100	100	100	**99**	
Reticulocytes (%)	0.7 ± 0.3	0.9 ± 0.3	0.9 ± 0.2	**1.1 ± 0.3***	**1.6 ± 0.7****	**2.5 ± 1.4****	2.2; 1.7-2.9
percentage of control	-	129	129	**157**	**229**	**357**	
Clinical chemistry; females, day 137; mean ± SD							
Cholesterol (mmol/L)	1.47 ± 0.48	1.61 ± 0.42	1.75 ± 0.32	**1.91 ± 0.30***	**2.26 ± 0.36****	**2.64 ± 0.44****	1.43; 1.13–1.86
percentage of control	-	110	119	**130**	**154**	**180**	
Total bilirubin by colorimetry (µmol/L)	1.79 ± 0.30	**1.50 ± 0.28***	1.83 ± 0.34	1.70 ± 0.38	**2.21 ± 0.31****	**2.36 ± 0.46****	-
percentage of control	-	**84**	102	95	**123**	**132**	-
γ-glutamyl transpeptidase, GGTP (nkat/L)	0 ± 0	0 ± 1	0 ± 0	1 ± 1	1 ± 2	**2** ± 3*	2; 0–10
Organ weight; mean ± SD							
Terminal body weight (g)	247.532 ± 15.668	242.292 ± 14.909	245.275 ± 17.713	241.816 ± 18.338	**234.588 ± 14.169****	**236.26 ± 15.870***	-
Absolute adrenal weight (mg)	78.92 ± 10.432	78.80 ± 7.036	**86.75 ± 10.85***	**96.16 ± 11.12****	**108.16 ± 12.36****	**121.12 ± 15.24****	77.189; 70.000–83.200
percentage of control	-	100	**110**	**122**	**137**	**153**	
Relative adrenal weight (%)	0.032 ± 0.004	0.033 ± 0.002	**0.035 ± 0.004****	**0.040 ± 0.004****	**0.046 ± 0.005****	**0.051 ± 0.007****	0.033; 0.028–0.037
percentage of control	-	102	**111**	**125**	**145**	**161**	

	Sex and dietary doses (ppm)						
	Females						
Dietary dose; (ppm)	**0**	**30**	**100**	**300**	**1500**	**15 000**	**HCD [a]**
Dose; lactation (ppm)	**0**	**15**	**50**	**150**	**750**	**7 500**	
Parameter							
Absolute ovary weight (g)	130.32 ± 20.497	130.88 ± 19.023	127.58 ± 18.743	**143.68 ± 21.906***	137.00 ± 23.873	**150.12 ± 28.875****	113.26; 100.00–126.72
percentage of control	-	100	98	**110**	105	**115**	
Relative ovary weight (%)	0.053 ± 0.008	0.054 ± 0.007	0.052 ± 0.008	**0.060 ± 0.008****	**0.058 ± 0.009***	**0.063 ± 0.011****	0.049; 0.042–0.054
percentage of control	-	103	99	**113**	110	**120**	
Gross pathology							
Number examined	25	25	25	25	25	25	-
Adrenal cortex: enlarged	0	0	0	3	11	25	-
Histopathology							
Adrenal Cortex							
Number Examined	25	25	25	25	25	25	-
Vacuolation increased	0	0	3	19	25	25	-
minimal	0	0	3	16	8	2	-
slight	0	0	0	3	16	5	-
moderate	0	0	0	0	1	17	-
severe	0	0	0	0	0	1	-
Hypertrophy, diffuse	0	0	0	4	10	16	-
minimal	0	0	0	4	9	11	-
slight	0	0	0	0	1	5	-
Ovary							
Number examined	24	25	25	25	25	24	-
Vacuolation of interstitial glands	0	0	5	10	19	22	-
minimal	0	0	3	5	6	5	-
slight	0	0	2	3	7	8	-
moderate	0	0	0	2	2	5	-
severe	0	0	0	0	4	4	-
	F1 pup data						
Body weight (g); mean ± SD							
Lactation day 1	6.4 ± 0.4	6.5 ± 0.6	6.6 ± 0.8	6.3 ± 0.5	6.3 ± 0.8	6.4 ± 0.6	-
Lactation day 4	9.8 ± 1.0	10.0 ± 1.0	10.0 ± 1.5	9.6 ± 1.0	9.6 ± 1.3	9.9 ± 1.2	-
Lactation day 7	16.0 ± 1.3	16.2 ± 1.4	16.3 ± 1.8	15.7 ± 1.1	15.6 ± 1.6	15.9 ± 1.5	-
Lactation day 14	33.9 ± 2.4	33.3 ± 2.3	33.8 ± 3.0	33.3 ± 1.9	32.5 ± 2.0	32.8 ± 2.4	-
Lactation day 21	53.3 ± 3.0	52.3 ± 3.2	53.0 ± 4.3	52.4 ± 3.2	**50.6 ± 3.1***	**49.6 ± 3.2****	-
percentage of control	-	98	99	98	**95**	**93**	-

	Sex and dietary doses (ppm)						
	Females						
Dietary dose; (ppm)	**0**	**30**	**100**	**300**	**1500**	**15000**	**HCD [a]**
Dose; lactation (ppm)	**0**	**15**	**50**	**150**	**750**	**7500**	
Parameter							
Body weight gain (g); mean ± SD							
Lactation days 14–21	19.5 ± 1.2	18.9 ± 1.5	19.2 ± 1.6	19.1 ± 1.6	**18.1 ± 1.6***	**16.8 ± 1.1****	
percentage of control	-	*97*	*98*	*98*	***93***	***86***	
Lactation days 1–21	46.9 ± 2.8	45.7 ± 2.9	46.4 ± 3.6	46.0 ± 3.2	**44.2** ± 2.7*	**43.2** ± 2.7**	-
percentage of control	-	97	99	98	94	92	-
	F1 parental animals						
Dose (mg/kg per day)							
Premating days 0–70	0	2.7	9.1	28.7	137.0	1391.3	-
Gestation	0	2.2	7.5	22.6	114.0	1174.4	-
Lactation	0	2.7	9.4	29.1	148.2	1420.5	-
Body weight gain (g); mean ± SD							
Premating days 0–70	140.5 ± 10.5	144.1 ± 18.1	137.4 ± 9.8	141.6 ± 15.4	139.8 ± 12.8	137.0 ± 13.6	-
Gestation days 0–7	26.3 ± 4.5	24.7 ± 4.8	**22.3 ± 4.6***	23.4 ± 5.4	**20.3 ± 4.4****	**20.3 ± 5.6****	-
percentage of control	-	94	85	89	**77**	**77**	-
Gestation days 7–14	25.5 ± 5.1	24.5 ± 4.1	24.8 ± 4.3	25.4 ± 4.6	24.1 ± 3.7	25.1 ± 5.0	-
Gestation days 0–20	112.6 ± 15.2	109.1 ± 15.6	104.6 ± 14.5	110.0 ± 15.9	106.6 ± 11.6	104.7 ± 18.0	-
Haematology; females, day 124; mean ± SD							
Red blood cells (10^9/L)	8.71 ± 0.43	8.69 ± 0.30	8.76 ± 0.35	8.60 ± 0.27	8.47 ± 0.32	**8.28 ± 0.34****	-
percentage of control	-	100	101	99	97	**95**	-
Haemoglobin (mmol/L)	10.3 ± 0.2	**10.1 ± 0.3***	10.3 ± 0.3	**10.1 ± 0.6****	**10.0 ± 0.2****	**10.0 ± 0.4***	-
percentage of control	-	**98**	100	**98**	**97**	**97**	-
Clinical chemistry; females, day 124; mean ± SD							
Cholesterol (mmol/L)	1.77 ± 0.26	1.86 ± 0.29	1.97 ± 0.34	**2.28 ± 0.42****	**2.67 ± 0.51****	**2.68 ± 0.50****	-
percentage of control	-	105	111	**129**	**151**	**151**	-
Organ weights; mean ± SD							
Terminal body weight (g)	237.0 ± 15.526	236.4 ± 19.0970	229.6 ± 10.4399	232.2 ± 22.864	226.26 ± 17.523	228.5 ± 15.688	-
Absolute adrenal weight (mg)	83.68 ± 9.724	81.48 ± 10.178	86.48 ± 10.071	**96.52 ± 10.481****	**108.12 ± 15.128****	**119.68 ± 13.158****	77.196; 73.400–82.360
percentage of control	-	97	103	**115**	**129**	**143**	
Relative adrenal weight (%)	0.035 ± 0.004	0.035 ± 0.004	0.038 ± 0.004	**0.042 ± 0.004****	**0.048 ± 0.006****	**0.052 ± 0.005****	0.033; 0.031–0.035
percentage of control	-	98	106	**118**	**135**	**148**	

	Sex and dietary doses (ppm)						
	Females						
Dietary dose; (ppm)	**0**	**30**	**100**	**300**	**1500**	**15 000**	**HCD [a]**
Dose; lactation (ppm)	**0**	**15**	**50**	**150**	**750**	**7 500**	
Parameter							
Absolute ovary weight (g)	106.84 ± 15.367	111.48 ± 17.441	106.92 ± 11.068	**122.92 ± 20.732****	**119.04 ± 21.841***	116.25 ± 18.753	115.191; 109.708–119.480
percentage of control	-	104	100	**115**	**111**	109	
Relative ovary weight (%)	0.045 ± 0.007	0.047 ± 0.006	0.047 ± 0.004	**0.053 ± 0.007****	**0.052 ± 0.007****	**0.051 ± 0.008***	0.049; 0.046–0.050
percentage of control	-	104	103	**117**	**116**	**113**	
Gross pathology							
Number of animals	25	25	25	25	25	25	-
Adrenal cortex: enlarged	0	0	0	1	17	24	
Histopathology							
Number of animals	25	25	25	25	25	25	-
Adrenal glands							
Vacuolation increased	1	2	23	25	25	25	-
minimal	1	2	21	8	0	2	-
slight	0	0	2	15	9	5	-
moderate	0	0	0	2	16	18	-
Hypertrophy, diffuse	0	0	0	0	2	10	-
minimal	0	0	0	0	2	7	-
slight	0	0	0	0	0	3	-
Ovary							
Vacuolation of interstitial glands	0	0	0	11	20	22	-
minimal	0	0	0	10	10	7	-
slight	0	0	0	1	7	8	-
moderate	0	0	0	0	3	7	-
F2 pup data							
Body weight (g); mean ± SD							
Lactation day 1	6.5 ± 0.7	6.3 ± 0.6	6.4 ± 0.7	6.2 ± 0.6	6.4 ± 0.6	6.3 ± 0.7	-
Lactation day 4	9.9 ± 1.5	9.9 ± 1.0	9.7 ± 1.3	9.5 ± 1.1	9.2 ± 1.0	9.3 ± 1.4	-
Lactation day 7	16.1 ± 1.8	16.1 ± 1.3	15.8 ± 1.7	15.6 ± 1.2	**14.7 ± 1.8***	**14.8 ± 1.7***	-
percentage of control	-	100	98	97	**91**	**92**	-
Lactation day 14	32.7 ± 2.9	32.5 ± 2.1	32.1 ± 2.7	31.8 ± 2.2	**30.6 ± 2.7***	**30.8 ± 2.8***	-
percentage of control	-	99	98	97	**94**	**94**	-
Lactation day 21	50.7 ± 3.9	50.4 ± 2.5	50.1 ± 3.4	49.7 ± 3.0	**47.4 ± 3.7****	**46.4 ± 3.8****	-
percentage of control	-	99	99	98	**93**	**92**	-

	Sex and dietary doses (ppm)						
	Females						
Dietary dose; (ppm)	**0**	**30**	**100**	**300**	**1500**	**15000**	**HCD [a]**
Dose; lactation (ppm)	**0**	**15**	**50**	**150**	**750**	**7500**	
Parameter							
Body weight gain (g); mean ± SD							
Lactation days 1-4	3.4 ± 0.8	3.6 ± 0.6	3.3 ± 0.7	3.2 ± 0.6	**2.9** ± 0.6*	3.0 ± 0.7	-
percentage of control	-	106	97	94	85	88	-
Lactation days 4-7	6.1 ± 0.5	6.1 ± 0.6	6.0 ± 0.7	6.1 ± 0.5	**5.5 ± 0.8****	**5.7 ± 0.7****	-
percentage of control	-	100	98	100	**90**	**93**	-
Lactation days 1–21	44.2 ± 3.4	44.0 ± 2.3	43.6 ± 3.0	43.4 ± 2.8	**41.1 ± 3.3****	**40.0 ± 3.3****	-
percentage of control	-	100	99	98	**93**	**90**	-

SD: Standard deviation; MCHC: mean corpuscular haemoglobin concentration; Source: Schneider et al., 2017

a Historical control data; shown as: mean; minimum–maximum of range;

Statistically significant at: * $P < 0.05$ ** $P < 0.01$;

Tests used as follows:
Body weight gain; Dunnett's test (two-sided),
Haematology; Wilcoxon test,
Clinical biochemistry; Kruskal–Wallis + Wilcoxon test (two-sided),
Organ weight; Kruskal-Wallis H and Wilcoxon test (two-sided),
Histopathology; Fisher`s exact test (two-sided)
Reproductive performance; Dunnett's two-sided or Fisher's exact test, one-sided;

The NOAEL for parental toxicity was 30 ppm (equal to 2.3 mg/kg bw per day), based on increased adrenal weights and vacuolation of the adrenal cortex in males and females of the F0 and F1 generations, and ovary interstitial gland vacuolation in females of the F0 generation at 100 ppm (equal to 7.5 mg/kg bw per day)

The NOAEL for reproductive toxicity was 15 000 ppm (equal to 1146.7 mg/kg bw per day), the highest dose tested.

The NOAEL for offspring toxicity was 300 ppm (equal to 22.6 mg/kg bw per day) based on decreased body weight in male and female pups of the F1 and F2 generations at 1500 ppm (equal to 111.3 mg/kg bw per day) (Schneider et al., 2017).

(b) Developmental toxicity

Rat

In a tolerability study that was neither GLP nor guideline-compliant, three nonpregnant female Wistar (Crl:WI(Han)) rats per dose group were administered broflanilide (purity 99.07%) for 14 days by gavage at 0, 250, 500 or 1000 mg/kg bw per day, as a suspension in 1% CMC in drinking water. Food consumption and body weight were determined regularly during the study. Animals were checked daily for any abnormal clinical signs. Blood samples for haematology and clinical chemistry assessment were taken from the fasted animals at the end of the administration period. All animals were necropsied and assessed for gross pathology including weight determination of their liver, adrenals and heart.

No effects on clinical signs, food consumption or body weights were reported.

There were no apparent treatment-related effects on haematological or clinical chemistry parameters. Increased adrenal weights (by up to about 20%) were observed in animals at 500 and 1000 mg/kg bw per day when compared to controls.

It was concluded that under the conditions of this study oral administration of broflanilide to nonpregnant Wistar rats for 14 days up to a limit dose of 1000 mg/kg bw per day was well tolerated (Flick, 2011a).

In a dose-finding study (not GLP), 10 pregnant, (Crl:WI(Han)) rats per dose group were orally administered (by gavage) broflanilide (purity 99.07%) at dose levels of 0, 100, 300 or 1000 mg/kg bw per day, as a suspension in 1% CMC in drinking water, from gestation GD 6 until GD 19. Food consumption was determined regularly during the study. Body weights were measured on these same days, as well as on study day 0. Animals were checked daily for any abnormal clinical signs. Blood samples were taken at the end of the administration period. At necropsy all animals were assessed for gross pathology and the following organs weighed: liver, spleen, adrenals, heart and unopened uterus.

There were no effects reported on clinical signs, food consumption or body weight.

No significant changes were reported when haematology parameters were investigated. A slight but not statistically significant reduction in RBC count was observed at 1000 mg/kg bw per day, by about 5% compared to controls. In animals at the high dose an increase in total bilirubin by 24% when compared to controls (not statistically significant) was observed. A dose-related increase in triglycerides (again, not statistically significant) by up to 23% when compared to control was observed at all dose levels. An increase in absolute and relative adrenal weights (not statistically significant) by 13% and 11% respectively when compared to controls was observed in animals at 1000 mg/kg bw per day. There were no treatment-related findings at gross pathology examination.

Based on the findings of the study, doses of 100, 300 and 1000 mg/kg bw per day were considered adequate for a definitive prenatal developmental toxicity study with broflanilide (Flick, 2011b).

In the main prenatal developmental toxicity study, broflanilide (purity 99.67%) was administered by gavage to 25 time-mated female Wistar rats per dose group at dose levels of 0, 100, 300 or 1000 mg/kg bw per day, suspended in 1% aqueous CMC in a dose volume of 10 mL/kg bw, from GD 6 until GD 19. Food consumption and body weights of the animals were recorded regularly throughout the study period. The state of the animals' clinical condition was checked each day. On GD 20, all females were sacrificed and assessed for gross pathology, which included weighing the unopened uterus and the placenta). For each dam, corpora lutea were counted and the number and distribution of implantation sites determined, differentiating between resorptions, live and dead fetuses. The fetuses were removed from the uterus, sexed, weighed and investigated for external findings. Thereafter, one half of the fetuses from each litter were examined for soft tissue findings and the remaining fetuses for skeletal (including cartilage) findings.

There were no clinical signs of toxicity or mortalities, nor any treatment-related changes in food consumption, body weights, weights of uterus and placenta, or observations at caesarian section examination.

At fetal examination no treatment-related external or visceral malformations or variations were observed. Skeletal examination did reveal variations of different bone structures, with or without effects on corresponding cartilages. The total incidences of skeletal variation showed no relation to dosing and related to several parts of the fetal skeletons. They did not reflect statistically, dose-related or biologically relevant differences between the groups and/or could be found at comparable or higher frequencies in the HCD provided. Thus, all skeletal variations were considered to be spontaneous in nature and not related to treatment.

Skeletal malformations were noted. Two fetuses in the control group were affected (5–01 M, misshapen tuberositas deltoidea; 17–03 F, thoracic hemivertebra, misshapen thoracic vertebra).Four fetuses in the group treated at 300 mg/kg bw per day were affected (54–03 M, shortened humerus; 55–09 M, misshapen thoracic vertebra; 71–06 F, misshapen tuberositas deltoidea; 75–06 F, fused mandible). Three fetuses in the group treated at 1000 mg/kg bw per day were affected (82–11 M, severely malformed sternum; 85–01 F, absent lumbar vertebra; 89–05 M, multiple skeletal malformations involving sternum, vertebral column, ribs and skull). These malformations were not considered to be related to treatment as their incidence was within normal background levels seen in the HCD.

Table 24. Key findings of the developmental toxicity study in rats

	Dose level (mg/kg bw per day)			
Finding	**0**	**100**	**300**	**1000**
Fetal abnormalities; skeletal				
Number of fetuses examined	129	137	133	131
Number of litters examined	25	25	25	25
Malformations (number [percentage])				
Fetal incidence	2 [1.6]	0 [0.0]	4 [3.0]	3 [2.3]
Litter incidence	2 [8.0]	0 [0.0]	4 [16]	3 [12]
Affected fetuses/litter; mean ± SD	1.6 ± 5.54	0.0 ± 0.00	2.8 ± 6.71	2.1 ± 5.92
Variations (number [percentage])				
Fetal incidence	123 [95]	132 [96]	131 [98]	126 [96]
Litter incidence	25 [100]	25 [100]	25 [100]	25 [100]
Affected fetuses/litter; mean ± SD	95.0 ± 10.80	96.5 ± 10.95	98.5 ± 5.10	96.3 ± 9.04

SD: Standard deviation; Source: Schneider, Grauert & van Ravenzwaay, 2016

The NOAEL for maternal toxicity was 1000 mg/kg bw per day, the highest dose tested.

The NOAEL for fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Schneider, Grauert & van Ravenzwaay, 2016).

Rabbit

In tolerability study that was neither GLP nor guideline-compliant, three nonpregnant female New Zealand White rabbits (Crl:KBL) per dose group were administered broflanilide (purity 99.58%) for 14 days by gavage at 0, 100, 300 or 1000 mg/kg bw per day, suspended in 1% CMC in drinking water. Food consumption and body weight were determined regularly during the study. Animals were checked daily for any abnormal clinical signs. All animals were necropsied and assessed for gross pathology.

No treatment-related clinical signs of toxicity, mortalities, or changes to food consumption or body weight, nor any gross pathology findings.

Under the conditions of this study, it was concluded that oral administration of broflanilide to nonpregnant rabbits for 14 days up to a limit dose of 1000 mg/kg bw per day was well tolerated (Flick, 2016a).

In a dose-finding study (not GLP), five presumed-pregnant (artificial insemination) New Zealand White rabbits (Crl:KBL) per dose group were orally (by gavage) administered broflanilide (purity 99.58%) at dose levels of 0, 100, 300 or 1000 mg/kg bw per day, suspended in 1% CMC in drinking water, from GD 6 until GD 28. Food consumption was determined regularly during the study, including on day 0. Animals were checked daily for any abnormal clinical signs. Blood samples were taken at the end of the administration period. At necropsy all animals were assessed for gross pathology and the following organs weighed: liver, spleen, heart and unopened uterus.

Pregnancy status examination showed that the number of pregnant animals was 4, 2, 4 and 3 at 0, 100, 300 and 1000 mg/kg bw per day, respectively. Two animals (a pregnant one on day 14 and a nonpregnant one on day 17) died prematurely due to gavage errors (both had changes in the lungs).

No treatment-related effects were apparent, however due to the small size of the dosed groups a quantitative evaluation of potential changes to the parameters examined was not possible. The study author concluded that oral administration of broflanilide up to the limit dose of 1000 mg/kg bw per day from GD 6 to 28 was well tolerated by pregnant rabbits (Flick, 2016b).

In the main prenatal developmental toxicity study, broflanilide (purity 98.67%) was administered by gavage to 25 presumed-pregnant (artificial insemination) female New Zealand White rabbits (Crl:KBL) per dose group at dose levels of 0, 100, 300 or 1000 mg/kg bw per day, suspended in 1%

aqueous CMC at a dose volume of 10 mL/kg bw, from GD 6 until GD 28. Food consumption and body weight were recorded regularly throughout the study period. The state of health of the animals was checked each day. On GD 29 all surviving females were sacrificed and assessed for gross pathology (including weight determinations of the unopened uterus and placenta). For each doe, corpora lutea were counted and the number and distribution of implantation sites determined, differentiating between resorptions, live and dead fetuses. Fetuses were removed from the uterus, sexed, weighed and further investigated for any external, soft tissue or skeletal (including cartilage) findings.

At terminal sacrifice on GD 29, between 20 and 24 females from each group showed implantation sites. There were no treatment-related or spontaneous mortalities. One female at 300 mg/kg bw per day was sacrificed after a gavage error and two females at 1000 mg/kg bw per day were found dead on GD 18 or 19. Gross pathological examination of these animals revealed findings indicative of gavage errors.

In clinical observations, completely reduced defaecation was observed in three control, five low-dose, eight mid-dose and six high-dose females. There were no treatment-related effects on food consumption. The mean body weights and the average body weight gain for the low-, mid- and high-dose groups were approximately comparable to the concurrent control group throughout the entire study period. However, there were some statistically significantly changes in mean body weight gain, in particular a decrease on GDs 11–14 at 100 and 1000 mg/kg bw per day. These were considered to be spontaneous events.

During the exposure period reduced body weight gain was observed in females at 300 and 1000 mg/kg bw per day, lower by 39% and 29% respectively compared to controls. It was noted that these changes were not dose-related or different from the control in a statistically significant sense. In the absence of any effects on body weight in these animals the observed body weight gains were considered of doubtful relation to treatment or any toxicological relevance.

The mean gravid uterus weights of the tested rabbits were not influenced by intake of the test substance. The differences between these groups and the control group showed no dose-dependency and were assessed to be without biological relevance.

There were no treatment-related or biological relevant changes compared to controls in the number of corpora lutea, implantations, pre- and post-implantation losses, live fetuses, sex ratio, intrauterine deaths or fetal weight. A statistically significant decrease in placental weight was reported at 100 and 300 mg/kg bw per day with a flat dose response between these two doses. Since placental weight at 1000 mg/kg bw per day was similar to the control values the decrease in placental weights were considered unrelated to treatment and of no biological relevance.

No external malformations were recorded or treatment-related external variations observed. There were no treatment-related visceral malformations or variations.

The overall incidence of skeletal variations did not show any relation to treatment. When individual skeletal variations are considered, a statistically significant incidence of fused sternebra (unchanged cartilage) in terms of litters affected and number of affected fetuses per litter, was reported at 300 mg/kg bw per day. These increases were greater than the upper limit of the HCD for both units, however as no increase at the next highest dose was observed the finding was considered unrelated to treatment. An apparent treatment-related increase in fetal and litter incidences, and in the number of fetuses per litter with incomplete ossification of the talus (cartilage present) was observed at all dose levels when compared to controls (which showed none), achieving statistical significance for number of fetuses per litter at 1000 mg/kg bw per day. The distributions of this finding were within the HCD in all cases. A slight increase (not statistically significant) in the incidence of litters and fetuses with fused rib cartilage was observed at 300 and 1000 mg/kg bw per day compared to controls (which showed none), consisting of one litter at 300 and two litters (one fetus each) at 1000 mg/kg bw per day. This finding was an unclassified cartilage finding without impact on the respective bony structure. All distributions were within the HCD values, except for the litter incidence at 1000 mg/kg bw per day which was slightly higher than the HCD upper limit. The occurrence of this isolated finding was considered of doubtful relation to treatment or biological relevance.

Skeletal malformations were observed at 100 and 300 mg/kg bw per day. At 300 mg/kg bw per day, the difference from controls (which showed none) was statistically significant. However, the respective value for affected fetuses per litter for those treated at 300 mg/kg bw per day (2.2%) was within the HCD values (mean 1.4%, range 0.0%–3.4%) and there was no dose–response relationship. The individual skeletal malformations were therefore considered to be incidental.

Table 25. Key findings of the developmental toxicity study in rabbits

	Dose (mg/kg bw per day)				HCD[a]
Finding	**0**	**100**	**300**	**1000**	**(mean; range)**
Skeletal					
Fetuses evaluated (*N*)	214	242	221	167	
Litters evaluated (*N*)	24	24	23	20	
Variations					
Fused sternebra, unchanged cartilage					
Fetal incidence (*N*) [%]	0 [0.0]	2 [0.8]	8 [3.6]	1 [0.6]	0.8; 0–1.9
Litter incidence (*N*) [%]	0 [0.0]	2 [8.3]	**4 [17.0]***	1 [5.0]	5.6; 0–11.5
Affected fetuses/litter Mean ± SD [%]	0.0 ± 0.0	0.7 ± 2.46	**4 ± 12.0***	0.5 ± 2.24	0.7; 0–1.4
Incomplete ossification of talus; cartilage present					
Fetal incidence (*N*) [%]	0 [0.0]	1 [0.4]	3 [1.4]	4 [2.4]	2.4; 0–8.2
Litter incidence (*N*) [%]	0 [0.0]	1 [4.2]	2 [8.7]	3 [15]	13.4; 0–45
Affected fetuses/litter Mean ± SD [%]	0.0 ± 0.0	0.4 ± 2.04	1.2 ± 4.16	**1.6 ± 4.17***	2.1; 0–7.4
Total variations					
Number of fetuses [%]	203 [95]	240 [99]	216 [98]	162 [97]	98.2; 95.2–100
Number of litters [%]	24 [100]	24 [100]	23 [100]	20 [100]	100; 100–100
Affected fetuses/litter (mean % ± SD)	93.4 ± 13.35	98.9 ± 3.62	97.9 ± 5.65	97.1 ± 5.64	98.4; 94.2–100
Unclassified cartilage observations					
Fused rib cartilage					
Fetal incidence (*N*) [%]	0 [0.0]	0 [0.0]	1 [0.5]	2 [1.2]	0.4; 0–2.0
Litter incidence (*N*) [%]	0 [0.0]	0 [0.0]	1 [4.3]	2 [10]	2.8; 0–9.1
Affected fetuses/litter (mean % ± SD)	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 1.9	1.1 ± 3.25	0.4; 0–1.9

bw body weight; N: Number affected; SD: Standard deviation Source: Flick, Grauert & van Ravenzwaay, 2018

[a] Historical control data: 12 studies from 2009 to 2013 for a total of 284 litters, 2346 fetuses; fetal, litter or fetal/litter incidence: % of total; % range (per study);

Statistically significant at: * $p < 0.05$; ** $p < 0.01$, using Fisher's exact test (one-sided) for *N* of malformations and variations and the Wilcoxon test (one-sided) for percentages

The NOAEL for maternal toxicity was 1000 mg/kg bw per day, the highest dose tested.

The NOAEL for fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Flick, Grauert & van Ravenzwaay, 2018)

2.6 Special studies

(a) Neurotoxicity

An acute neurotoxicity study that was neither GLP nor guideline-compliant was carried out to identify time to peak effect. Broflanilide (purity 98.7%) was administered by gavage to a single group of six (three of each sex) (Crl:WI(Han)) rats at a dose level of 2000 mg/kg bw. Drinking water containing 0.5% CMC served as the vehicle. All animals were checked at least daily for any clinical signs of toxicity, for moribund and dead animals. Detailed clinical observations were conducted prior to administration and then 15, 30 and 60 minutes, 2, 3, 4, 5 and 6 hours after administration, and then on days 2 and 3. Body weights were recorded once before administration.

It was concluded that the single administration of broflanilide at a dose level of 2000 mg/kg bw did not cause any substance-related signs of toxicity nor any mortalities (Buesen, 2017).

In an acute neurotoxicity study, 10 Wistar (Crl:WI(Han)) rats/sex per group were administered broflanilide (purity 98.7%) by gavage at dose levels of 0, 200, 600 or 2000 mg/kg bw. Drinking water containing 0.5% CMC served as the vehicle. The animals were observed up to two weeks after dosing. Body weight was determined before administration and on a weekly basis thereafter, and FOB and motor activity (MA) measurements were carried out seven days prior to administration and on the day of administration (day 0, approximately one hour after substance administration to correspond to T_{max}) as well as 7 and 14 days thereafter. At the end of the observation period, five animals per sex and test group were subjected to neuropathological examinations (brain weight, brain cross-sections, eyes with retina and optical nerve, spinal cord and peripheral nervous system). The remaining animals were sacrificed without further examination.

No mortalities or clinical signs of toxicity were recorded during the observation period. Body weights were not affected in either sex. There were no substance-related effects on the results of FOB examinations.

Motor activity values were decreased to a statistically significant extent in males at 200 and 2000 mg/kg bw at interval 3 on day 0, and a significant increase was reported in males at 600 mg/kg bw at interval 6 on day 0. On study day 7, motor activity was significantly decreased in males at all dose levels at interval 4, in females at 200 mg/kg bw at interval 7 and 8, and in females at 2000 mg/kg bw at interval 8. These changes were not considered related to treatment as only single intervals were affected and overall motor activity was not affected. No significant changes in motor activity were observed in any group on study day 14.

There were no treatment-related effects on brain weight, gross lesions or histopathology findings in tissues of the nervous system.

The NOAEL for systemic toxicity was 2000 mg/kg bw, the highest dose tested.

The NOAEL for neurotoxicity was 2000 mg/kg bw, the highest dose tested (Buesen et al., 2017c).

In a 90-day neurotoxicity study, 10 Wistar (Crl:WI(Han)) rats/sex per group were administered broflanilide (purity 98.7%) in the diet at dose levels of 0, 1500, 5000 or 15 000 ppm (equal to 0, 99, 320 and 1041 mg/kg bw per day for males, 0, 118, 423 and 1137 mg/kg bw per day for females). Food consumption was determined once a week. Body weight was measured once a week and on days when FOB tests were performed. Animals were examined for signs of toxicity or mortality at least once a day. Detailed clinical examinations in an open field were conducted prior to the start of the administration period and weekly thereafter. Functional observational batteries and MA measurements were carried out on study days −7, 1, 22, 50 and 85. Five animals per sex from each test group were subjected to neuropathological examinations (brain weight, brain cross-sections, eyes with retina and optical nerve, spinal cord and peripheral nervous system).

No mortalities or clinical signs of toxicity were recorded during the observation period. Body weights nor food consumption were affected in either sex. There were no substance-related effects on FOB observations.

No significant treatment-related effects on MA results were seen overall or in any single interval. Occasional statistically significant changes compared to controls were reported for single intervals:

on study day 50, interval 10 increased MA was reported in females at 5000 ppm, and on study day 85, decreased MA was reported in males at interval 12 at 1500 and 5000 ppm, and in females at 1500 ppm at interval 7. These changes were not considered treatment-related. Considering the MA overall, a slight reduction in MA scores was noted in males at 15 000 ppm on day 85 compared to controls and pretreatment values. Differences in these MA scores were not statistically significant when compared with controls; in the absence of other neurobehavioural findings they were considered not toxicologically relevant.

There were no treatment-related effects on brain weight, gross lesions or histopathology findings in tissues of the nervous system.

The NOAEL for systemic toxicity was 15 000 ppm (equal to 1041 mg/kg bw per day), the highest dose tested.

The NOAEL for neurotoxicity was 15 000 ppm (equal to 1041 mg/kg bw per day), the highest dose tested (Buesen et al., 2015a).

(b) Immunotoxicity

In a 28-day immunotoxicity study, groups of 10 male (Crl:WI (Han)) Wistar rats were administered broflanilide (purity 98.67%) at dietary concentrations of 0, 1200 ppm, 4000 ppm or 12 000 ppm (equal to 0, 104, 344 and 1020 mg/kg bw per day). At the same time, a positive control group for assessing immunotoxicity by T-lymphocyte-dependent antibody response (TDAR), consisting of 10 male Wistar rats received 4.5 mg/kg bw per day of cyclophosphamide monohydrate (CP) by gavage. All animals were immunized six days before blood sampling and necropsy using 0.5 mL of sheep red blood cells (sRBCs) at 4×10^8 cells/mL, administered intraperitoneally. Food consumption was determined weekly and body weight twice weekly. The animals were examined for signs of toxicity or mortality at least once a day. Detailed clinical examinations in an open field were conducted prior to the start of the administration period and weekly thereafter. The sRBC immunoglobulin (IgM) titre was measured at the end of the study. Additionally, all animals were assessed for any gross lesions and their spleen and thymus were weighed.

No treatment-related mortalities, clinical signs of toxicity, or effects on body weight or food consumption were observed.

Mean sRBC IgM antibody levels measured in the 1200, 4000 and 12 000 ppm groups were greater than the mean control group titre by 59%, 37% and 25%, respectively. The administration of the positive control substance led to effects indicative of immunotoxicity; reduced absolute and relative spleen and thymus weights, as well as reduced sRBC IgM antibody levels.

The NOAELs for systemic toxicity and for immunotoxicity were both 12 000 ppm (1020 mg/kg bw per day), the highest dose tested (Buesen et al., 2015e).

(c) Mechanistic studies

Hormone activity

Broflanilide and its metabolite DM-8007 were evaluated for their endocrine disrupting potential in a number of in vitro assays that assessed their ability to bind rat and human estrogen receptors, a rat androgen receptor, to transactivate androgen/estrogen receptors, and to affect steroidogenesis.

Table 26. Results of hormonal activity studies performed on broflanilide and its metabolite DM-8007

Type of study	Organism/cells	Test item and concentration	Conclusion	Reference
Rat androgen receptor (AR) binding competitive assay [a] (EPA OCSPP 890.1150) Study acceptable	SD Rat prostate cytosol	***Broflanilide*** 1×10^{-10} M to 1×10^{-4} M ***DM-8007*** 3.16×10^{-12} M to 3.16×10^{-5} M	Broflanilide: non-binder to the AR Metabolite DM-8007: non-binder to the AR	Rijk, 2019a
Androgen receptor (AR) transcriptional activation assay [b] (OECD TG 458) Study acceptable	Chinese hamster ovary cells (CHO-K1)	***Broflanilide and DM-8007*** Agonist: from 1 pM to 1 µM Antagonist: from 10 pM to 1 µM	Broflanilide and DM-8007 did not show androgenic agonist or antagonist activity	Rijk, 2020

Type of study	Organism/cells	Test item and concentration	Conclusion	Reference
Rat estrogen receptor (ER) binding competitive assay [c] (EPA OCSPP 890.1250) Study acceptable	SD rat uterine cytosol	***Broflanilide*** 1×10^{-10} to 31.6×10^{-6} M ***DM-8007*** 1×10^{-10} to 1×10^{-5} M	Broflanilide and DM-8007 did not interact with ER	Rijk, 2019b
Human estrogen receptor (hrERα) binding competitive assay [d] (OECD TG 493) Study acceptable	Human estrogen receptor-α, (hrERα)	***Broflanilide*** 1×10^{-11} to 10^{-4} M ***DM-8007*** 1×10^{-11} to $10^{-4.5}$ M	Broflanilide and DM-8007 were non-binders towards hrERα	Rijk, 2019c
Estrogen receptor (hrERα) transcriptional activation assay [e] (OCSPP 890.1300; OECD 455) Study acceptable	hERα-HeLa-9903	***Broflanilide and DM-8007*** Agonist: from 1 pM to 1 µM Antagonist: from 10 pM to 1 µM	Broflanilide and DM-8007 showed no activity as estrogenic agonists or antagonists.	Verkaart, 2020a
Modulation of steroidogenesis [f] (OCSPP 890.1550; OECD 456) Study acceptable	H295R human adrenocortical carcinoma cells	***Broflanilide*** Experiment 1: 0.01, 0.1, 1, 10, 100 & 1000 nM Experiments 2 and 3: 0.1, 1, 10, 100, 316 & 3160 nM ***DM-8007*** Experiment 1: 0.01, 0.1, 1, 10, 100, 1000 and 10 000 nM Experiments 2 and 3: 0.1, 1, 10, 100, 1000, 3160 and 10 000 nM	Broflanilide and DM-8007 decreased estradiol release; No effect on testosterone release	Verkaart, 2020b

[a] The study was conducted using ventral prostate cytosol from Sprague Dawley rats as a source of androgen receptor. Each experiment consisted of three independent runs, and each run contained three replicates at each concentration. The competitive binding assay was conducted with increasing concentrations of either broflanilide from 10^{-10} to 10^{-4} M or its metabolite DM-8007 from 3.16×10^{-12} to 3.16×10^{-5} M as determined by solubility. Ethanol was used as a vehicle (3.2% v/v). The assay included adequate positive and negative controls and ligand reference standard. An initial saturation assay showed AR to be present in a reasonable number (B_{max}) and with an adequate affinity to radiolabelled reference ligand (K_d). Marginally lower K_d values were obtained compared to the test guideline, however this demonstrates that rat prostate cytosol batch used had an higher affinity for [^{3}H]R-1881 and therefore was considered acceptable. Under the experimental conditions broflanilide and DM-8007 did not show displacement of [^{3}H]R1881 from the androgen receptor, no IC_{50} could be calculated and the average lowest point on the response curves across experiments was 93% for both broflanilide and DM-8007. Therefore, broflanilide and DM-8007 were both classified as 'non-binders' in the AR binding assay.

[b] The study was conducted using AR-EcoScreen™ cell line (CHO-K1-derived cell line). After solubility assessment broflanilide or DM-8007 (in DMSO) were tested at final concentrations ranging from 1 pM to 1 µM in the agonist assay and from 10 pM to 1 µM in the antagonist assay. Duration of exposure range from 21 to 24 hours. Two independent, valid experiments were considered for the agonist assay. For the antagonist assay, three independent, valid experiments were considered. In every experiment each broflanilide or DM-8007 concentration was tested in triplicate. Adequate reference chemicals were adopted for agonist and antagonist experiments. Cytotoxicity was evaluated by determining the *Renilla* luciferase activity in the antagonist assays. In the two agonist experiments the RPC_{max} (the maximum level of response induced by a test chemical, expressed as a percentage of the response induced by 1 nM E2 on the same plate) values were 0.7% and 0.2% for broflanilide and 0.1% and 0.3%, for DM-8007, respectively. In the antagonist experiments, broflanilide and DM-8007 did not show an androgenic antagonistic response up to a concentration of 1 µM, and as a result no log IC_{30} (inhibition at 30% of maximum activity) could be determined for all experiments. Under the experimental conditions, it was concluded that broflanilide and DM-8007 were negative in the AR agonist and antagonist assay.

[c] The study was conducted using ventral uterine cytosol from Sprague Dawley rats as source of estrogen. Each experiment consisted of three independent runs, and each run containing three replicates at each concentration. The competitive binding assay was conducted with increasing concentrations of either broflanilide or DM-2008 from 1×10^{-10} to 1×10^{-3} M. Based on solubility, the maximum final concentration evaluated was 31.6×10^{-6} M for broflanilide and 1×10^{-5} M for DM-8007. DMSO was used as the vehicle at a final concentration of 2% (v/v). The assay included adequate positive and negative controls and ligand reference standard. Results from the saturation assay showed that the ER was present in a reasonable number (B_{max}) and with an adequate affinity to radiolabelled reference ligand (K_d). For 19-norethindrone (NE), slope values were just outside the guidance criteria. Since the curve showed a decrease consistent with one-site competitive binding, this

minor deviation was accepted. The values for the bottom of the curve (%) were outside the guidance criteria. However, a clear sigmoidal dose–response curve was obtained with a definite IC_{50} value, and this resulted in a positive judgment of the weak positive control NE in the ER binding assay, thereby demonstrating sufficient responsiveness of the assay for weak positive test items. For this reason this deviation was accepted. Under the experimental conditions broflanilide and DM-8007 did not show displacement of [^{3}H]E2 from the androgen receptor; no IC_{50} could be calculated and the lowest point on the response curves across experiments was above 75% for both broflanilide and DM-8007. Therefore, broflanilide and DM-8007 were both classified as non-interactive in the ER binding assay.

[d] The study was conducted using the human estrogen receptor-α (hrERα), according to the Freyberger–Wilson in vitro method. The competitive binding experiment was conducted with increasing concentrations of broflanilide from 1×10^{-11} to 10^{-4} M (DM-8007 from 1×10^{-11} to $10^{-4.5}$ M) in ethanol (1%, v/v final concentration) as a vehicle based on solubility determinations. Three independent runs were conducted, each contained three replicates at each concentration. The assay included adequate positive and negative control and ligand reference standard. An initial saturation assay met the acceptance criteria and showed adequate number (B_{max}) of ER and affinity to with radiolabelled reference ligand (K_d). Acceptance criteria for the positive and negative reference compounds were met with the exception of the logIC_{50} for NE that were outside the guidance criteria, however, since the NE was observed to be more potent than required for the guidance criteria, (indicating sufficient responsiveness) the results were accepted. Concentration response curves for broflanilide and DM-8007 did not show displacement of [^{3}H]E2 from hrERα at increasing concentrations up to 100 µM and 31.6 µM, respectively. Under the experimental conditions both broflanilide and DM-8007 were considered non-binders to hrERα.

[e] The estrogen receptor transcriptional activation agonist and antagonist assay was conducted using hERα-HeLa-9903 cells. After solubility assessment broflanilide and DM-8007 were tested (in DMSO) at final concentrations ranging from 1 pM to 1 µM (agonist assay) and from 10 pM to 1 µM (antagonist assay) in three independent experiments in each of which test items concentrations were each tested in triplicate. Duration of exposure ranged from 21.5 to 23 hours across experiments. Cytotoxicity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test under agonist and antagonistic culture conditions. Adequate positive and negative reference compounds were used for the agonist (E2, 17α-estradiol, 17α-methyltestosterone and corticosterone) and antagonist (tamoxifen and flutamide, 4-hydroxytamoxifen and digitonin) experiments. Acceptability criteria were met by the agonist and antagonist experiments. A deviation compared to the guideline was noted in the log PC_{50} and log PC_{10} for 17α-methyltestosterone (weak positive control) in one experiment. As these values were consistent compared to a laboratory internal validation study and allowed a consistent correct classification of the reference compound as a weak positive, the deviation did not invalidate the experiment. In both independent agonist experiments RPC_{max} were consistently below 10% for both broflanilide and DM-8007. For the antagonist experiments, broflanilide and DM-8007 did not show an estrogen antagonistic response up to a concentration of 1 µM and as a result no log IC_{30} could be determined. Under the experimental conditions, it was concluded that broflanilide and DM-8007did not show any estrogenic agonist or antagonist activity.

[f] The steroidogenesis assay was conducted with H295R cells incubated with broflanilide at concentrations of 1000, 100, 10, 1, 0.1 and 0.01 nM (Experiment 1), and 3160, 1000, 316, 100, 10, 1 and 0.1 nM (Experiments 2 and 3) and DM-8007 at concentrations of 10 000, 1000, 100, 10, 1, 0.1 and 0.01 nM (Experiment), and 10 000, 3160, 1000, 100, 10, 1 and 0.1 nM (Experiments 2 and 3) in triplicate for 48 hours. Doses were established based on solubility and cytotoxicity. The vehicle was 0.1% DMSO. Quality control (QC) plates were run concurrently with each independent run and adequate positive and negative controls included. The assay acceptability criteria were met. In Experiment 1, estradiol was significantly depressed by broflanilide at 1000 nM and DM-8007 at 10 000 nM. Testosterone was significantly increased by broflanilide at 0.1 nM and significantly decreased by DM-8007 at 10 000 nM. In Experiment 2 the high end of the concentration curve was investigated in more detail. Estradiol was significantly depressed by broflanilide at 1000 and 3160 nM and by DM-8007 at 0.1, 1, 100, 3160 and 10 000 nM. No significant effects on testosterone release were observed in Experiment 2. Experiment 3 used the same concentrations as in Experiment 2. Estradiol was significantly depressed by broflanilide at 10, 100, 316, 1000 and 3160 nM. With DM-8007, estradiol was significantly increased at concentrations 0.1, 1, and 10 nM, but depressed at 3160 and 10 000 nM. No significant effects on testosterone release was observed in Experiment 3. Overall, broflanilide and DM-8007 induced a dose-related decrease in estradiol. The reported testosterone changes were considered incidental given the absence of dose–response relationship and poor reproducibility of results. It was concluded that broflanilide and DM-8007 altered estradiol release in H295R cells and therefore were considered to be positive in the steroidogenesis assay.

In an investigative study (not guideline-compliant), broflanilide (purity 98.67%) was administered for 90 days through the diet to 16 Wistar (Crl:WI(Han)) rats of each sex per group at dose levels of 0, 500 or 15 000 ppm (equal to 0, 32 and 972 mg/kg bw per day for males, 0, 36 and 1127 mg/kg bw per day for females). Additionally, eight satellite animals of each sex per group were used for ACTH challenge. All animals were observed for clinical signs daily, their body weight and food consumption recorded weekly. Estrous cycle was determined during pretest, on blood sampling days, after urine collection and on the day of necropsy. Blood was sampled from all main group animals for quantification of hormones at days 10, 45 and 91 as follows: follicle stimulating hormone (FSH; females only), luteinizing hormone (LH), progesterone (females only), testosterone (males only), estradiol (females only), and prolactin (females, males at 91 days only). Urine was collected on days 8, 42 and 85/88 for aldosterone/creatinine analysis. At necropsy all animals were subject to macroscopic examination. For the main

group animals the following organ weights were recorded: adrenal glands, pituitary, epididymides, ovaries, prostate, seminal vesicles, testes, thymus and uterus. For all satellite animals the weights of the adrenals only (along with terminal body weights) were recorded. Histopathological examination was conducted on adrenal glands, epididymides, ovaries, pituitary glands, thymus, testes and uterus from the main group animals but only on adrenal glands of satellite groups animals. Adrenal and ovary samples from 10 animals of each sex per main group were subject to differential oil red O (lipid stain) and filipin (cholesterol stain) staining to assess vacuole content. In addition, an ACTH challenge with subsequent measurement of corticosterone levels was performed on all satellite animals on days 6 and 90, with blood collection approximately 45 minutes after exogenous administration of ACTH via intramuscular injection (as well as before ACTH dosing on day 90). In an immunohistochemistry analysis, formalin-fixed, paraffin-embedded pituitary samples from 12 animals of each sex per main group were stained (rabbit anti-LH antibody) to detect LH expression in the pituitary gland, using the test facility validated method and control materials: positive (human placenta trophoblasts; rat epithelial cells in the adenohypophysis), and negative (human placenta stromal fibroblasts; rat pituitary non-LH-expressing epithelial cells). Pituitary slides were scored to assess staining intensity and frequency in epithelial pars distalis cells. Vacuoles were also assessed by grade.

All animals survived to necropsy and no treatment-related clinical signs were observed. There were no changes in body weight of males or females compared to controls. Body weight gains in males and females of the 500 ppm group were depressed by 7% and 5% , respectively. In the 15 000 ppm groups weight gain for males and females was depressed by 7% compared to controls. These changes were considered non-adverse. There were no treatment-related changes in food consumption.

Most females had regular estrous cycles of four to five days. Extended diestrus occurred in three control females; one female at 15 000 ppm had an irregular cycle. In addition, shortened diestrus occurred in a single animal at 500 ppm with an irregular cycle. The low incidence of these findings did not indicate any relationship to treatment.

There were no notable differences between treated groups and controls with respect to FSH levels. The FSH data set showed high variability. No statistically significant differences were identified.

Progesterone levels were generally depressed in all females, and those in diestrus only at 500 and 15 000 ppm at 10 and 91 days, as well as in animals at 15 000 ppm at 45 days. The difference was statistically significant only at 15 000 ppm on day 10. Females (including diestrus only) at 500 ppm showed an increase in progesterone level at 45 days.

There were no notable differences in estradiol levels between treated groups and controls in females or females in diestrus.

Prolactin data was highly variable. Prolactin was increased in males at 500 and 15 000 ppm at 91 days compared with controls. In females, when data from all animals examined was included, prolactin levels at 500 and 15 000 ppm showed changes relative to controls in opposite directions; at 15 000 ppm levels were increased (not statistically significant) at 45 days and decreased at 91 days, whilst at 500 ppm a decrease to 45 days was followed by a statistically significant increase after 91 days. In females in diestrus, decreased prolactin levels were observed after 45 and 91 days at 500 and 15 000 ppm, achieving statistical significance at the highest dose after 91 days.

Luteinizing hormone was generally increased in a dose-related manner in both treated groups in males, females and females in diestrus. The difference from controls achieved statistical significance in males, females and females in diestrus at 15 000 ppm, in females at 500 ppm on day 45, and in females and females in diestrus at 15 000 ppm on day 91. It was noted that of all hormone parameters investigated the LH data set (particularly the male data) showed the least variability. When LH levels are evaluated in relation to exposure duration, an increase is observed in females at the high dose. For the males, the control and low-dose LH values were similar.

Increased testosterone (statistically significant) was seen in males at 500 and 15 000 ppm on day 10, with a flat dose–response curve. Testosterone levels after 45 and 91 days were lower than for controls, with no dose–response relationship at day 91. When compared with controls these changes were not statistically significant. The trend over the time course of exposure showed that in control animals testosterone increased over time, whilst in treated animals testosterone decreased over time.

Urine levels of aldosterone normalized on creatinine were generally increased in all groups in both sexes compared to controls. The increase was statistically significant on several occasions: in males at 15 000 ppm on day 85, in females at 500 ppm on days 8 and 42, and in females at 15 000 ppm on days 85/88, and in females in diestrus at 500 ppm on day 42.

Corticosterone data was highly variable in all animals, with and without ACTH activation. In males of the main group a decrease was reported at 500 and 15 000 ppm at day 45, achieving statistical significance at the high dose, although an increase that was not dose-related was reported in the same animals after 91 days. In females and females in diestrus of the main group, changes in corticosterone levels at 10 and 45 days were generally in opposite directions at different dose levels. At 91 days, corticosterone was increased in females and females in diestrus at 15 000 ppm. It was noted that corticosterone values in these animals were similar to values for females of the satellite group before ACTH activation. An apparent corticosterone increase with a relatively flat dose–response relationship was observed in males of the satellite group before ACTH activation at 500 and 15 000 ppm at 90 days, however it was noted these corticosterone values were similar to the control values for the main group after 91 days.

In all satellite groups (control, 500 ppm and 15 000 ppm, males and females) group mean corticosterone values were higher after the ACTH challenge than before, indicating corticosterone synthesis and secretion had not been inhibited. However, following ACTH activation a dose-related increase compared to controls was observed after 90 days in males and females given broflanilide at 500 and 15 000 ppm, achieving statistical significance at the high dose.

Overall, the interpretation of potential broflanilide effects on analyzed hormones was hampered by a high degree of variability in the results, making the data inadequate for quantitative evaluation. However, effects were noted on progesterone, prolactin, LH, testosterone, aldosterone and corticosterone secretion.

Macroscopic examination showed treatment-related findings in the adrenal glands of all animals (main and satellite groups) and both sexes. These changes consisted of an increased incidence of enlarged adrenal glands at 500 and 15 000 ppm, achieving statistical significance at the high dose, and a statistically significant increase in pale discolouration at 500 and 15 000 ppm. The findings correlated with a statistically significant increase in adrenal gland weights seen in both sexes. In females of the main group, increases in ovaries weight were observed at 500 and 15 000 ppm, achieving statistical significance at 500 ppm for relative weight, and at 15 000 ppm for absolute and relative weights. A statistically significant increase in pituitary weights was reported in main-group females at 15 000 ppm. An increase in uterus weights (not statistically significant) was observed at 15 000 ppm.

Microscopic examination revealed a treatment-related increased in the incidence and severity of cortical vacuolation in the adrenal glands of all animals (mainly in the zona glomerulosa and zona fasciculata) at 500 ppm (severity up to slight), and at 15 000 ppm (severity up to severe). A dose-dependent increased incidence and severity in oil red O staining (lipid stain) was observed in the adrenal cortex of males and females at 500 and 15 000 ppm (severity up to moderate). Positive filipin staining (for cholesterol) was observed in all treated animals.

In a similar way to the cortical vacuolation already outlined, a treatment-related increase in incidence and severity of vacuolation of the interstitial cells (and thecal cells) was recorded in females at 500 ppm (severity up to slight) and at 15 000 ppm (severity up to moderate). A dose-dependent increased incidence and severity (up to severe degree) in oil red O staining (mainly in the interstitial and thecal cells) was observed at 500 and 15 000 ppm. No substantial differences in filipin staining were noted between cells from control and treated females.

Immunohistochemistry analysis revealed a reduction in the overall intensity of staining and in the number of LH-positive epithelial cells in males at 500 and 15 000 ppm. Increases in cytoplasmic vacuolation were also observed in treated males. No differences between groups were evident in the female animals. The immunohistochemistry data were interpreted by the study author to indicate that the male rats had a compound-mediated, dose-responsive increase in the synthesis (increased vacuolation) and secretion (decreased staining) of LH by the LH-positive cells in the pars distalis of the pituitary gland (Hoekstra, 2017; Lourens, 2017).

Table 27. Key findings in the 90-day investigative toxicity study in rats

	Sex and dietary dose (ppm)					
	Males			Females		
Parameter	**0**	**500**	**15 000**	**0**	**500**	**15 000**
Body weight (g)						
Day 1	203 ± 11	206 ± 13	208 ± 16	149 ± 7	150 ± 7	151 ± 7
Day 91	419 ± 31	412 ± 28	415 ± 47	239 ± 13	236 ± 14	235 ± 15
% of control	-	98	99	-	99	98
Main group hormone levels						
FSH in all females; mean ± SD (ng/mL)						
Number examined	-	-	-	16	16	16
Day 10	-	-	-	5.82 ± 5.01	3.77 ± 2.28	5.19 ± 2.97
% of control	-	-	-	-	65	89
Day 45	-	-	-	4.85 ± 2.95	5.45 ± 3.80	4.02 ± 2.54
% of control	-	-	-	-	112	83
Day 91	-	-	-	4.61 ± 3.19	6.83 ± 6.91	4.13 ± 3.32
% of control	-	-	-	-	148	90
FSH in diestrus females only; mean ± SD (ng/mL)						
Number examined	-	-	-	13	14	10
Day 10	-	-	-	3.64 ± 1.23	3.02 ± 0.40	3.19 ± 0.66
% of control	-	-	-	-	83	88
Number examined	-	-	-	12	12	12
Day 45	-	-	-	3.42 ± 1.10	4.45 ± 3.21	3.31 ± 1.77
% of control	-	-	-	-	130	97
Number examined	-	-	-	12	11	13
Day 91	-	-	-	3.01 ± 1.12	2.87 ± 0.95	2.74 ± 1.11
% of control	-	-	-	-	95	91
Progesterone in all females; mean ± SD (nmol/L)						
Number examined	-	-	-	16	16	16
Day 10	-	-	-	44.8 ± 20.14	37.3 ± 21.20	**32.3 ± 15.84***
% of control	-	-	-	-	83	**72**
Day 45	-	-	-	53.7 ± 21.24	58.8 ± 20.10	41.2 ± 18.04
% of control	-	-	-	-	109	77
Day 91	-	-	-	61.5 ± 30.88	55.2 ± 27.86	52.1 ± 27.88
% of control	-	-	-	-	90	85
Progesterone in diestrus females only; mean ± SD (nmol/mL)						
Number examined	-	-	-	13	14	10
Day 10	-	-	-	47.01 ± 21.77	36.99 ± 22.72	33.02 ± 18.72
% of control	-	-	-	-	*79*	*70*
Number examined	-	-	-	12	12	12
Day 45	-	-	-	59.03 ± 22.00	64.24 ± 20.34	41.25 ± 16.55
% of control	-	-	-	-	109	70
Number examined	-	-	-	12	11	13

	Sex and dietary dose (ppm)					
	Males			Females		
Parameter	**0**	**500**	**15000**	**0**	**500**	**15000**
Day 91	-	-	-	66.43 ± 33.55	53.21 ± 29.30	55.55 ± 29.96
% of control	-	-	-	-	*80*	*84*
Estradiol; mean ± SD (pmol/L)						
Number examined	-	-	-	16	16	16
Day 10	-	-	-	202.59 ± 51.22	195.91 ± 31.23	188.81 ± 25.81
% of control	-	-	-	-	97	93
Day 45	-	-	-	206.82 ± 69.29	205.51 ± 72.63	217.37 ± 59.00
% of control	-	-	-	-	99	105
Day 91	-	-	-	204.52 ± 87.35	241.65 ± 96.18	226.21 ± 68.79
% of control	-	-	-	-	118	111
Estradiol in diestrus females only; mean ± SD (pmol/L)						
Number examined	-	-	-	13	14	10
Day 10	-	-	-	208.43 ± 55.15	195.98 ± 33.42	196.87 ± 28.60
% of control	-	-	-	-	94	94
Number examined	-	-	-	12	12	12
Day 45	-	-	-	216.30 ± 77.32	205.16 ± 74.71	217.63 ± 65.54
% of control	-	-	-	-	95	101
Number examined	-	-	-	12	11	13
Day 91	-	-	-	218.85 ± 97.33	273.91 ± 98.66	233.79 ± 73.46
% of control	-	-	-	-	125	107
Prolactin; mean ± SD (ng/mL)						
Number examined	16	16	16	16	16	16
Day 10	-	-	-	15.5 ± 19.87	9.3 ± 10.77	21.4 ± 34.39
% of control	-	-	-	-	60	138
Day 45	-	-	-	22.2 ± 41.48	14.3 ± 16.59	39.2 ± 88.13
% of control	-	-	-	-	*64*	*177*
Day 91	3.9 ± 3.95	6.7 ± 11.73	8.2 ± 12.86	11.0 ± 10.07	15.8 ± 24.88	**7.5 ± 15.47***
% of control	-	172	210	-	144	**68**
Prolactin (ng/mL) in diestrus females only; mean ± SD (ng/mL)						
Number examined	-	-	-	13	14	10
Day 10	-	-	-	12.18 ± 13.72	6.51 ± 6.67	14.46 ± 16.94
% of control	-	-	-	-	53	119
Number examined	-	-	-	12	12	12
Day 45	-	-	-	22.05 ± 46.70	10.88 ± 8.16	9.66 ± 12.07
% of control	-	-	-	-	49	44

	Sex and dietary dose (ppm)					
	Males			Females		
Parameter	**0**	**500**	**15000**	**0**	**500**	**15000**
Number examined	-	-	-	12	11	13
Day 91	-	-	-	11.64 ± 10.90	6.56 ± 6.28	**8.01 ± 17.10***
% of control	-	-	-	-	57	69
Luteinizing hormone, LH; mean ± SD (ng/mL)						
Number examined	16	16	16	16	16	16
Day 10	2.3 ± 0.33	2.5 ± 0.32	2.6 ± 0.39	2.2 ± 0.67	1.9 ± 0.35	2.1 ± 0.45
% of control	-	109	113	-	86	95
Day 45	2.0 ± 0.38	2.3 ± 0.49	**2.6 ± 0.84***	1.8 ± 0.34	**2.2 ± 0.61***	**2.4 ± 0.53***
% of control	-	115	**130**	-	**122**	**133**
Day 91	1.2 ± 0.49	1.2 ± 0.53	1.8 ± 0.98	1.8 ± 0.79	1.9 ± 0.38	**2.6 ± 0.68***
% of control	-	100	150	-	106	**144**
Luteinizing hormone, LH in diestrus females only; mean ± SD (ng/mL)						
Number examined	-	-	-	13	14	10
Day 10	-	-	-	2.24 ± 0.72	1.86 ± 0.38	2.18 ± 0.53
% of control	-	-	-	-	86	100
Number examined	-	-	-	12	12	12
Day 45	-	-	-	1.82 ± 0.38	2.28 ± 0.69	**2.35 ± 0.49***
% of control	-	-	-	-	128	**133**
Number examined	-	-	-	12	11	13
Day 91	-	-	-	1.94 ± 0.85	2.08 ± 0.37	**2.80 ± 0.65***
% of control	-	-	-	-	111	**147**
Testosterone; mean ± SD (nmol/L)						
Number examined	16	16	16	-	-	-
Day 10	11.3 ± 4.42	**29.4 ± 27.34***	**29.4 ± 28.14***	-	-	-
% of control	-	**260**	**260**	-	-	-
Day 45	16.2 ± 12.72	13.0 ± 6.95	12.5 ± 5.83	-	-	-
% of control	-	80	77	-	-	-
Day 91	15.7 ± 11.29	9.8 ± 2.63	12.1 ± 7.20	-	-	-
% of control	-	62	77	-	-	-
Aldosterone/creatine ratio, all males and females; mean ± SD (× 10^{-7})						
Number examined	16	16	16	16	16	16
Day 8	1.8 ± 0.55	2.0 ± 0.45	2.1 ± 0.72	4.1 ± 1.60	**6.4 ± 2.33***	5.0 ± 2.03
% of control	-	111	117	-	**156**	122
Day 42	1.2 ± 0.34	1.4 ± 0.36	1.8 ± 0.81	3.2 ± 1.06	**4.0 ± 0.96***	4.6 ± 1.99
% of control	-	117	150	-	**125**	144
Day 85/88	1.2 ± 0.38	1.5 ± 0.35	**2.1 ± 1.07***	3.2 ± 1.35	3.6 ± 1.34	**3.5 ± 1.23***
% of control	-	125	**175**	-	113	**109**

	Sex and dietary dose (ppm)					
	Males			Females		
Parameter	**0**	**500**	**15000**	**0**	**500**	**15000**
Aldosterone/creatinine ratio in diestrus females only; mean ± SD (× 10^{-7})						
Number examined	-	-	-	13	14	10
Day 8	-	-	-	3.62 ± 1.08	4.05 ± 0.64	3.99 ± 1.45
% of control	-	-	-	-	114	111
Number examined	-	-	-	12	12	12
Day 42	-	-	-	3.10 ± 1.15	**3.93 ± 0.77***	3.60 ± 1.16
% of control	-	-	-	-	**126**	116
Number examined	-	-	-	12	11	13
Day 85/88	-	-	-	2.58 ± 0.74	3.44 ± 1.43	3.19 ± 1.10
% of control	-	-	-	-	131	123
Corticosterone, all males and females; mean ± SD (ng/mL)						
Number examined	16	16	16	16	16	16
Day 10	143.2 ± 101.4	141.4 ± 106.9	156.2 ± 114.9	343.8 ± 185.4	250.1 ± 135.1	280.9 ± 151.5
% of control	-	99	109	-	73	119
Day 45	212.2 ± 74.8	187.5 ± 81.2	**145.4 ± 77.0***	422.3 ± 200.5	471.0 ± 240.4	357.3 ± 244.5
% of control	-	88	**69**	-	112	85
Day 91	129.3 ± 82.9	190.1 ± 107.4	154.4 ± 83.4	344.8 ± 174.5	390.6 ± 231.2	470.1 ± 291.8
% of control	-	147	119	-	113	136
Corticosterone in diestrus females only; mean ± SD (ng/mL)						
Number examined	-	-	-	13	14	10
Day 10	-	-	-	326.9 ± 189.4	258.5 ± 131.5	345.5 ± 138.7
% of control	-	-	-	-	79	106
Number examined	-	-	-	12	12	12
Day 45	-	-	-	471.9 ± 190.0	460.4 ± 227.8	341.3 ± 256.9
% of control	-	-	-	-	98	72
Number examined	-	-	-	12	11	13
Day 91	-	-	-	345.4 ± 185.3	345.6 ± 202.4	524.1 ± 296.5
% of control	-	-	-	-	-	152
Satellite group hormone levels (8 rats/sex per group unless otherwise specified)						
Corticosterone pre ACTH challenge; mean ± SD (ng/mL)						
Day 90	108.8 ± 4.92	123.8 ± 119.69	123.1 ± 62.92	453.7 ± 196.48	445.5 ± 252.36	635.9 ± 398.60
% of control	-	114	113	-	98	140
Corticosterone 45 minutes post ACTH challenge; mean ± SD (ng/mL)						
Day 6	209.7 ± 69.6	161.8 ± 117.0	160.0 ± 72.9	222.5 ± 171.8	314.2 ± 220.2	236.8 ± 113.4
% of control	-	77	76	-	141	106

	Sex and dietary dose (ppm)					
	Males			Females		
Parameter	**0**	**500**	**15000**	**0**	**500**	**15000**
Day 90	420.0 ± 188.1	618.1 ± 282.4	**738.2 ± 180.0***	667.3 ± 156.4	873.3 ± 354.2	**1198.3 ± 377.1***
% of control	-	147	**176**	-	131	**178**
Organ weight; absolute; mean ± SD (g)						
Terminal body weight	397 ± 29	390 ± 25	393 ± 44	224 ± 13	221 ± 12	219 ± 12
% of control	-	98	99	-	99	98
N examined	24	24	24	24	24	24
Adrenals	0.053 ± 0.010	**0.062 ± 0.009****	**0.074 ± 0.008****	0.067 ± 0.008	**0.078 ± 0.011****	**0.112 ± 0.011****
% of control	-	**117**	**140**	-	**116**	**167**
N examined	-	-	-	16	16	16
Ovaries	-	-	-	0.143 ± 0.025	0.157 ± 0.019	**0.168 ± 0.021****
% of control	-	-	-	-	110	**117**
N examined	16	16	16	16	16	16
Pituitary	0.009 ± 0.001	0.009 ± 0.001	0.009 ± 0.000	0.012 ± 0.002	0.013 ± 0.002	**0.014 ± 0.002****
% of control	-	100	100	-	108	**117**
N examined	16	16	16	16	16	16
Uterus	-	-	-	0.744 ± 0.4	0.754 ± 0.3	1.09 ± 0.6
% of control	-	-	-	-	101	147
Organ weight; relative to body weight; mean ± SD (ng/mL)						
Adrenal (%)	0.013 ± 0.002	**0.016 ± 0.003****	**0.019 ± 0.003****	0.030 ± 0.003	**0.035 ± 0.005****	**0.051 ± 0.004****
% of control	-	**123**	**146**	-	**117**	**170**
Ovaries (%)	-	-	-	0.064 ± 0.010	**0.072 ± 0.010***	**0.078 ± 0.007****
% of control	-	-	-	-	**113**	**122**
Pituitary (%)	0.002 ± 0.000	0.002 ± 0.000	0.002 ± 0.000	0.005 ± 0.001	0.006 ± 0.001	**0.006 ± 0.001****
% of control	-	100	100	-	120	**120**
Uterus (%)	-	-	-	0.351 ± 0.18	0.345 ± 0.14	0.506 ± 0.57
% of control	-	-	-	-	98	144
Gross pathology						
Number examined	24	24	24	24	24	**24**
Adrenal glands						
Enlarged	0	3	**12****	1	6	**24****
Discolouration	0	**8****	**17****	0	7**	**24****
Histopathology						
Adrenal glands						
Vacuolation, cortical						
Number examined	24	24	24	24	24	24
total	1	8	17	0	10	24

	Sex and dietary dose (ppm)					
	Males			Females		
Parameter	**0**	**500**	**15000**	**0**	**500**	**15000**
minimal	1	6	8	0	8	3
slight	0	2	5	0	2	6
moderate	0	0	3	0	0	13
severe	0	0	1	0	0	2
Hypertrophy						
Number examined	24	24	24	24	24	24
total	0	0	0	0	6	22
minimal	0	0	0	0	4	8
slight	0	0	0	0	2	6
moderate	0	0	0	0	0	8
Oil red O staining						
Number examined	10	10	10	10	10	10
total	0	8	10	0	6	10
minimal	0	5	1	0	2	0
slight	0	2	4	0	3	3
moderate	0	1	5	0	1	7
Filipin staining						
Number examined	10	9	10	10	10	7
total	0	6	9	1	8	7
minimal	0	3	3	1	4	1
slight	0	3	6	0	4	6
Ovaries						
Number examined	-	-	-	16	16	16
Vacuolation of interstitial cells						
total	-	-	-	0	4	16
minimal	-	-	-	0	2	0
slight	-	-	-	0	2	10
moderate	-	-	-	0	0	6
Oil red O staining						
total	-	-	-	10	10	10
minimal	-	-	-	5	1	0
slight	-	-	-	5	4	0
moderate	-	-	-	0	5	4
severe	-	-	-	0	0	6
Filipin staining						
total	-	-	-	10	10	8
slight	-	-	-	10	10	8

Ctrl control group; *N*: Number of animals examined or stained; SD: Standard deviation; Source: Lourens, 2017.

Statistically significant at: * $p < 0.05$; ** $p < 0.01$, using tests as follows:
Body weight: Dunnett's test(two-sided)
Organ weight: Kruskal–Wallis and Wilcoxon tests (two-sided)
Hormone data: Wilcoxon rank test, minimal, slight; slight, moderate; moderate, severe; marked

Enzyme and cholesterol studies

Study 1

A GLP in vitro study investigated the inhibitory properties of broflanilide and its metabolite DM-8007 towards CYP11A1, the key enzyme that converts cholesterol to pregnenolone in the first step of steroidogenesis. The detection of possible CYP11A1 inhibition was performed using the substrate/metabolite combination [^{14}C]cholesterol/[^{14}C]pregnenolone and appropriate metabolic conditions for incubation and analysis of mitochondrial lysosome fractions from the male rat adrenal gland. Final protein concentration was 0.5 mg protein/mL and incubation time 60 minutes. Quantitative analysis of the substrate and metabolite, [^{14}C]cholesterol) and [^{14}C]pregnenolone, was performed using a liquid chromatography (LC) method with photodiode array and radioactivity detection (LC-PDA-RAD). The experiment consisted of five replicate incubations in the presence or absence of a positive control inhibitor (ketoconazole) at concentrations ranging from 0.01 to 30 µM (in acetonitrile), or broflanilide and DM-8007 at a concentration of 30 µM (in a DMSO : acetonitrile mixture ratio 2 : 3 by volume).

The doses of ketoconazole inhibited CYP11A1 in a dose-related manner, with a highest inhibition of 99% at the highest dose of 30 µM, confirming this positive control inhibitor of CYP11A1 activity. Neither broflanilide nor DM-8007 at a concentration of 30 µM induced inhibition of CYP11A1 activity in the mitochondrial-lysosomal fraction from male rat adrenal glands (Bachelor, 2020a).

Study 2

A GLP-compliant in vitro study investigated the inhibitory properties of broflanilide and its metabolite DM-8007 towards neutral cholesterase enzyme (nCEase), the rate limiting enzyme converting cholesterol esters (derived mainly from stored sources or derived from high-density lipoproteins) to cholesterol in rats. The nCEase activity was determined using the substrate/metabolite combination [^{3}H]cholesteryl oleate/[^{3}H]cholesterol. Following method development, a validation phase and a pilot experiment, the main experiment was conducted using cytosolic fractions of rat ovary and female rat adrenal glands, incubated at 37°C for 15 minutes with broflanilide or DM-8007 at concentrations ranging from 0.03 to 100 µM (five replicates) in a DMSO : acetonitrile mixture ratio 2 : 3 by volume, or in the presence or absence of the positive control inhibitor simvastatin at concentrations ranging from 0.005 to 200 µM (five replicates). The recovered of radioactivity was determined by LSC and samples were analyzed by LC-PDA-RAD.

The average recoveries of radioactivity in the cytosol fractions of female rat adrenal glands were 91%, 79%, 78% and 87% for the vehicle, simvastatin (positive control), broflanilide and DM-8007, respectively, with an overall recovery of 83%, which was considered acceptable. Inhibitory activity of simvastatin on nCEase (by 57.9% and 55.5%) was observed at concentrations of 50 and 200 µM respectively, with no inhibition observed at doses of 10 µM or less. The half-maximal inhibitory concentration (IC_{50}) for simvastatin was 71 µM, confirming its ability to inhibit nCEase activity in cytosolic fractions from female rat adrenal glands. Inhibition of nCEase activity by broflanilide was observed at 3, 10 and 30 µM, by 10%, 35.3% and 47.2% respectively. No inhibition due to broflanilide was observed at doses of 1 µM or less. Metabolite DM-8007 inhibition of nCEase was observed at 3, 10, 30 and 100 µM, by 9.9%, 28.2%, 42.3% and 47.2% respectively. No inhibition due to DM-8007 was observed at doses of 1 µM or less. The IC_{50} values for broflanilide and DM-8007 were 24 µM and 73 µM respectively.

The average recovery of radioactivity in the cytosol fractions of female rat ovary was 75%, 74%, 68% and 67% for the vehicle, simvastatin (positive control), broflanilide and DM-8007, respectively, with an overall recovery of 69%. No clear explanation for the lower recoveries obtained in the main experiment using the cytosolic fractions from rat ovary could be found. However, the lower recovery had no effect on positive control (simvastatin) inhibition results which were comparable to the inhibition results observed during the positive control inhibition pilot experiments. The IC_{50} values for broflanilide and DM-8007 for cytosolic fractions of female rat ovary were not determined because a clear dose-dependent response was not observed under these study conditions.

It was concluded that broflanilide and DM-8007 inhibited nCEase in the cytosolic fraction of female rat adrenal glands. Comparing of the IC_{50} values for broflanilide and DM-8007 with the IC_{50} for the positive control (simvastatin), broflanilide was shown to possess greater inhibitory potency than the control and metabolite DM-8007 equal potency to the positive control. No dose-related inhibition of nCEase was noted in cytosolic fractions from female rat ovary (Bachelor, 2020b).

Study 3

A GLP-compliant in vitro study investigated the inhibitory properties of broflanilide and DM-8007 towards the acid cholinesterase enzyme (aCEase) the rate limiting enzyme converting cholesterol-esters (derived mainly from low-density lipoproteins and de novo synthesis) to cholesterol in non-rodents. The aCEase activity was determined using the substrate/metabolite combination [^{3}H]cholesteryl oleate/[^{3}H]cholesterol. Following method development, a validation phase and a pilot experiment, the main experiment was conducted using mitochondrial-lysosomal fractions of rat testes incubated at 37°C for 60 minutes with either broflanilide or DM-8007 (in triplicate) at concentrations from 0.03 to 100 µM (in a DMSO : acetonitrile mixture, ratio 2 : 3 by volume), or in the presence or absence of the positive control inhibitor simvastatin (in triplicate) at concentrations from 0.005 to 200 µM. The recovered of radioactivity was determined by LSC and samples were analyzed by LC-PDA-RAD.

The average recoveries of radioactivity in samples were 87%, 88%, 83% and 83% for the vehicle, simvastatin (positive control), broflanilide and DM-8007, respectively. Inhibition of nCEase activity by simvastatin was observed at concentrations of 50 µM (by 27.4%) and 200 µM (by 61.6%). No inhibition by simvastatin was observed at doses of 2 µM or less. The IC_{50} of simvastatin (positive control) was 144 µM. Neither broflanilide nor its metabolite DM-8007 induced significant inhibition of aCEase activity in the mitochondrial lysosomal fractions from rat testes at concentrations range from 0.03 to 100 µM. Among all doses tested, inhibition of aCEase activity was not greater than 10% and 22% in samples treated with broflanilide and DM-8007 respectively.

Under the experimental conditions, it was concluded that broflanilide and its metabolite DM-8007 did not inhibit aCEase activity in rat testis mitochondrial lysosomes (Bachelor, 2020c).

Study 4

An investigative (not GLP-compliant) study was conducted to elucidate the histopathology findings from the study by Lourens, (2017) regarding ovary and adrenal tissues from Wistar (Crl:WI(Han) rats administered broflanilide for 90 days at dose levels of 0, 500 and 15 000 ppm (equal to 0, 32 and 972 mg/kg bw per day in males, 0, 36 and 1127 mg/kg bw per day in females). Adrenal (left) and ovary (left) tissue samples from six animals per sex from the control, 500 and 15 000 ppm dose groups were analyzed to determine tissue concentrations of cholesterol and cholesterol-ester using an ultra-high performance liquid chromatography separation system followed by tandem mass spectrometric analysis (ultraHPLC-MS/MS) for identification of the target molecules. Besides cholesterol, 11 cholesterol esters (CEs) were analyzed and evaluated in adrenal gland tissue (CE16:0, CE18:1, CE18:2, CE20:2, CE20:3, CE20:4, CE20:5, CE22:4, CE22:5, CE22:6 and CE24:5) and in the ovary (CE16:0, CE18:1, CE18:2, CE20:1, CE20:3, CE20:4, CE22:4, CE22:5, CE22:6, CE24:4, CE24:5). Analyte content results were expressed as change in analyte content relative to control per mg of tissue (wet tissue), except for cholesterol, CE16:0 and CE18:1 for which tissue concentrations (µg compound per mg tissue) and organ content (mg per organ, not using wet weight-normalized values) were used.

Results showed statistically significant treatment-related and dose-dependent increases in cholesterol esters in adrenal and ovarian tissues from both male and female animals in the high-dose level (15 000 ppm) tissues. Changes in tissues at the low dose (500 ppm) were statistically significant for most analytes, confirming the treatment-related and dose-dependent nature of cholesterol ester content in the assessed organs.

An increase in cholesterol content, albeit lower compared to cholesterol ester levels, was observed in adrenal and ovarian tissue. This was of statistical significance at the high dose (15 000 ppm) on a per-gram-of-tissue basis and on a per-organ basis. At the low dose (500 ppm) statistical significance was observed in male adrenal tissue on a per-organ basis.

In conclusion, subchronic treatment of male and female Wistar rats for 90-days with broflanilide via the diet leads to a treatment-related and dose-dependent increase in cholesterol and the main cholesterol esters in adrenal and ovarian tissues (Herold, 2020).

Pharmacology studies

Investigative and preliminary studies

In a number of investigative, in vitro pharmacology assays (not GLP-compliant) broflanilide and its metabolite DM-8007 were assessed for their ability to interact (as agonists or antagonists) with dopamine receptor D1- and D2-like families, transporter, or to interfere with dopamine transport (uptake assay).

Agonist and antagonist effects on the D1-like receptor were evaluated in human recombinant Chinese hamster ovary cells (CHO) exposed broflanilide or DM-8007 at concentrations ranging from 7.81×10^{-7} M to 1.0×10^{-4} M for 30 minutes at room temperature, in the absence (agonist effect) or presence (antagonist effect) of dopamine stimulus. The D1 receptor functional response was evaluated measuring cyclic adenosine monophosphate (cAMP) by homogenous time-resolved fluorescence (HTRF) assay. The cellular agonist effect was calculated as a percentage of the control response to a known reference agonist; the cellular antagonist effect for each target was calculated as a percentage inhibition in the presence of a control reference agonist response.

Activity with respect to D2-like receptors was evaluated in human recombinant embryonic kidney cells (HEK-293 cells) expressing the short dopamine receptor for agonist activity and the long dopamine receptor for antagonist activity. Cells were exposed to concentrations of broflanilide or DM-8007 ranging from 7.81×10^{-7} M to 1.0×10^{-4} M for 60 minutes at room temperature in the presence of 1 nM of the natural receptor ligand [^{3}H]7-hydroxy-*N*,*N*-dipropyl-2-aminotetralin (7-OH-DPAT) for the agonist assay, or of 0.3 nM [^{3}H]*N*-methyl-spiperone for the antagonist assay, and 10 µM of butaclamol (a dopamine receptor antagonist). Scintillation counting was used as the detection method. Compound binding was calculated as a percentage inhibition of the binding of a radioactively-labelled, ligand that was specific for each target.

Effects on dopamine transporter were evaluated using human recombinant CHO cells exposed to concentrations of broflanilide or DM-8007 ranging from 7.81×10^{-7} M to 1.0×10^{-4} M for 120 minutes at 4°C in the presence of 4 nM [^{3}H]*N*-[1-(2-benzo[*b*]thiophenyl)piperidine (BTCP), which is a dopamine receptor antagonist. Scintillation counting was used as the detection method. Compound protein inhibition effect was calculated as a percentage inhibition of control protein activity.

Functional dopamine uptake was evaluated using synaptosomes from rat striatum exposed to concentrations of broflanilide or DM-8007 ranging from 7.81×10^{-7} M to 1.0×10^{-4} M for 15 minutes at 37° C and to 0.2 µCi/mL [^{3}H]dopamine. Scintillation counting was used as the detection method.

All the foregoing assays were conducted in duplicate for each concentration tested.

Under the experimental conditions used, broflanilide and DM-8007 showed little capability as orthosteric agonists or antagonists of the radioligand receptors tested (D1-receptor, D2-receptor, dopamine transporter). In the dopamine uptake assay, a small effect was observed (25% mean inhibition of dopamine uptake in synaptosomes) for broflanilide, but not for DM-8007 (Brock, 2018).

Two follow-up, bioactivity, in vitro assays (not GLP-compliant) were performed to further investigate the findings observed by Brock (2018).

One bioactivity assay was conducted to evaluate the potential agonist and/or antagonist activity of broflanilide and DM-8007 on peripheral D1-like receptors in the vas deferens of mouse testes. The method employed in this assay had been adapted from the scientific literature (Carratù et al., 1989) to maximize reliability and reproducibility. A longitudinal section of vas deferens was obtained from ICR-derived male mice weighing 30 ± 5 g. and placed under field-stimulated condition (70% of maximum voltage, 0.1 Hz, 220 ms for train and 1 ms pulse duration at 10 Hz) by means of 1 g tension and located in a 10 mL bath containing the selective D2-like peripheral receptor antagonist domperidone at 1 µM, in Krebs solution at pH 7.4 and 32°C. According to the methodology, the criteria for classifying a substance as a possible agonist or antagonist of peripheral dopamine D1-like receptors is its ability to induce a relaxant response (isometrically recorded) or an inhibition of the relaxant response by 50% or more relative to the selective peripheral D1-like receptor agonist control (1 µM SKF38393) within 20 minutes of exposure.

The second bioactivity assay was conducted to evaluate the potential agonist and/or antagonist activity of broflanilide and DM-8007 on peripheral D2-like receptors in mouse distal colon circular smooth muscle. The methodology employed in this assay had been adapted from the scientific literature (Auteri et al., 2017) to maximize reliability and reproducibility. Distal colon circular smooth muscle was removed and placed under 0.5 g tension in a 10 mL bath containing Krebs solution at pH 7.4 and 37°C. The carbachol-precontracted tissues were contracted with the 1 µM isoproterenol then exposed to a submaximal concentration of the reference agonist bromocriptine (10 µM) to verify responsiveness and to obtain a control relaxation. According to the methodology the criteria for classifying a substance as a possible agonist or antagonist of peripheral dopamine D2-like receptors is its ability to induce a relaxant response (isometrically recorded) or an inhibition of the relaxant response by 50% or more relative to the selective peripheral D2-like receptor agonist control (10 µM bromocriptine) within 30 minutes of exposure.

In both experiments broflanilide and its metabolite DM-8007 were assayed in 0.10% DMSO as a vehicle at concentrations of 1 µM and 10 µM.

In the D1 tissue bioassay, neither of the tested compounds showed agonist or antagonist activity.

In the D2-like tissue bioassay, broflanilide showed dose-dependent dopamine antagonistic activity. Likewise, DM-8007 showed dopamine antagonistic activity as well as a slight agonistic response that did not quite reach the criteria for a positive response (50% or greater) (Chen , 2019).

Additional pharmacology studies were conducted to obtain information about the potential onset and characterization of acute poisoning by broflanilide, and the medical treatment in the event of such an intoxication.

In a preliminary pharmacology study that was neither GLP- nor guideline-compliant, a single oral administration of broflanilide (purity 98.67%) was given by gavage to three Wistar rats and three ICR mice at dose levels of 0, 500, 1000 or 2000 mg/kg bw, in a constant dose volume of 20 mL/kg bw of 1% aqueous methylcellulose. All animals were observed for morbidity, mortality and general health condition for seven days post administration. Body weight was routinely recorded during the course of the study.

There were no treatment-related effects in either rats or mice up to 2000 mg/kg bw, the highest dose tested (Motomura, 2014).

Main study 1

In a first main pharmacology study broflanilide (purity 98.67%) suspended in a 1% aqueous solution of methylcellulose was administered once by gavage to three ICR mice/sex/group and five Wistar rats of each sex per group at dose levels of 0, 500, 1000 or 2000 mg/kg bw for both mice and rats. The experimental design was constructed in four parts:

- Part A; examination of clinical signs (multidimensional observation; Irwin method) in mice, performed before administration, and 1 hour, 4 hours, 1, 2, and 3 days after administration.
- Part B; examination of clinical signs in rats (multidimensional observation; FOB method) performed before administration, and 1 hour, 4 hours, 1, 2, and 3 days after administration.
- Part C; examination of respiratory pattern and measurement of respiratory rates in male rats performed before administration, and 1 hour, 4 hours, and 1 day after administration
- Part D: examination of cardiovascular effects in male rats (Part D) by means of measurement of blood pressure (mean systolic pressure) and heart rate (pulse), performed before administration, and 1 hour, 4 hours, and 1 day after administration.

All animals were observed for morbidity and mortality at least twice a day up to three days after administration, and their body weights recorded before administration and at termination on day 3.

The were no mortalities during the course of the study, nor treatment-related body weight changes in mice. One male mouse at 2000 mg/kg bw showed a marginal decrease in reactivity. In another male at the high dose tested reactivity was marginally reduced and irritability/aggressiveness marginally increased. These observations appeared one hour after administration and resolved within one day following administration.

In rats there were no mortalities during the course of the study and no treatment-related effects were noted on body weigh change in either sex nor in multidimensional clinical observations. Examination of the respiratory and cardiovascular system did not reveal treatment-related effects.

The NOAEL for acute effects in mice was 1000 mg/kg bw, based on a marginal, transient decrease in reactivity and increased irritability in males at 2000 mg/kg bw.

The NOAEL for acute toxicity in rats was 2000 mg/kg bw, the highest dose tested (Motomura, 2015).

Main study 2

In a second pharmacology study, broflanilide (purity 98.7%) was assessed for its effects on the central nervous system (CNS), renal, urinary and digestive system in rats, effects on body temperature, respiratory and cardiovascular system in dogs, and the autonomic nervous system in Guinea pig ileum.

For all experiments conducted in rats the dosing regime consisted of a single gavage dose of broflanilide at dose levels of 0, 125, 500 or 2000 mg/kg bw, dissolved in 1% aqueous methyl cellulose. Except for motor activity assessments in which five male Crlj:WI rats per dose group were used, all other experiments employed 10 male Crlj:WI rats per dose group. Effects on the CNS were investigated in five experiments.

Two experiments investigated the potential antagonistic or synergistic effects of broflanilide on induced convulsions animals. For the antagonistic and synergistic effects, convulsions were induced by an intraperitoneal administration of 80 or 44 mg/kg bw of pentylenetetrazole, respectively, one hour after administration of the vehicle or test substance. The presence or absence of clonic convulsions, tonic extensor convulsions and death were observed for up to 30 minutes after administration of pentylenetetrazole.

A third experiment investigated effects on the pentobarbital-induced sleeping time in animals. Animals received an intravenous dose of pentobarbital at 30 mg/kg bw one hour after administration of vehicle or test substance, and sleeping time (time from supine posture to a spontaneous return to abdominal position) was measured.

A fourth experiment evaluated spontaneous motor activity from immediately after administration to the end of measurement (four hours after administration).

The fifth experiment evaluated effects on pain threshold using Randall–Sellitto's method at one, two and four hours after the administration of the vehicle or test substance.

A sixth experiment investigated the effects on the renal and urinary systems. Urine volume, specific gravity, osmotic pressure, and concentrations of sodium ions (Na^+), potassium (K^+) and chloride (Cl^-) ions were measured in samples collected for six hours after administration.

Potential effects on the digestive system (small intestine transit) were assessed in a seventh experiment in which rats were orally administered 5% (w/v) of activated charcoal suspension one hour after administration of the vehicle or the test substance. Transit and inhibition rates were measured in isolated small intestines 20 minutes after administration of the charcoal suspension.

The investigation of potential broflanilide effects on the respiratory and cardiovascular systems and body temperature was conducted in four male beagle dogs, orally (gelatine capsules) administered broflanilide as a single dose at levels of 0, 100, 300 or 1000 mg/kg bw. Each dog was given single doses of the vehicle (0) followed by a single dose of broflanilide at increasing doses of 100, 300 or 1000 mg/kg bw with an interval of six days or more between each administration. Evaluated respiratory parameters were: respiratory rate, tidal volume and minute volume. Evaluated cardiovascular parameters were: systolic, diastolic and mean blood pressure, heart rate, and electrocardiogram. Data on body temperature, respiratory and cardiovascular parameters were acquired using a telemetry transmitter from two hours or more before administration until 24 hours or more after administration.

As part of the examination of effects on the autonomic nervous system, the effects of broflanilide was assessed on the contraction of the isolated Guinea pig ileum induced by agonists (acetylcholine, histamine, barium chloride and serotonin). Ileum preparations were first precontracted with acetylcholine, histamine, barium chloride or serotonin. Next vehicle or broflanilide (in 1% DMSO)

at 0, 6×10^{-5}, 6×10^{-4} and 6×10^{-3} mg/mL were applied to five preparations per group, to each pre-contracted preparation. Finally the agonists were applied in the presence of the vehicle or broflanilide at its range of concentrations. Contractile force was measured at each step and the ratio of contractile forces produced by the contractile agent before and after application of the vehicle or broflanilide was calculated (Imaizumi, 2019).

Table 28. Summary of results from the multipart pharmacology study of Imaizumi, 2019

End-point/test item	Animal [group size]	Route	Dose (mg/kg bw)			Results
			Broflanilide dose level	Maximum no effect	Effective dose	
Pentylenetetrazole-induced convulsions (antagonistic effects)	Male rats [10]	Oral, gavage	125 500 2000	2000	-	No effects
Pentylenetetrazole-induced convulsions (synergistic effects)	Male rats [10]	Oral, gavage	125 500 2000	500	2000	Decrease in the number of animals with tonic extensor convulsions or death induced by administration of pentylenetetrazole
Pentobarbital induced sleeping time	Male rats [10]	Oral, gavage	125 500 2000	2000	-	No effects
Spontaneous locomotor activity	Male rats [5]	Oral, gavage	125 500 2000	2000	-	No effects
Pain threshold	Male rats [10]	Oral, gavage	125 500 2000	2000	-	No effects
Urine volume and urinary excretion of electrolytes	Male rats [10]	Oral, gavage	125 500 2000	500	2000	Significant increase in the urinary excretion of K^+
Small intestine transit	Male rats [10]	Oral, gavage	125 500 2000	2000	-	No effects
Body temperature	Male dogs [4]	Oral, gelatin capsule	100 300 1000	1000	-	No effects
Respiratory and cardiovascular systems [a]	Male dogs [4]	Oral, gelatin capsule	100 300 1000	1000	-	No effects
Isolated ileum (acetylcholine- and barium chloride-induced contraction)	Male Guinea pigs [5]	Applied to magnus tube	6×10^{-5} 6×10^{-4} 6×10^{-3} mg/mL	6×10^{-4} mg/mL	6×10^{-3} mg/mL	Significant decrease of acetylcholine- and barium chloride-induced contraction

[a] Respiratory rate, tidal volume, minute volume, blood pressure, heart rate, ECG; Source: Imaizumi, 2019

2.7 Studies on metabolites

(a) Metabolite DM-8007 (3-benzamido-*N*-[2-bromo-4-(perfluoropropan-2-yl)-6-(trifluoromethyl)phenyl]-2-fluorobenzamide

DM-8007 is an animal as well as plant metabolite of broflanilide; its structure is shown below.

Figure 11. Chemical structures of DM-8007

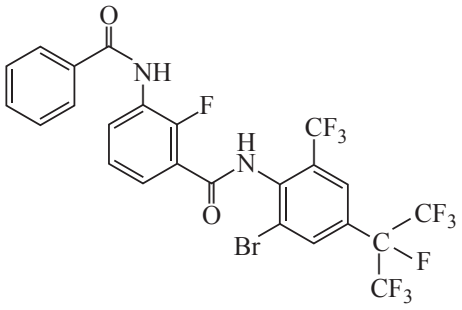


Table 29. Summary of results from toxicity studies with metabolite DM-8007

Study	Purity	Result	Reference
Acute oral (rat)	98.8%	LD_{50} > 2000 mg/kg bw	Park, 2015
Ames test	98.8%	Negative, with and without metabolic (S9) activation	Kim, 2015a
14-day dietary toxicity study (rat); 0, 5000, 15 000 ppm, equal to: (M) 0, 495, 1527 mg/kg bw per day, (F) 0, 511, 1594 mg/kg bw per day	99.6%	NOAEL = 15 000 ppm equal to 1527 mg/kg bw per day, the highest dose tested	Buesen et al., 2016b
28-day oral toxicity study (rat); 0, 300, 1000, 3 000 ppm, equal to: (M) 0, 33, 85, 278 mg/kg bw per day, (F) 0, 31, 94, 378 mg/kg bw per day	99.7%	NOAEL = 3 000 ppm equal to 278 mg/kg bw per day, the highest dose tested	Buesen et al., 2017d
90-day dietary toxicity study (rat); 0, 125, 500, and 3 000 ppm, equal to: (M) 0, 7.8, 31, 190 mg/kg bw per day, (F) 0, 8.8, 36, 215 mg/kg bw per day	99.7%	NOAEL = 3 000 ppm equal to 190 mg/kg bw per day, the highest dose tested	Buesen et al., 2017e

M: Males; F: Females;

In an acute oral toxicity study, five female fasted Wistar rats (RccHan®:WIST) were given a single oral gavage dose of DM-8007 (purity 98.84%) in 0.5% (w/v) aqueous methylcellulose at doses of 0, 175, 550 or 2000 mg/kg bw using the up-and-down procedure. Animals were then observed for 14 days.

There were no deaths and no clinical signs in animals at 175 or 550 mg/kg bw. At 2000 mg/kg bw on day 1 animals produced stools after dosing of a similar colour to the test compound; this colour had disappeared on day 2 after dosing. No test substance-related effects were evident in any animal on body weight data or necropsy findings.

The LD_{50} of metabolite DM-8007 was greater than 2000 mg/kg bw (Park, 2015).

In a reverse bacterial gene mutation assay, *Salmonella typhimurium* strains TA1535, TA1537, TA98, TA100 of and *Escherichia coli* strain WP2*uvr*A(pKM101) were exposed to DM 8007 (purity 98.8%) in DMSO at concentrations ranging from 2.44 to 5000 µg/plate in the presence and absence of an induced rat liver metabolic activation using the preincubation procedure. Two independent experiments were conducted and adequate positive controls employed. A dose range-finding study was conducted to establish the high dose for the main study.

Growth inhibition was evident at 78.1 µg/plate and above in TA98, at 1250 µg/plate and above in TA100 and at 313 µg/plate and above in TA1537 in the absence of metabolic activation. Precipitation was evident at 78.1 µg/plate and above in the absence and presence of metabolic activation. There were no relevant increases in revertant colonies induced by the compound.

It was concluded that metabolite DM-8007 was not mutagenic under these experimental conditions (Kim, 2015a).

In a 14-day oral range finding study that met GLP standards but was not guideline compliant, DM-8007 (purity 99.6%) was administered to groups of three male and three female Wistar (Crl:WI(HAN)) rats via the diet at concentrations of 0, 5000 and 15 000 ppm (equal to 0, 495 and 1527 mg/kg bw per day for males, 0, 511 and 1594 mg/kg bw per day for females). Food consumption and body weights were determined twice weekly. Animals were examined for signs of toxicity or mortality at least once a day. Detailed, clinical examinations in an open field were conducted prior to the start of exposure and weekly thereafter. At the end of the exposure period all animals were sacrificed and assessed for gross pathology.

No animal died during the study period. There were no adverse effects on clinical observations, body weight, food and water consumption or gross necropsy at any dose level.

Under the experimental conditions the NOAEL was 15 000 ppm (equal to 1517 mg/kg bw per day), the highest dose tested (Buesen et al., 2016b).

In a 28-day oral toxicity study, DM-8007 (purity 99.7%) was administered to groups of five male and five female Crl:WI(Han) rats via the diet at concentrations of 0, 300, 1000 or 3000 ppm (equal to 0, 33, 85 and 278 mg/kg bw per day for males, 0, 31, 94 and 378 mg/kg bw per day for females). The animals were examined for signs of toxicity or mortality at least once a day and subjected to weekly detailed clinical examinations. Food consumption and body weight were determined weekly. Haematology, clinical chemistry and urinalysis examinations were performed in week 4. At the end of the treatment period, the animals were sacrificed and necropsied, organ weights were determined and tissues preserved for examination. The limited number of tissues evaluated by light microscopy were: all gross lesions, adrenal glands, cervix, kidneys, liver, ovaries, spleen, thyroid glands, uterus and vagina).

No animals died during the study period. There were no remarkable clinical observations noted during the course of the study. No test substance-related changes in mean body weight or change in mean body weights were observed in either male or female animals. There were no changes in food consumption that were considered related to treatment.

No treatment related-effects were noted in haematology or clinical chemistry parameters.

In males at 3000 ppm, increases (not statistically significant) in absolute and relative weights of prostate (with seminal vesicles) by 14%–16% and 23%–26% respectively, were noted when compared to controls. In animals of this dose group absolute and relative weights of testes were lower than for controls, by14% in both cases. Differences in testis weights from controls were not statistically significant. In the absence of microscopic investigation of these organs, the toxicological relevance of their weight changes could not be disregarded. However it was noted that no treatment-related effects on these organs weights or microscopic findings were seen in the 90-day toxicity study of Buesen et al. (2017e) at the highest dose tested in that study of 190 mg/kg bw per day.

In microscopic examination one male from the high-dose group showed very minimally increased vacuolation in the cortical cells of the adrenal glands. This finding was considered to be within control range of other studies.

The NOAEL was 3000 ppm (equal to 278 mg/kg bw per day), the highest dose tested (Buesen et al., 2017d).

In a 90-day oral toxicity study, DM-8007 (purity 99.7%) was administered to groups of 10 male and 10 female of Crl:WI(Han) rats per dose group via the diet at concentrations of 0, 125, 500 or 3000 ppm (equal to 0, 7.8, 31 and 190 mg/kg bw per day for males, 0, 8.8, 36 and 215 mg/kg bw per day for females). Food consumption and body weight were determined weekly. The animals were examined for signs of toxicity or mortality at least once a day. Detailed clinical examinations were conducted in an open field before start of the administration period and then weekly. Ophthalmological examinations were performed before the start of, and at the end of the administration period. Towards the end of

the administration period FOB and MA assessments were conducted, as well as clinic chemistry and haematological examinations and urinalysis. On completion of the administration period, all animals were sacrificed and assessed for gross pathology. Adrenal glands, brain, epididymides, heart, kidneys, liver, ovaries, spleen, testes, thymus, thyroid and uterus were weighed. Histopathological examinations were conducted on a standard set of tissues from the control and high-dose animals and from all gross lesions.

There were no deaths during the course of the study, nor were any treatment-related clinical signs of toxicity noted. No test substance-related changes inn water or food consumption, mean body weight or mean body weight change were observed in either males or females from any dose group.

No treatment-related effects were noted during FOB assessments in either sex. No treatment-related effects were noted regarding the motor activity measurements. A statistically significant decrease in counts was reported for a single interval (55 minutes) in males at all dose levels, with no dose–response relationship. In females, a statistically significant increase in the counts (10 minute interval) was reported at 500 ppm. The overall MA scores of treated males and females was similar to controls.

No treatment-related effects were noted as a result of ophthalmological examinations.

In females, haematology analysis revealed lowered Hb at 125, 500 and 3000 ppm (by 5%, 4% and 2% compared to controls respectively) which was statistically significant but not dose-related. Haematocrit was lower (statistically significant) in females at 125, 500 and 3000 ppm (by 4%, 3% and 3% respectively compared to control. These changes were statistically significant at the low and high doses. Given the absence of alteration in any other correlated parameters, these marginal changes were considered of no toxicological significance.

Clinical chemistry analysis revealed a statistically significant decrease in potassium in males at 500 and 3000 ppm, by 3% and 5% respectively compared to control. In the absence of correlating effects in other physiopathological parameters this marginal change was not considered of toxicological relevance. The mean potassium values for these animals were within the laboratory HCD range.

Absolute and relative spleen weights were increased in females to a statistically significant extent at 125, 500 and 3000 ppm (by 12–13%, 11–14% and 9–11% respectively compared to controls) with a relatively flat dose response. In the absence of treatment-related histopathological effects these changes were considered of no toxicological significance. It was noted that the mean control values were lower than HCD range and that values for all treatment groups were within the HCD range.

There were no histopathology alterations ascribable to the administration of the test item.

The NOAEL was 3000 ppm (equal to 190 mg/kg bw per day), the highest dose tested (Buesen et al., 2017e).

(b) Metabolite DC-DM-8007 (3-amino-N-[2-bromo-4-(perfluoropropan-2-yl)-6-(trifluoromethyl)phenyl]-2-fluorobenzamide)

Metabolite DC-DM-8007 is a poultry and ruminant metabolite of broflanilide, the structure of which is shown below.

Figure 12. Chemical structures of DC-DM-8007

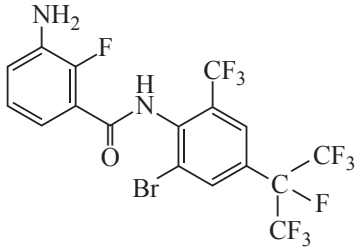


Table 30. Summary of results of toxicity studies with the metabolite DC-DM-8007

Study	Purity	Result	Reference
Acute oral (rat)	98.6%	LD_{50} > 2000 mg/kg bw	Lee, 2016
Ames test	98.6%	Negative, both with and without metabolic activation (S9)	Kim, 2015b
Preliminary 14-day dietary toxicity study (rat); 0, 5000, 15 000 ppm equal to: (M) 0, 196, 699 mg/kg bw per day (F) 0, 274, 654 mg/kg bw per day	97.2%	NOAEL not determined LOAEL = 5000 ppm (equal to 196 mg/kg bw per day)	Buesen, 2016
14-day dietary toxicity study (rat); 0, 100, 300, 1500 ppm equal to: (M) 0, 10, 30, 150 mg/kg bw per day (F) 0, 11, 32, 152 mg/kg bw per day	97.2%	NOAEL not determined LOAEL = 100 ppm (equal to 10 mg/kg bw per day)	Buesen, et al., 2016c
28-day oral toxicity study (rat); 0, 100, 500, 1500 ppm equal to: (M) 0, 11, 47, 156 mg/kg bw per day (F) 0, 12, 44, 145 mg/kg bw per day	99.8%	NOAEL not determined LOAEL = 100 ppm (equal to 11 mg/kg bw per day)	Buesen et al., 2018
90-day dietary toxicity study (rat); 0, 30, 75, 750 ppm equal to: (M) 0, 1.9, 5.3, 54 mg/kg bw per day (F) 0, 2.2, 5.7, 56 mg/kg bw per day	99.8%	NOAEL = 30 ppm (equal to 2.2 mg/kg bw per day) LOAEL = 75 ppm (equal to 5.7 mg/kg bw per day)	Buesen et al., 2017f

M: Males; F: Females;

In an acute oral toxicity study, five female fasted (RccHan:WIST) rats were given a single oral dose by gavage of DC-DM-8007 (purity 98.58%) in 0.5% (w/v) aqueous methylcellulose solution at doses of 175, 550 or 2000 mg/kg bw using the up-and-down procedure. Animals were then observed for 14 days.

There were no deaths and no clinical signs in animals at 175 or 550 mg/kg bw. At 2000 mg/kg bw on day 1 animals produced stools after dosing of a similar colour to the test compound; this colour had disappeared after dosing on day 2. No test substance-related effects were evident in any animal on body weight data or necropsy findings.

The LD_{50} of metabolite DC-DM-8007 was greater than 2000 mg/kg bw (Lee, 2016).

In a reverse bacterial gene mutation assay, *Salmonella typhimurium* strains TA1535, TA1537, TA98, TA100 of and *Escherichia coli* strain WP2*uvr*A(pKM101) were exposed to DC-DM-8007 (purity 98.6%) in DMSO, at concentrations ranging from 39.1 to 5000 µg/plate in the presence and absence of an induced rat liver metabolic activation using the preincubation procedure. Two independent experiments were conducted and adequate positive controls employed. A dose range-finding study was conducted to establish the high dose for the main study.

At 5000 µg/plate growth inhibition was evident in TA98, TA100 and TA1537 in the presence of metabolic activation, at 1250 µg/plate and above in TA98, and at 78.1 µg/plate in TA100, Ta1535 and TA 1537 in the absence of metabolic activation. No relevant increases in revertant colonies were induced by the compound.

Metabolite DC-DM-8007 was not mutagenic under these experimental conditions (Kim, 2015b).

In a preliminary 14-day dose range-finding study that was neither guideline nor GLP-compliant, metabolite DC-DM-8007 (purity 97.2%) was administered to groups of three males and three female Wistar (Crl:WI(HAN)) rats via the diet at dose levels of 0, 5000 and 15 000 ppm (equal to 0 196 and 699 mg/kg bw per day for males, 0, 274 and 654 mg/kg bw per day for females). Due to toxicity (see below), administration continued for only for five days. Food and water consumption were determined on day 3. Body weights were determined on days 0, 3 and 5. Animals were examined for signs of

toxicity and mortality at least once a day. Due to severe clinical findings, treatment was stopped on day 5. On day 6, all animals were sacrificed and assessed for gross pathology.

In both test groups, severe clinical findings including poor general condition, piloerection and tremors, were observed. Food and water consumption were reduced to a statistically significant extent and body weight losses were observed in males and females. Pathological changes in the adrenal gland (grey–white discolouration of the cortex) and liver (enlargement) were observed in all treated animals. A black focus was observed in the glandular stomach of one male at 15 000 ppm.

No histopathology examination was conducted.

In this study a NOAEL could not be identified due to effects at 5000 ppm (equal to 196 mg/kg bw per day) (Buesen, 2016).

In a 14-day oral range-finding study that was neither guideline nor GLP-compliant, DC-DM-8007 (purity 97.2%) was administered to groups of five male and five female Wister (Crl:WI (Han)) rats via the diet at concentrations of 0, 100, 300 or 1500 ppm (equal to 0, 10, 30 and 150 mg/kg bw per day for males, 0, 11, 32 and 152 mg/kg bw per day for females). Food consumption was determined daily during the first week and on days 10 and 14. Drinking water consumption was determined on days 3, 7, 10 and 14 and body weights were determined on days 0, 3, 7, 10 and 14. Animals were examined for signs of toxicity at least once a day. After the administration period all animals were sacrificed and assessed for gross pathology. The adrenals, liver, kidneys and spleen of all animals was weighed.

No mortalities or clinical signs of toxicity were observed during the course of the study.

In males at 1500 ppm, body weight gain was significantly depressed on days 3 (by 30%), 7 (20%) and 10 (14%). There were no relevant or treatment-related effects on food or water consumption, or on body weight parameters of males or females.

Absolute and relative adrenal weights were increased in both sexes at all dose levels when compared with controls. In males, the increases in absolute and relative adrenal weights were by 13%–14%, 16%–20% and 41%–48% at 100, 300 and 1500 ppm respectively, achieving statistical significance at the highest dose. In females the increases in absolute and relative adrenal weights were by 10%–11%, 23%–21% and 29%–30% at 100, 300 and 1500 ppm respectively, achieving statistical significance at the mid and high dose in the case of absolute weight, and at all dose levels for relative weight.

Absolute and relative liver weights were increased (not statistically significant) by 17% and 22% respectively in males at 1500 ppm. In females, absolute and relative liver weight were increased by 6%–7%, 9%–6% and 35%–36% at 100, 300 and 1500 ppm respectively compared to controls, achieving statistical significance for the absolute and relative weights at the high dose and for the relative weight also at the low and mid doses.

Absolute and relative spleen weights were significantly increased (by about 140%) compared to controls in males and females at 1500 ppm. Increases in spleen weight by 16%–12% compared to controls were also observed in females at 300 ppm. Spleen weights at the mid dose were not significantly different from control.

Gross pathology examination revealed an enlarged adrenal cortex in the majority of males and all females at 1500 ppm, an enlarged liver in one male and a liver focus in one female at 1500 ppm, as well as discoloured spleens in females at 300 ppm and similar discolouration in all males and females at 1500 ppm. Enlarged spleens were observed in all males and females at 1500 ppm.

No histopathology examination was conducted.

In this study no NOAEL could be identified due to increased adrenal weights at 100 ppm (equal to 10 mg/kg bw per day) (Buesen et al., 2016c).

In a 28-day oral toxicity study, DC-DM-8007 (purity 99.83%) was administered to groups of five male and five female Wistar (Crl:WI(HAN)) rats via the diet at concentrations of 0, 100, 500 or 1500 ppm (equal to 0, 11, 47 and 156 mg/kg bw per day for males, 0, 12, 44 and 145 mg/kg bw per day for females. Food consumption and body weight were determined weekly. Animals were examined for signs of toxicity or mortality at least once a day. Detailed clinical examinations in an open field

were conducted prior to the administration period and weekly thereafter. Clinical chemistry and haematological examinations as well as urinalysis were carried out towards the end of the administration period. After the administration period, all animals were sacrificed, and organs weighed (adrenal glands, brain, epididymides, heart, liver, kidney, ovaries, prostate, seminal vesicles and coagulating gland, spleen, testes, thymus, thyroid gland, uterus and cervix). Gross and histopathological examinations were conducted on a complete set of organs and tissues.

No mortalities or clinical signs of toxicity were observed during the course of the study.

There were no treatment-related effects on water or food consumption or on body weight parameters in either sexes.

Haematology examination showed for males at 500 and 1500 ppm, a statistically significant reduction in RBC counts, by 10% and 12% respectively compared to controls, and of Hb by 5% and 6% compared to controls. Red blood cells and Hb were also significantly decreased (by 13% and 9% compared to controls) in females at 1500 ppm. Mean corpuscular volume was significantly increased in males and females at 500 and 1500 ppm by approximately 6% and 9%, respectively. A statistically significant increase in absolute reticulocyte counts by 113% and 188% compared to controls was reported for males at 500 and 1500ppm respectively, and by 73%, 95% and 321% compared to controls for females at 100, 500 and 1500 ppm respectively.

Clinical biochemistry assessment revealed a statistically significant increase in urea and triglycerides (by 26% and 97% respectively) in females at 1500 ppm. It was noted that mean triglyceride values for females at 1500 ppm were in the range of the HCD and that the mean value of the concurrent controls was on the low end of the historical control range.

With respect to organ weights, absolute adrenal weight was significantly increased by 45% compared to control in males at 1500 ppm and by 29% and 30% in females at 500 and 1500 ppm respectively. Relative adrenal weights were significantly increased compared to controls, by 46% in males at 1500 ppm and by 31% and 26% in females at 500 and 1500 ppm respectively. In the absence of correlating histopathology findings for the adrenals of females, the observed adrenal weight changes were not considered of toxicological importance.

Absolute liver weights were increased by 20% and 33% compared to controls statistically significant) in males and females at 1500 ppm respectively. Relative liver weights were increased by 9% and 21% compared to controls in males at 500 and 1500 ppm respectively, achieving statistical significance at 500 ppm only. In females, relative liver weight was significantly increased compared to controls by 12% and 27% at 500 and 1500 ppm respectively. Liver weight changes observed at 500 ppm were not considered toxicologically relevant as there were no treatment-related histopathology findings.

Absolute and relative spleen weights were greater by about 28% and 99% compared to controls (statistically significant) in males at 500 and 1500 ppm respectively. In females, absolute and relative spleen weights were significantly greater by about 25%, 22% and 100% compared to controls at 100, 500 and 1500 ppm respectively. Increases in ovarian absolute and relative weights of 21–24% and 29%–35% compared to controls were seen at 500 and 1500 ppm respectively, achieving statistical significance for absolute weight at the high dose. In the absence of any related effects observed at histopathological examination, the increased ovary weights observed at 500 ppm were not considered toxicologically relevant.

No treatment-related effects were observed in examinations of gross pathology.

Histopathology examinations revealed two males in the 1500 ppm group with slight adrenal cortical diffuse hypertrophy and these were the ones with the highest individual adrenal weights. A single male in the 1500 ppm group showed minimally increased vacuolation in cortical adrenal cells, but these were considered to fall within concurrent control levels seen in other studies. In females, no histopathological observations correlated with these organ weight increases. Minimal centrilobular hypertrophy was observed in the livers of males and a single female at 1500 ppm. Minimal vacuolation of the interstitial glands of the ovaries was noted in females of the 1500 ppm group. Extramedullary haematopoiesis (mainly reticulocytes) was noted in males and females of all dose groups, with a dose-dependent increase in incidence and severity. Ectatic vessels/sinusoids were noted in the 1500 ppm groups.

Table 31. Key findings from the DC-DM-8007 28-day toxicity study in rats

	Sex and dietary dose (ppm)				
	Males				
Parameters	0	100	500	1500	HCD (mean; range)
Haematology; mean ± standard deviation					
Red blood cell count (10^{12}/L)	7.93 ± 0.28	7.62 ± 0.26	**7.14 ± 0.19****	**7.00 ± 0.52***	
percentage of control	-	96	**90**	**88**	-
Haemoglobin, Hb (mmol/L)	8.8 ± 0.1	8.7 ± 0.2	**8.4 ± 0.2****	**8.3 ± 0.3**	-
percentage of control	-	99	**95**	**94**	-
Haematocrit, Ht (L/L)	0.417 ± 0.008	0.405 ± 0.008	0.397 ± 0.008	0.401 ± 0.014	-
percentage of control	-	97	95	96	-
Mean corpuscular volume, MCV (fL)	52.6 ± 1.4	53.2 ± 1.9	**55.7 ± 2.2***	**57.4 ± 3.2***	-
percentage of control	-	101	**106**	**109**	-
Reticulocytes (10^9/L)	132.8 ± 21.6	160.1 ± 31.9	**283.3 ± 27.2****	**383.0 ± 120.4****	-
percentage of control	-	121	**213**	**288**	-
Clinical biochemistry; mean ± standard deviation					
Urea (mmol/L)	5.91 ± 0.56	**7.16 ± 0.26****	5.76 ± 0.49	6.18 ± 0.43	
percentage of control	-	**121**	97	105	-
Triglycerides (mmol/L)	0.88 ± 0.15	0.60 ± 0.17	0.75 ± 0.19	0.86 ± 0.37	-
percentage of control	-	68	85	98	-
Absolute organ weights; mean ± standard deviation					
Adrenal glands (mg)	56.6 ± 7.436	59.2 ± 3.421	60.8 ± 9.834	**82.0 ± 16.956****	-
percentage of control	-	105	107	**145**	-
Liver (g)	6.658 ± 0.455	6.92 ± 0.595	7.232 ± 0.486	**8.014 ± 0.659***	7.084; 6.244–10.131
percentage of control	-	104	109	**120**	-
Ovaries (mg)	-	-	-	-	-
percentage of control	-	-	-	-	-
Spleen (g)	0.48 ± 0.044	0.506 ± 0.089	**0.608 ± 0.041****	**0.95 ± 0.146****	-
percentage of control	-	105	**127**	**198**	-
Relative organ weights; mean ± standard deviation					
Adrenal glands (%)	0.022 ± 0.003	0.023 ± 0.001	0.024 ± 0.004	**0.032 ± 0.008***	-
percentage of control	-	104	108	**146**	-
Liver (%)	2.588 ± 0.055	2.661 ***± 0.046***	**2.828 ± 0.133****	3.155 ± 0.346	2.639; 2.472–2.911
percentage of control	-	103	**109**	121	-

Parameters	Sex and dietary dose (ppm)				
	Males				
	0	**100**	**500**	**1500**	**HCD (mean; range)**
Spleen (%)	0.186 ± 0.009	0.193 ± 0.018	**0.238 ± 0.02****	**0.372 ± 0.062****	-
percentage of control	-	104	**128**	**199**	-
Histopathology					
Number examined	5	5	5	5	-
Adrenal glands					
Cortical diffuse hypertrophy	0	0	0	2	-
minimal	0	0	0	0	-
slight	0	0	0	2	-
Cortical vacuolation	0	0	0	1	-
minimal	0	0	0	1	-
Liver					
Hypertrophy, centrilobular	0	0	0	4	-
minimal	0	0	0	4	-
Spleen					
Haematopoiesis, extramedullary (mainly reticulocytes)	0	2	5	5	-
minimal	0	2	1	0	-
slight	0	0	1	0	-
moderate	0	0	3	5	-
severe	0	0	0	0	-
Ectasis, vessels/sinusoids present	0	0	0	5	-
	Females				
	0 ppm	**100 ppm**	**500 ppm**	**1500 ppm**	**HCD (mean; range)**
Haematology; mean ± standard deviation					
Red blood cell count (10^{12}/L)	7.19 ± 0.11	7.03 ± 0.33	6.85 ± 0.31	**6.27 ± 0.24****	-
percentage of control	-	98	95	**87**	-
Haemoglobin, Hb (mmol/L)	8.2 ± 0.2	8.2 ± 0.3	8.1 ± 0.1	**7.5 ± 0.3****	-
percentage of control	-	100	99	**91**	-
Haematocrit, Ht (L/L)	0.377 ± 0.007	0.379 ± 0.015	0.377 ± 0.008	**0.354 ± 0.016***	-
percentage of control	-	101	100	**94**	-
Mean corpuscular volume, MCV (fL)	52.4 ± 0.6	54.0 ± 2.0	**55.0 ± 1.5 ***	**56.5 ± 2.2 ****	-
percentage of control	-	103	**105**	**108**	-
Reticulocytes (10^9/L)	114.8 ± 18.4	**199.1 ± 55.2****	**223.5 ± 28.6****	**483.2 ± 92.9****	153.2; 102.2–208.2
percentage of control	-	**173**	**195**	**421**	-

	Females				
	0 ppm	**100 ppm**	**500 ppm**	**1500 ppm**	**HCD (mean; range)**
Clinical biochemistry ; mean ± standard deviation					
Urea (mmol/L)	6.21 ± 0.45	6.96 ± 0.24	7.46 ± 1.07	**7.82** ± 0.74**	
percentage of control	-	112	119	**126**	-
Triglycerides (mmol/L)	0.30 ± 0.05	0.43 ± 0.15	0.35 ± 0.08	**0.59** ± 0.21**	0.44; 0.29–0.63
percentage of control	-	143	117	**197**	-
Absolute organ weights; mean ± standard deviation					
Adrenal glands (mg)	65.6 ± 9.45	69.2 ± 9.257	**84.4 ± 7.668***	**85.4 ± 9.29***	-
percentage of control	-	*105*	***129***	***130***	-
Liver (g)	4.4 ± 0.458	4.458 ± 0.604	4.858 ± 0.391	**5.844 ± 0.706****	4.617; 4.146–6.697
percentage of control	-	*101*	*110*	***133***	-
Ovaries (mg)	79.4 ± 13.221	79.8 ± 19.588	96.0 ± 23.313	**106.8 ± 13.18****	-
percentage of control	-	101	121	**135**	-
Spleen (g)	0.336 ± 0.073	0.42 ± 0.05	0.406 ± 0.065	**0.672 ± 0.082****	-
percentage of control	-	125	121	**200**	-
Relative organ weights; mean ± standard deviation					
Adrenal glands (%)	0.041 ± 0.004	0.042 ± 0.003	**0.053 ± 0.006****	**0.051 ± 0.007***	-
percentage of control	-	105	**131**	**126**	-
Liver (%)	2.728 ± 0.085	2.733 ± 0.136	**3.048 ± 0.147****	**3.473 ± 0.242****	2.702; 2.54–2.98
percentage of control	-	100	**112**	**127**	-
Spleen (%)	0.208 ± 0.037	**0.258 ± 0.021***	0.253 ± 0.024	**0.4 ± 0.031****	-
percentage of control	-	124	122	**192**	-
Ovaries (%)	0.049 ± 0.008	0.049 ± 0.008	0.061 ± 0.018	0.064 ± 0.007	
percentage of control		98	124	129	
Histopathology					
Number examined	5	5	5	5	-
Adrenal glands					
Cortical diffuse hypertrophy	0	0	0	0	-
minimal	0	0	0	0	-
slight	0	0	0	0	-
Cortical vacuolation	0	0	0	0	-
minimal	0	0	0	0	-
Liver					
Hypertrophy, centrilobular	0	0	0	1	-
minimal	-	-	-	1	-

	Females				
	0 ppm	**100 ppm**	**500 ppm**	**1500 ppm**	**HCD (mean; range)**
Ovaries					
Vacuolation of interstitial glands	0	0	0	4	-
minimal	0	0	0	4	-
Spleen					
Haematopoiesis, extramedullary (mainly reticulocytes)	0	3	3	5	-
minimal	0	1	2	0	-
slight	0	1	1	2	-
moderate	0	1	0	2	-
severe	0	0	0	1	-
Ectasis, vessels/sinusoids present	0	0	0	4	-

HCD: Historical control data; Source: Buesen et al., 2018

Statistically significant at: * $p < 0.05$; ** $p < 0.01$

No NOAEL could be identified due to increased extramedullary haematopoiesis in the spleen, and increased reticulocyte counts in both sexes, and increased spleen weights in females at the lowest dose tested of 100 ppm (equal to 11 mg/kg bw per day) (Buesen et al., 2018).

In a 90-day repeat-dose toxicity study, groups of 10 male and 10 female Crl:WI(Han) Wister rats were administered DC-DM-8007 (purity 99.83%) via the diet at concentrations of 0, 30, 75 or 750 ppm (equal to 0, 1.9, 5.3 and 54 mg/kg bw per day for males, 0, 2.2, 5.7 and 56 mg/kg bw per day for females). Food consumption and body weight were determined weekly. The animals were examined for signs of toxicity or mortality at least once a day. Detailed clinical examinations in an open field were conducted prior to the start of the administration period and weekly thereafter. Ophthalmological examinations were performed before the beginning and at the end of the administration period. In addition an FOB and MA assessments were carried out at the end of the administration period. Clinical chemistry and haematological examinations together with urinalyses were performed towards the end of the administration period. At the end of the administration period all animals were sacrificed and assessed for gross pathology. Adrenal glands, brain, epididymides, heart, kidneys, liver, ovaries, spleen, testes, thymus, thyroid gland and uterus were weighed .Histopathological examinations were conducted on a complete set of organs/tissues from control and high-dose animals, and gross lesions, adrenal glands, liver, ovaries and spleen examined in all groups.

No deaths or clinical signs of toxicity were observed during the course of the study. A slight increase in food consumption throughout the study period (by up to 20% compared with controls) was observed in males at 75 and 750 ppm. However, these increases were not always dose-related and did not attain statistical significance. Body weight and body weight gain were reduced compared with controls in males at 750 ppm throughout the study period, by about 5% and 10% respectively. However these weights were not statistically different from those of controls. Body weight and body weight gain were greater than for controls throughout the study period in females at 30 ppm, achieving statistical significance for body weight gain. These body weights effects were considered unrelated to treatment. No effects on water consumption were observed.

No treatment related-effects were observed in FOB examinations. Regarding the overall motor activity, no test substance-related deviations were noted for male or female animals treated at any dose level when compared to the control animals. Motor activity at single a interval (45 minutes) was significantly depressed in males at 30 ppm. However, this deviation was regarded as being unrelated to treatment.

No treatment-related findings were observed at ophthalmological examination.

Haematology examination revealed, compared to controls, a statistically significant decrease

in RBCs, Hb, Ht, MCHC hb concentration (by 12%, 9%, 4% and 5% respectively), and an increase in MCV (by 8%), all in males at 750 ppm. In females, a statistically significant reduction at 75 and 750 ppm in RBC counts (by 5% and 18% respectively), Hb (by 3% and 12%), Ht (by 3% and −8%) and increase in MCV (by 2% and 12%) compared to controls were observed. A statistically significant decrease in mean corpuscular haemoglobin concentration (by 4%) and increase in mean corpuscular haemoglobin content (by 7%) compared to controls were observed in females at 750 ppm. Significantly increased absolute reticulocyte counts were observed in males and females at 750 ppm (by 184% and 187%, respectively) and in females at 75 ppm (by 29%) when compared to controls.

Absolute and relative neutrophil counts were increased in males at 750 ppm, by 34% and 28% respectively compared to controls, achieving statistical significance for the absolute neutrophils only. In the absence of any treatment-related effects on WBCs and lymphocytes these changes were not considered toxicologically relevant. Absolute and relative monocyte counts were depressed in males at 750 ppm, by 22% and 25% respectively compared to controls, achieving statistical significance for relative counts only. Reduced absolute and relative monocyte counts were also observed in females at 75 and 750 ppm, in their case by about 15% and 20% respectively compared to controls. These differences from control values were not statistically significant. Absolute and relative eosinophil counts were depressed by 27% and 33% respectively when compared to control in males at 750 ppm, but did not achieve statistical significance. In females at 750 ppm absolute and relative eosinophil counts were reduced by 37% and 44% respectively compared to controls. Statistically significant decreases in relative eosinophil counts were also observed in females at 30 and 75 ppm, but with no apparent dose–response relationship. Due to the small size of changes seen in subpopulations of leukocytes they were considered of no toxicological relevance.

In females at 75 and 750 ppm urine volume was increased when compared to controls, whereas its specific gravity was lower by 2%. Specific gravity at 75 ppm and urine volume at 750 ppm were changed, but not to a statistically significant extent. These findings were not accompanied by any other change in kidney parameters and they were therefore regarded as treatment-related but not adverse.

With respect to organ weights, adrenal weights for the 750ppm dose groups increased (with statistical significance) by 21% and 29% (absolute), and by 29% and 34% (relative) compared to controls in males and females respectively. A statistically significant increase in absolute and relative heart weights (by 16% and 10% respectively compared to controls) was observed in females at 750 ppm. In males at 750 ppm, a significant increase in relative heart weight (by 6% compared to control) was observed. No difference in the absolute heart weight was noted for males at the high dose. In the absence of histopathological findings, effects on heart weight were considered of no toxicological relevance. A statistically significant, but not dose-related, increase in absolute liver weight (by 13% compared to controls) was observed in males at 30 and 750 ppm. A statistically significant increase in relative liver weight (by 20% compared to controls) was observed in males only at 750 ppm. Absolute and relative liver weights were significantly increased (by 32% and 26% respectively compared to controls) in females at 750 ppm. Absolute and relative ovary weights were also significantly increased at 750 ppm, by 31% and 25% respectively compared to controls. Statistically significant increases in absolute and relative spleen weights (by 46% and 54% respectively compared to controls), were observed in males at 750 ppm. In females the increases in absolute spleen weight were 12% and 105%, and in relative spleen weight 12% and 96% compared to controls, at 75 and 750 ppm respectively, achieving statistical significance at the high dose. An increase in absolute thyroid weight (by 18%), and in relative thyroid weight (by 16% and 25% respectively) compared to controls was observed in males at 75 and 750 ppm, achieving statistical significance at the high dose. There were no histopathology findings relating to the thyroid. A statistically significant decrease in absolute weight (by 7% compared to controls) was observed for epididymides and testes in males at 750 ppm; relative weight changes were unaffected in these organs. No histopathology findings were noted for the testes and epididymides.

Gross pathology examinations revealed one female at 750 ppm with an enlarged spleen which correlated to increased weights and histopathology for this animal.

Treatment-related histopathology findings were noted for the adrenal glands and ovaries in female animals, livers in males and spleens of both sexes.

Minimal microvesicular and macrovesicular vacuolation in the adrenal glands (predominantly in

zona fasciculata and zona glomerulosa) was observed in all females at 750 ppm. The histopathological findings correlated with the increased adrenal gland weights recorded for females at 750 ppm. No histopathological correlate was seen to the increased adrenal gland weights of males at 750 ppm.

An increased incidence of minimal centrilobular hepatocellular hypertrophy was observed in the livers of males at 750 ppm. The histopathological findings correlated with the increased liver weights recorded for males receiving 750 ppm. No histopathological correlate was seen to the increased liver weights of females treated at this same dose level.

Increased incidence of minimal to slight microvesicular vacuolation in interstitial glands was observed in the ovaries of almost all females at 750 ppm. The histopathological findings correlated with increased ovary weights in the high-dose females.

An increased incidence of moderate to marked extramedullary haematopoiesis (mainly reticulocytes) and ectasia of vessels (dilated sinusoids in the red pulp of the spleen) were observed in the spleen of males at 750 ppm. This histopathological finding correlated with the increased spleen weights and reticulocyte counts seen in males at 750 ppm. In females, an increased incidence and severity (from slight to marked) of extramedullary haematopoiesis was observed in the spleen of animals at 75 and 750 ppm, correlating with increased spleen weights and reticulocyte counts at the same dose levels. Two females at 30 ppm exhibited slight extramedullary haematopoiesis in the spleen, however, this did not correlate with other physiopathological parameters.

Table 32. Key findings for metabolite DC-DM-8007 in the 90-day toxicity study in rats.

	Sex and dietary dose (ppm)				
	Males				
Parameters	**0**	**30**	**75**	**750**	**HCD (mean; range)**
Haematology; mean ± standard deviation					
Red blood cell count (10^{12}/L)	8.49 ± 0.29	8.32 ± 0.27	8.39 ± 0.21	7.49 ± 0.22**	-
percentage of control	-	98	99	88	-
Haemoglobin, Hb (mmol/L)	9.0 ± 0.4	8.7 ± 0.3	8.8 ± 0.1	**8.2 ± 0.2****	-
percentage of control	-	97	98	**91**	-
Haematocrit, Ht (L/L)	0.431 ± 0.016	0.419 ± 0.014	0.429 ± 0.005	**0.412 ± 0.008****	-
percentage of control	-	97	99	**96**	-
Mean corpuscular volume, MCV (fL)	50.8 ± 1.6	50.4 ± 1.2	51.1 ± 1.4	**55.0 ± 1.5****	-
percentage of control	-	99	101	**108**	-
Mean corpuscular haemoglobin, MCH(fmol)	1.06 ± 0.05	1.05 ± 0.03	1.05 ± 0.03	1.09 ± 0.03	-
percentage of control	-	99	99	103	-
Mean corpuscular haemoglobin concentration, MCHC (mmol/L)	20.85 ± 0.53	20.77 ± 0.41	20.58 ± 0.23	**19.85 ± 0.29****	-
percentage of control	-	100	99	**95**	-
Reticulocytes (10^9/L)	136.7 ± 24.7	148.3 ± 23.8	136.9 ± 21.0	**387.7 ± 74.4****	-
percentage of control	-	108	100	**284**	-

Parameters	Sex and dietary dose (ppm)				
	Males				
	0	**30**	**75**	**750**	**HCD (mean; range)**
Absolute organ weights; mean ± standard deviation					
Terminal body weight (g)	367.95 ± 25.242	397.71 ± 32.894*	371.62 ± 32.217	347.25 ± 26.002	-
percentage of control	-	108	101	94	-
Adrenal glands (mg)	59.5 ± 4.170	61.1 ± 7.047	56.9 ± 5.486	**72.1 ± 5.685****	59.965; 54.100–66.700
percentage of control	-	103	96	**121**	
Liver (g)	8.024 ± 0.649	9.054 ± 0.955**	8.282 ± 1.084	**9.061 ± 0.688****	-
percentage of control	-	113	103	113	-
Spleen (g)	0.600 ± 0.067	0.595 ± 0.104	0.573 ± 0.063	**0.874 ± 0.119****	-
percentage of control		99	95	**146**	-
Relative organ weights; mean ± standard deviation					
Adrenal glands (%)	0.016 ± 0.001	0.015 ± 0.002	0.015 ± 0.001	**0.021 ± 0.002****	0.016; 0.014–0.018
percentage of control	-	95	95	**129**	
Liver (%)	2.181 ± 0.105	2.275 ± 0.121	2.227 ± 0.203	**2.611 ± 0.104****	-
percentage of control	-	104	102	**120**	-
Spleen (%)	0.163 ± 0.016	0.149 ± 0.021	0.155 ± 0.021	**0.252 ± 0.033****	-
percentage of control	-	92	95	**154**	-
Histopathology					
Number examined	10	10	10	10	-
Adrenal glands					
Vacuolation, increased	0	0	0	0	-
minimal	0	0	0	0	-
Liver					
Hypertrophy, centrilobular	0	0	0	6	-
minimal	0	0	0	6	-
Spleen					
Haematopoiesis, extramedullary	3	3	4	10	-
minimal	2	3	4	0	-
slight	1	0	0	2	-
moderate	0	0	0	7	-
marked	0	0	0	1	-
Ectasis, vessel, present	0	0	0	10	-

Parameters	Females				
	0	30	75	750	HCD (mean; range)
Haematology; mean ± standard deviation					
Red blood cell count (10^{12}/L)	7.72 ± 0.15	7.61 ± 0.29	**7.30 ± 0.30****	**6.36 ± 0.27****	7.85; 7.51–8.26
percentage of control	-	99	**95**	**82**	
Haemoglobin, Hb (mmol/L)	8.6 ± 0.2	8.5 ± 0.2	**8.3 ± 0.2****	**7.6 ± 0.3****	8.7; 8.2– 9.5
percentage of control	-	99	**97**	**88**	
Haematocrit, Ht (L/L)	0.400 ± 0.010	0.399 ± 0.009	**0.387 ± 0.013***	**0.368 ± 0.009****	-
percentage of control	-	100	**97**	**92**	-
Mean corpuscular volume, MCV (fL)	51.9 ± 1.0	52.5 ± 1.5	**53.0 ± 1.0***	**57.9 ± 2.0****	-
percentage of control	-	101	**102**	**112**	-
Mean corpuscular haemoglobin, MCH(fmol)	1.11 ± 0.03	1.12 ± 0.03	1.14 ± 0.03	**1.19 ± 0.05****	-
percentage of control	-	101	103	**107**	-
Mean corpuscular haemoglobin concentration, MCHC (mmol/L)	21.44 ± 0.27	21.34 ± 0.23	21.46 ± 0.40	**20.48 ± 0.32****	-
percentage of control	-	100	100	**96**	-
Reticulocytes (10^9/L)	146.6 ± 39.4	147.7 ± 24.6	**189.7 ± 26.7***	**421.4 ± 53.5****	2.1; 1.7–2.9
percentage of control	-	101	**129**	**287**	
Absolute organ weights; mean ± standard deviation					
Terminal body weight (g)	230.13 ± 16.312	237.04 ± 13.921	230.96 ± 22.164	241.43 ± 11.834	-
percentage of control	-	103	100	105	-
Adrenal glands (mg)	84.6 ± 9.924	83.2 ± 13.959	85.7 ± 8.757	**113.7 ± 9.922****	-
percentage of control	-	98	101	**134**	-
Liver (g)	5.474 ± 0.490	5.368 ± 0.291	5.443 ± 0.514	**7.235 ± 0.584****	5.139; 4.722–5.474
percentage of control	-	98	99	**132**	
Ovaries (mg)	102.2 ± 12.97	108.6 ± 13.43	110.8 ± 16.12	**133.9 ± 15.62****	-
percentage of control	-	106	108	**131**	-
Spleen (g)	0.410 ± 0.054	0.435 ± 0.056	0.458 ± 0.058	**0.841 ± 0.163****	-
percentage of control	-	106	112	**205**	-

Parameters	Females				
	0	**30**	**75**	**750**	**HCD (mean; range)**
Relative organ weights; mean ± standard deviation					
Adrenal glands (%)	0.037 ± 0.003	0.035 ± 0.006	0.037 ± 0.004	**0.047 ± 0.005****	-
percentage of control	-	96	100	**129**	-
Liver (%)	2.380 ± 0.158	2.266 ± 0.082*	2.361 ± 0.132	**2.997 ± 0.198****	2.364; 2.246–2.498
percentage of control	-	*95*	*99*	**126**	
Ovaries (%)	0.044 ± 0.005	0.046 ± 0.005	0.048 ± 0.004	**0.056 ± 0.007****	-
percentage of control	-	103	108	**125**	-
Spleen (%)	0.178 ± 0.018	0.183 ± 0.020	0.200 ± 0.029	**0.349 ± 0.067****	-
percentage of control	-	103	112	**196**	-
Histopathology					
Number examined	10	10	10	10	-
Adrenal glands					
Vacuolation, increased	0	0	0	10	-
Minimal	0	0	0	10	-
Liver					
Hypertrophy, centrilobular	0	0	0	0	-
Minimal	0	0	0	0	-
Ovaries					
Vacuolation, interstitial glands	0	0	0	9	-
minimal	0	0	0	7	-
slight	0	0	0	2	-
Spleen					
Haematopoiesis, extramedullary	3	5	6	10	-
minimal	3	3	4	0	-
slight	0	2	2	5	-
moderate	0	0	0	4	-
marked	0	0	0	1	-
Ectasis, vessel, present	0	0	0	10	-

HCD: Historical control data; Source: Buesen et al., 2017f

Statistically significant at: * $p < 0.05$; ** $p < 0.01$

The NOAEL was 30 ppm (equal to 2.2 mg/kg bw per day) based on decreases in red blood cell count, haematocrit and haemoglobin, increases in reticulocyte count and extramedullary haematopoiesis in the spleen, and increased spleen weights in females, at 75 ppm (equal to 5.7 mg/kg bw per day) (Buesen et al., 2017f).

(c) Metabolite S(PFP-OH)-8007 (*N*-[2-bromo-4-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)-6-(trifluoromethyl)phenyl]-2-fluoro-3-(*N*-methylbenzamido)benzamide)

S(PFP-OH)-8007 is a plant metabolite, the structure of which is shown below.

Figure 13. Chemical structure of S(PFP-OH)-8007

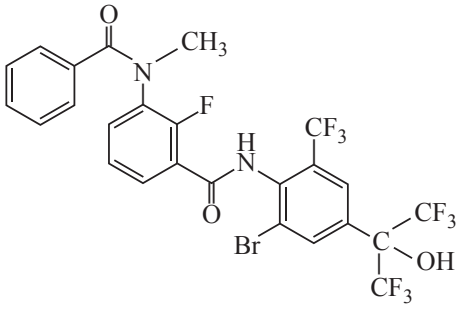


Table 33. Summary of studies with metabolite S(PFP-OH)-8007

Study	Purity	Result	Reference
Acute oral (rat)	99.1%	LD_{50} > 2000 mg/kg bw	Na, 2016
Ames test	99.1%	Negative, both with and without metabolic (S9) activation	Kim, 2015c
14-day dietary toxicity study (rat); 0, 5000, 15 000 ppm, equal to: (M) 0, 472 and 1577 mg/kg bw per day (F) 0, 503 and 1450 mg/kg bw per day	99.9%	NOAEL not determined LOAEL = 5000 ppm (equal to 472 mg/kg bw per day)	Buesen et al., 2016d
28-day dietary toxicity study (rat); 0, 300, 1000, 3000 ppm, equal to: (M) 0, 26, 81 and 243 mg/kg bw per day (F) 0, 30, 109 and 265 mg/kg bw per day	99.9%	NOAEL = 300 ppm (equal to 26 mg/kg bw per day) LOAEL = 1000 ppm (equal to 81 mg/kg bw per day)	Buesen et al., 2017g
90-day dietary toxicity study (rat); 0, 125, 500, 3000 ppm, equal to: (M) 0, 8.3, 32 and 193 mg/kg bw per day (F) 0, 9.1, 37 and 219 mg/kg bw per day	99.9	NOAEL not determined LOAEL = 125 ppm (equal to 8.3 mg/kg bw per day)	Buesen et al., 2017h

In an acute oral toxicity study, five female fasted Wistar (RccHan®: WIST) rats were given a single oral dose by gavage of S(PFP-OH)-8007 (purity 99.06%) in 0.5% (w/v) aqueous methylcellulose solution at doses of 175, 550 or 2000 mg/kg bw using the up-and-down procedure. Animals were then observed for 14 days.

There were no deaths and no clinical signs of toxicity were observed in animals at 175 or 550 mg/kg bw. There were no deaths and no clinical signs in animals at 175 or 550 mg/kg bw. At 2000 mg/kg bw on day 1 animals produced stools after dosing of a similar colour to the test compound; this colour had disappeared after dosing on day 2. Test substance-related effects on body weight parameters or necropsy findings were not apparent in any animal.

The LD_{50} of metabolite S(PFP-OH)-8007 was greater than 2000 mg/kg bw (Na, 2016).

In a reverse bacterial gene mutation assay, *Salmonella typhimurium* strains TA1535, TA1537, TA98, TA100 of and *Escherichia coli* strain WP2*uvr*A(pKM101) were exposed to S(PFP-OH)-8007 (purity 99.06%) in DMSO at concentrations ranging from 9.77 to 5000 µg/plate in the presence and absence of an induced rat liver metabolic activation using the preincubation procedure. Two independent experiments were conducted and adequate positive controls employed. A dose range-finding study was conducted to establish the high dose for the main study.

Growth inhibition due to the test substance was evident at concentrations of 156 µg/plate and above in strain TA100 in the absence of metabolic activation. Inhibition was not evident at any dose level in strains TA98, TA100, TA1535, TA1537 and WP2uvrA(pKM101) in the presence of metabolic

activation and in TA98, TA1535, TA1537 and WP2uvrA(pKM101) strains in the absence of metabolic activation. The deposition of the test substance was evident at more than 625 and 313 μg/plate in the presence and absence of metabolic activation respectively. However, it did not interfere with the colony counting. There were no relevant increases of revertant colonies induced by the compound.

Metabolite S(PFP-OH)-8007 was not mutagenic under these experimental conditions (Kim, 2015c).

In a 14-day dose-range finding study that was GLP, but not guideline-compliant, groups of three male and three female Crl:WI(Han) Wistar rats were administered S(PFP-OH)-8007 (purity 99.96%) via the diet, at concentrations of 0, 5000 or 15 000 ppm (equal to 0, 472 and 1577 mg/kg bw per day for males, 0, 503 and 1450 mg/kg bw per day for females). The rats were examined for mortality or signs of toxicity at least once a day. Food consumption and body weight were determined twice weekly and detailed open field clinical examinations were conducted before the start of dosing, and then weekly. After the dosing period all animals were sacrificed and assessed for gross pathology.

No animal died during the study period and no clinical signs of toxicity were observed during the examination period. No test substance-related effects on body weight parameters were observed on any animal. Gross pathology examination revealed discoloured adrenal glands in all animals at 5000 and 15 000 ppm. Additionally, enlarged adrenal glands were observed in all females 15 000 ppm.

Under the experimental conditions of the study no NOAEL was identified due to gross pathology findings in the adrenal glands of both sexes at the lowest dose tested of 5000 ppm (equal to 472 mg/kg bw per day) (Buesen et al., 2016d).

In a 28-day oral toxicity study, S(PFP-OH)-8007 (purity 99.96%) was administered to groups of five male and five female (Crl:WI(Han)) rats via the diet at concentrations of 0, 300, 1000 or 3000 ppm (equal to 0, 26, 81 and 243 mg/kg bw per day for males, 0, 30, 109 and 265 mg/kg bw per day for females). Food consumption and body weight were determined weekly. Animals were examined for signs of toxicity or mortality at least once a day. Detailed clinical examinations in an open field were conducted prior to the administration period and weekly thereafter. Clinical biochemistry and haematological examinations and urinalysis were performed towards the end of the administration period. After the administration period, all animals were sacrificed, and organs weighed (adrenal glands, brain, epididymides, heart, kidneys, liver, ovaries, prostate, seminal vesicles with coagulating gland, spleen, testes, thymus, thyroid gland and uterus with cervix). The following organs in all groups were subject to gross and histopathological examination: adrenal glands, cervix, kidneys, liver, ovaries, spleen, thyroid glands, uterus and vagina.

No animal died during the study period and no clinical signs of toxicity were observed during the examination period. There was no treatment-related effects on food or water consumption, nor on body weight.

Haematology examination revealed a statistically significant increase in absolute reticulocyte counts in males at all dose levels. As these changes lacked any dose–response relationship they were considered unrelated to treatment, but in any case, no alteration in other correlating physiopathological parameters was observed. There were no treatment-related effects on clinical biochemistry or urinalyses parameters.

An increase in adrenal gland weights was observed in males at 1000 and 3000 ppm, by approximately 20% and 30% respectively compared to control. Increases in adrenal glands weight were also noted in females at all dose levels, (by 12% to 25% compared to controls) but with no dose–response relationship. The adrenal weight changes were not statistically significant but could correlate with histopathological findings seen in males and females at 1000 and 3000 ppm.

Kidney weights were lower compared to controls in males and females at 3000 ppm, by approximately 10% in males and 10%–13% in females, achieving statistical significance for the relative weights in both sexes. In the absence of histopathological correlates, kidneys weight changes were considered of no toxicological relevance.

Statistically significant increases in absolute and relative thymus weights were observed in males at 300 and 3000 ppm, by 12%–14% and by 11% compared to controls, respectively. An increase in absolute and relative thymus weights was also observed in females at 1000 and 3000 ppm, by 22%–25% and by 15%–19% respectively. These thymus changes were considered of doubtful relation to treatment.

Gross pathology examination revealed a light brown discolouration of the adrenal glands in males and females at 1000 and 3000 ppm, and in females at 300 ppm.

Treatment-related histopathology findings were observed in the adrenal glands of males and females and in the ovaries. Minimal microvesicular vacuolation in all zones of the adrenal cortical cells was noted in males and females at 1000 and 3000 ppm along with minimal microvesicular vacuolation of the interstitial glands of the ovaries at 1000 and 3000 ppm.

Table 34. Key findings for S(PFP-OH)-8007 from the 28-day toxicity study in rats

	Sex and dietary dose (ppm)				
	Males				
Parameters	**0**	**300**	**1000**	**3000**	**HCD (mean; range)**
Haematology; mean ± standard deviation					
Reticulocytes (10^9/L)	128.5 ± 13.3	**153.2 ± 12.1***	**160.1 ± 13.0***	**146.9 ± 4.7***	150,6; 102.1–194.4
percentage of control	-	**119**	**125**	**114**	-
Absolute organ weights; mean ± standard deviation					
Adrenal glands (mg)	63.6 ± 8.503	69.6 ± 4.037	78.6 ± 10.691	81.8 ± 17.456	-
percentage of control	-	109	124	129	-
Relative organ weights; mean ± standard deviation					
Adrenal glands (%)	0.022 ± 0.003	0.024 ± 0.001	0.027 ± 0.003	0.028 ± 0.006	-
percentage of control	-	111	122	129	-
Gross pathology					
Number examined	5	5	5	5	-
Adrenal glands					
Gross lesions: discolouration	0	0	1	3	-
Histopathology					
Number examined	5	5	5	5	-
Adrenal glands					
Vacuolation, increased	0	0	2	5	-
minimal	0	0	2	5	-
Ovaries					
Vacuolation, interstitial glands	-	-	-	-	-
minimal	-	-	-	-	-
	Females				
Parameters	**0**	**300**	**1000**	**3000**	**HCD (mean; range)**
Haematology ± standard deviation					
Reticulocytes (10^9/L)	132.9 ± 18.9	160.7 ± 43.7	171.5 ± 38.1	141.6 ± 22.0	-
percentage of control	-	121	129	107	-
Absolute organ weights; mean ± standard deviation					
Adrenal glands (mg)	75.0 ± 7.583	94.0 ± 17.19	94.8 ± 11.345	87.2 ± 14.822	-
percentage of control	–	125	126	116	-

Parameters	Females				
	0	300	1000	3000	HCD (mean; range)
Relative organ weights; mean ± standard deviation					
Adrenal glands (%)	0.041 ± 0.004	0.05 ± 0.008	0.05 ± 0.005	0.046 ± 0.005	-
percentage of control	–	121	123	112	-
Gross pathology					
Number examined	5	5	5	5	-
Adrenal glands					
Gross lesions: discolouration	0	3	4	5	-
Histopathology					
Number examined	5	5	5	5	-
Adrenal glands					
Vacuolation, increased	0	0	1	2	-
minimal	0	0	1	2	-
Ovaries					
Vacuolation, interstitial glands	0	0	2	3	-
minimal	0	0	2	3	-

HCD: Historical control data; Statistically significant at: * $p < 0.05$; ** $p < 0.01$; Source: Buesen et al., 2017g

The NOAEL in this study was 300 ppm (equal to 26 mg/kg bw per day) based on increased adrenal weights and increased vacuolation in the adrenals of both sexes and increased vacuolation in interstitial glands of the ovary at 1000 ppm (equal to 81 mg/kg bw per day) (Buesen et al., 2017g).

In a 90-day repeat-dose toxicity study, groups of 10 male and 10 female Crl:WI(Han) Wistar rats were administered S(PFP-OH)-8007 (purity 99.96%) via the diet at concentrations of 0, 125, 500 or 3000 ppm (equivalent to 0, 8.3, 32, 193 mg/kg bw per day for males, 0. 9.1, 37 and 219 mg/kg bw per day for females). Food consumption and body weights were determined weekly. The animals were examined for signs of toxicity and mortality at least once a day. Moreover, detailed clinical examinations in an open field were conducted prior to the start of the administration period and weekly thereafter. Ophthalmological examinations were performed before the beginning of, and at the end of the administration period. A FOB as well as measurement of MA were carried out towards the end of the administration period. Clinical biochemistry and haematological examinations as well as urinalysis were performed towards the end of the administration period. After the administration period all animals were sacrificed and assessed for gross pathology. Adrenal glands, brain, epididymides, heart, kidneys, liver, ovaries, spleen, testes, thymus, thyroid glands and uterus were weighed. Histopathological examinations were conducted on a complete set of organs/tissues in the case of all animals in the control and high-dose groups, whilst adrenal glands, ovaries and spleen from all groups were examined.

There were no compound-related clinical signs of toxicity, nor effects on mortality, body weight parameters or food consumption.

During FOB and MA assessments the mean number of rearings of males at 3000 ppm was significantly lower. However, this change was assessed to be incidental since no other findings occurred during the performance of the FOB. Slightly increased MA was noted in males at 3000 ppm at intervals 20, 25 and 30 minutes. The overall MA of these animals was slightly higher than that of controls. These differences from controls were not statistically significant and in the absence of other neurobehavioural findings were considered not to be toxicologically relevant.

There were no treatment-related findings from ophthalmological examinations nor differences in haematological and urinalysis parameters.

Clinical biochemistry revealed a statistically significant and dose-related decrease in ALP

in females at 500 and 3000 ppm, by 16% and 24% respectively compared to controls. Statistically significant decreases in glucose, by 11% and 14% compared to controls, were observed in females at 125 and 3000 ppm respectively. It was noted that both these values were within the laboratory historical control range. In the absence of treatment-related effects on correlating physiopathological parameters these changes in biochemistry were considered of no toxicological relevance.

Absolute and relative adrenal weights were significantly increased in males and females at 125, 500 and 3000 ppm. Absolute weights were greater than controls by 23%, 24% and 48%, for males, and 17%, 18% and 24% for females, respectively. Corresponding relative adrenal weights were greater by 22%, 27% and 51% for males, and 15%, 15% and 19% for females. Differences in relative adrenal weight were statistically significant in males at all doses and in females at the high dose. Absolute and relative ovary weights were also significantly increased at 125, 500 and 3000 ppm. When compared to controls the absolute weight increases were by 12%, 18% and 15% and the for relative weights this was by 11%, 15% and 12%, respectively.

There were no treatment-related effects on gross pathology. Histopathology examination revealed treatment-related effects in the adrenal glands of males and females and in the ovaries. Adrenal glands showed an increased incidence and severity (from minimal to severe) of microvesicular and macrovesicular vacuolation, predominantly in the zona fasciculata and glomerulosa, which was present in males and females at all dose levels. This histopathological finding correlated with the increased adrenal gland weights seen in all treated groups.

In the ovary, increased incidence and severity (from minimal to slight) of microvesicular vacuolation of interstitial glands was observed in females at all dose levels. This histopathological finding correlated with the increased ovary weights seen in all treated groups.

Table 35. Key findings for S(PFP-OH)-8007 from the 90-day toxicity study in rats

	Sex and dietary dose (ppm)							
	Males				**Females**			
Parameters	**0**	**125**	**500**	**3000**	**0**	**125**	**500**	**3000**
Body and organ weights; mean ± standard deviation								
Terminal body weight (g)	374.35 ± 33.79	375.22 ± 28.60	362.80 ± 30.54	370.74 ± 30.98	219.63 ± 14.36	222.25 ± 9.53	225.73 ± 9.80	228.12 ± 12.71
Adrenal glands	55.7	**68.4****	**68.8****	**82.7****	69.5	**81.3***	**82.2****	**86.2****
Absolute weight (g)	± 4.809	**± 13.39**	**± 7.16**	**± 14.16**	± 9.348	**± 10.75**	**± 7.97**	**± 10.67**
percentage of control	-	**123**	**124**	**148**	-	**117**	**118**	**124**
Relative weight (%)	0.015 ± 0.002	**0.018**** **± 0.003**	**0.019**** **± 0.002**	**0.023**** **± 0.005**	0.032 ± 0.005	0.037 ± 0.005	0.036 ± 0.004	**0.038*** **± 0.004**
percentage of control	-	**122**	**127**	**151**	-	115	115	**119**
Ovary	-	-	-	-	93.5	**104.6***	**110.5****	**107.9****
Absolute weight (g)					± 8.96	**± 10.20**	**± 12.47**	**± 12.20**
percentage of control	-	-	-	-	-	**112**	**118**	**115**
Relative weight (%)	-	-	-	-	0.043 ± 0.003	**0.047*** **± 0.005**	**0.049**** **± 0.005**	**0.047*** **± 0.006**
percentage of control	-	-	-	-	-	**111**	**115**	**112**
Histopathology								
Number examined	10	10	10	10	10	10	10	10
Adrenal cortex; vacuolation increased								
All degrees	0	9	9	9	0	7	7	9
minimal	0	6	2	1	0	6	4	5
slight	0	3	5	4	0	1	2	4
moderate	0	0	2	3	0	0	1	0
severe	0	0	0	1	0	0	0	0

	Sex and dietary dose (ppm)							
	Males				Females			
Parameters	**0**	**125**	**500**	**3000**	**0**	**125**	**500**	**3000**
Ovaries; vacuolation, interstitial glands								
All degrees	-	-	-	-	0	5	8	9
minimal	-	-	-	-	0	5	7	6
slight	-	-	-	-	0	0	1	3

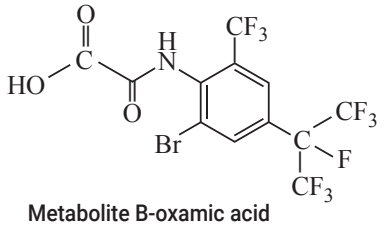
HCD: Historical control data; Statistically significant at: * $p < 0.05$; ** $p < 0.01$ Source: Buesen et al., 2017h

No NOAEL could be identified due to increased adrenal weights and increased vacuolation in the adrenals of both sexes, and increased ovary weight and vacuolation in interstitial glands of the ovary at the lowest dose tested of 125 ppm (equal to 8.3 mg/kg bw per day) (Buesen et al., 2017h).

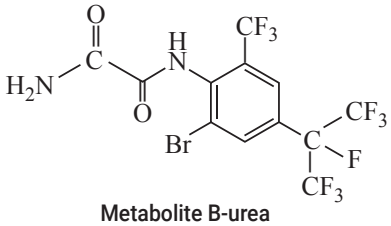
(d) Metabolites B-oxamic acid and B-urea

(*N*-[2-bromo-4-(perfluoropropan-2-yl)-6-(trifluoromethyl)phenyl]oxamic acid and
(*N*-[2-bromo-4-(perfluoropropan-2-yl)-6-(trifluoromethyl)phenyl]urea, respectively

Figure 14. Chemical structures of B-oxamic acid and B-urea



Metabolite B-oxamic acid



Metabolite B-urea

The metabolites B-oxamic acid and B-urea were assessed for their genotoxic properties (Ames end-point) using the quantitative structure–activity relationship (QSAR) statistical models (Case Ultra) and a rule-based expert model (DEREK Nexus), with reference to the parent compound for which genotoxicity data was available. The results with the DEREK Nexus model did not identify any structural alert for genotoxicity (Ames) or any unclassified or misclassified features for the metabolites B-oxamic acid and B-urea and the parent compound.

Metabolites B-urea and B-oxamic acid were predicted to be negative and in domain for AMES mutagenicity in the commercial model GT1_BMUT, both without any alerts associated with mutagenicity. For broflanilide, despite the detection of a weak alert the relevancy of which is dependent on activated/inactivated features, the prediction showed a negative in domain outcome. In this case no further discussion about the parent compound alert was necessary since an experimental Ames assay had been conducted and its results were negative.

It was concluded that metabolites B-oxamic acid and B-urea are unlikely to possess a mutagenic potential (Urbisch & Sanches-da-Rocha, 2020).

3. Observations in humans

No information was provided on the health of workers involved in the manufacture or use of broflanilide. No information on accidental or intentional poisoning in humans was available.

4. Microbial aspects

The impact of broflanilide residues on the human intestinal microbiome was evaluated through a decision-tree approach, adopted by the sixty-sixth meeting of the JECFA Committee for food producing animal drugs, which complies with VICH GL36(R)2 (VICH, 2013), and can also be used for pesticides.

Since there was no information/data found in the sponsor submission in this regard, and no information was available in the public domain, it was not possible for the Committee to determine if there is a need for the calculation of a microbiological acceptable daily intake (mADI) for broflanilide. This conclusion also applies for the need to determine a microbiological acute reference dose (mARfD).

Comments

Biochemical aspects

In disposition studies, intact and cannulated rats were administered [^{14}C]broflanilide labelled at either the phenyl (B label) or benzamide ring (C label). Both cannulated and intact rats were administered single oral doses of 5 mg/kg body weight (B or C label), or of 500 mg/kg body weight (C label only). Intact rats only were repeat-dosed at 5 mg/kg body weight (bw) with B label for up to 14 days. The oral absorption, calculated in bile duct-cannulated rats as the sum of the percentages of dose recovered in bile, urine, liver and carcass, was 14–23% of the administered dose (AD) following the single low dose, and 2% of AD at the single high dose.

Following oral doses, radioactivity was widely distributed. The total radioactivity retained in tissues accounted for less than 2% of AD following a single low dose, 0.1% of AD following a single high dose, and 4% or less of AD after repeated doses. Irrespective of dose or label position, the highest concentrations were measured in fat (1.8–3.0% AD from repeated dosing). There was no notable sex difference in the distribution or concentration of radioactivity.

There were no significant sex differences in either the rates or routes of excretion. After single oral doses excretion was rapid with more than 92% (urine, faeces and cage wash) of the AD excreted at 48 hours. The major route of excretion was through the faeces, accounting in general for more than 90% of AD independent of dose level or exposure period. In bile duct-cannulated rats, excreted radioactivity (bile, urine, faeces and cage wash) was greater than 85% of AD at 48 hours following a single low (B and C) or high dose (C label). There were no major differences in the patterns of excretion between sexes.

In toxicokinetic studies designed to assess absorption or bioavailability saturation effects after a single oral exposure, rats were administered [*B-ring*-^{14}C]broflanilide or [*C-ring*-^{14}C]broflanilide at doses ranging from 5 to 500 mg/kg bw. In general there were no significant differences in the toxicokinetic profile between males and females. Peak concentration (C_{max}) for plasma and area under the concentration–time curve (AUC) values broadly increased with increasing dose between 20 and 100 mg/kg bw, while at doses above 100 mg/kg bw the increases were not dose-proportional: values increased approximately three-fold against an expected five-fold increase. In general, the time to reach C_{max} (T_{max}) and the terminal half-life ($t_{½}$) were longer in animals given B-labelled broflanilide. Following consecutive daily oral low doses the C_{max} and $AUC_{(0–t)}$ values were approximately three-fold higher than those after a single low dose, with no substantial sex differences. The T_{max} values after repeated dosing were comparable to those after a single low dose.

After single or repeated low doses, unchanged broflanilide was the major radioactive component in faecal extracts, accounting for 52–77% of AD in intact or bile duct-cannulated rats, and for 88–94% of AD after a single high dose to intact or cannulated rats. In faecal samples from single or repeated low-dose rats, six metabolites were identified. Among these, metabolite S(PFPOH)8007), (M8) and DM-8007 (M11) accounted for a maximum of ca 2% and 5% of AD, respectively. Other metabolites accounted for a maximum of 4% of AD each. In the urine the major metabolite was hippuric acid which accounted for 6–11% of AD (C label) at the low dose in intact or bile duct-cannulated rats, and 0.7–0.8% at the high dose in intact rats. In bile samples, seven metabolites were identified, accounting for a maximum of 3% of AD each. The most abundant components in plasma, liver, kidney and fat were metabolite DM-8007 which accounted for 8–58% of total radioactivity (low dose and high dose), metabolite DC-DM-8007 which accounted for up to 17% of total radioactivity, and metabolite S(PFP-OH)8007 which accounted for up to 13% of total radioactivity (low dose).

In an in vitro study with human, rat and mouse hepatocytes, metabolism of broflanilide was extensive. Biodegradation in mice and rats was slightly faster than in humans. No human-specific metabolites of broflanilide were identified. In an identical study performed with metabolite DM-8007, the concentration of metabolite DM-8007 remained largely unchanged in human hepatocytes, while its biodegradation was apparent with mouse hepatocytes. No human-specific metabolites of DM-8007 were identified (Rabe, 2020a, b; Stroud, 2017; Townley 2017a, b, c; Wenker, 2012).

Toxicological data

The acute oral median lethal dose (LD_{50}) in rats was greater than 5000 mg/kg bw, the dermal LD_{50} was greater than 5000 mg/kg bw and the acute inhalation lethal median concentration (LC_{50}) was greater than 2.20 mg/L. Broflanilide was not irritating to the skin or eyes of rabbits. Broflanilide was not a skin sensitizer in mouse or Guinea pig (Dreher, 2012a–e; Remmele & Landsiedel, 2012; Ueda, 2014,2015, Wako, 2014)

In all species, the most common effects were increased adrenal gland weight and/or ovarian weight correlating with an increased incidence of vacuolation in the adrenal gland cortex and ovary interstitial cells, with rat being the most sensitive species.

In a 90-day toxicity study in mice in which broflanilide was administered at dietary concentrations of 0, 200, 1500 or 7000 ppm (equal to 0, 26.3, 199 and 955 mg/kg bw per day for males, 0, 32.3, 230 and 1148 mg/kg bw per day for females) the NOAEL was 1500 ppm (equal to 230 mg/kg bw per day) based on effects on the adrenal glands (increased adrenal weight and increased incidence of minimal cortical vacuolation) in females at 7000 ppm (equal to 1148 mg/kg bw per day) (Heal, 2016a).

In a 90-day toxicity study in rats in which broflanilide was administered at dietary concentrations of 0, 500, 1500, 5000 or 15 000 ppm (equal to 0, 35, 104, 345 and 1109 mg/kg bw per day for males, 0, 41, 126, 418 and 1239 mg/kg bw per day for females) no NOAEL could be identified due to effects on adrenal glands (increased weights correlated with increased vacuolation in both sexes and hypertrophy in females in the adrenal cortex), ovaries (increased incidence of vacuolation in interstitial cells) at 500 ppm (equal to 35 mg/kg bw per day), the lowest dose tested (Buesen, 2017a; Richter, Taraschewski & Wotske, 2015).

In a non-GLP and non-guideline complementary 90-day study in rats, aimed at specifically investigating broflanilide's target organ toxicity (in the adrenal gland and ovary), broflanilide was administered at dietary concentrations of 0 or 30 ppm (equal to 0 and 2.0 mg/kg bw per day for males, 0 and 2.2 mg/kg bw per day for females). The NOAEL was 30 ppm (equal to 2.0 mg/kg bw per day), the only dose tested (Buesen, 2017a).

In a 28-day and a 90-day toxicity study on dogs in which broflanilide was administered by capsule at dose levels of 0, 100, 300 or 1000 mg/kg bw per day, the NOAEL was 1000 mg/kg bw per day, the highest dose tested (Buesen et al., 2015c, 2016a).

In a one-year toxicity study on dogs in which broflanilide was administered by capsule at dose levels of 0, 100, 300 or 1000 mg/kg bw per day, no NOAEL could be identified due to reduced body weight in females, increased adrenal weight and adrenal gland enlargement in males, cortical cell hypertrophy in males, and vacuolation of the zona fasciculata of the adrenal gland in females at 100 mg/kg bw per day, the lowest dose tested (Keller, 2106).

In a carcinogenicity study in mice, broflanilide was administered at dietary concentrations of 0, 200, 1500 or 7000 ppm (equal to 0, 21, 157 and 745 mg/kg bw per day for males, 0, 22, 172 and 820 mg/kg bw per day for females). The NOAEL was 1500 ppm (equal to 157 mg/kg bw per day) based on pale and abnormal teeth in both sexes, increased absolute and relative adrenal weights, enlarged adrenal, marginally increased incidence of haematopoiesis, cortical vacuolation, corticomedullary vacuolation and inflammatory cell foci in the adrenal gland in females, slightly increased ovarian cysts, and a slight increase in cystic tubules in the kidneys of males, at 7000 ppm (equal to 745 mg/kg bw per day). No treatment-related increases in tumour incidence were observed in this study (Heal, 2016b).

In a two-year toxicity and carcinogenicity study in rats, broflanilide was administered at dietary concentrations of 0, 100, 300, 1500 or 15 000 ppm (equivalent to 0, 4.5, 14, 70 and 709 mg/kg bw per day for males, 0, 5.9, 19, 95 and 953 mg/kg bw per day for females). Satellite animals were given broflanilide at doses of 0, 30, 100, 300, 1500 or 15 000 ppm (equivalent to 0, 1.7, 5.7, 16, 84 and 822 mg/kg bw per day for males, 0, 2.1, 7.2, 20, 104 and 1128 mg/kg bw per day for females) for 12 months. No NOAEL could be identified for females due to an increased incidence of ovarian vacuolation at 100 ppm (equal to 5.9 mg/kg bw per day), the lowest dose tested, after 24 months of treatment. The NOAEL for carcinogenicity was 300 ppm (equal to 19 mg/kg bw per day) based on increased incidence of ovarian tumours of sex cord stromal origin and uterus adenocarcinomas at 1500 ppm (equal to 95 mg/kg bw

per day). A treatment-related increase in the incidence of Leydig cell adenomas was observed at the highest dose of 15 000 ppm (equal to 953 mg/kg bw per day) (Buesen et al., 2017b).

The Meeting concluded that broflanilide is not carcinogenic in mice but is carcinogenic in rats.

Broflanilide was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found (Fabian & Landsiedel, 2017; Kasamoto, 2019; Ruelker, 2020; Schultz & Landsiedel, 2013, 2014).

The Meeting concluded that broflanilide is unlikely to be genotoxic.

In view of the lack of genotoxicity, the absence of carcinogenicity in mice and the fact that tumours were only observed at moderate to high dose levels in rats, the Meeting concluded that broflanilide is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation reproductive toxicity study in rats, broflanilide was administered at dietary concentrations of 0, 30, 100, 300, 1500 or 15 000 ppm (equal to 0, 2.3, 7.5, 22.6, 112 and 1147 mg/kg bw/day for males, 0, 2.3, 7.5, 22.8, 111 and 1153 mg/kg bw per day for females). The NOAEL for parental toxicity was 30 ppm (equal to 2.3 mg/kg bw per day), based on increased adrenal weights and vacuolation of the adrenal cortex in males and females of the F0 and F1 generations, and ovary interstitial glands vacuolation in females of the F0 generation at 100 ppm (equal to 7.5 mg/kg bw per day). The NOAEL for reproductive toxicity was 15 000 ppm (equal to 1147 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 300 ppm (equal to 22.6 mg/kg bw per day) based on decreased body weights of pups at 1500 ppm (equal to 112 mg/kg bw per day) (Schneider et al., 2017).

In a prenatal developmental toxicity study in rats, broflanilide was administered by gavage at dose levels of 0, 100, 300 or 1000 mg/kg bw per day, from gestation day (GD) 6 until GD 19. The NOAEL for maternal toxicity was 1000 mg/kg bw per day, the highest dose tested. The NOAEL for embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Schneider, Grauert & van Ravenzwaay, 2016).

In a prenatal developmental toxicity study in rabbits, broflanilide was administered by gavage at dose levels of 0, 100, 300 or 1000 mg/kg bw per day, from GDs 6 to 28. The NOAEL for maternal toxicity was 1000 mg/kg bw per day, the highest dose tested. The NOAEL for embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Flick, Grauert & van Ravenzwaay, 2018).

The Meeting concluded that broflanilide is not teratogenic.

In an acute neurotoxicity study in rats, broflanilide was administered by gavage at dose levels of 0, 200, 600 or 2000 mg/kg bw. The NOAEL for both systemic toxicity and neurotoxicity was 2000 mg/kg bw, the highest dose tested (Buesen et al., 2017c). In a 90-day neurotoxicity study in rats, broflanilide was administered at dietary concentrations of 0, 1500, 5000 or 15 000 ppm (equal to 0, 99, 320 and 1041 mg/kg bw per day for males, 0, 118, 423 and 1137 mg/kg bw per day for females). The NOAEL for both systemic toxicity and neurotoxicity was 15 000 ppm (equal to 1041 mg/kg bw per day), the highest dose tested (Buesen et al., 2015a).

The Meeting concluded that broflanilide is not neurotoxic.

In a 28-day immunotoxicity study in rats, broflanilide was administered at dietary concentrations of 0, 1200, 4000 or 12 000 ppm (equal to 0, 104, 344, and 1020 mg/kg bw per day). The NOAEL for systemic toxicity and immunotoxicity was 12 000 ppm (equal to 1020 mg/kg bw per day), the highest dose tested (Buesen, 2015e).

The Meeting concluded that broflanilide is not immunotoxic.

In a number of in vitro assays broflanilide and its metabolite DM-8007 (M11) did not bind to rat or human estrogen receptors, rat androgen receptors, nor did they transactivate androgen/estrogen receptors (Rijk, 2019a, b, c, 2020; Verkaart, 2020a).

A number of in vitro and in vivo investigative studies were conducted to explore several possible MOAs for the vacuolation seen in the adrenal cortex and ovary, and for Leydig cell, ovary and uterus tumours in the rat. No MOA was established unequivocally for any of these effects and therefore their human relevance could not be discounted (Bachelor, 2020a, b, c; Herold, 2020; Hoekstra, 2017; Lourens, 2017; Verkaart, 2020a).

Toxicological data on metabolites and/or degradates

Metabolite DM-8007 (M11)

Metabolite DM-8007 is a rat (less than 5% of AD in faeces; approximately 50% of total radioactivity in plasma, liver and kidney), livestock and plant metabolite. Exposure in the rat was demonstrated in several toxicological studies.

The acute oral LD_{50} of DM-8007 was greater than 2000 mg/kg bw (Park, 2015).

Metabolite DM-8007 was tested in a gene mutation assay in bacteria. There was no evidence of mutagenicity (Kim, 2015a).

In a 28-day toxicity study in rats, metabolite DM-8007 was administered at dietary concentrations of 0, 300, 1000 or 3000 ppm (equal to 0, 33, 85 and 278 mg/kg bw per day for males, 0, 31, 94 or 378 mg/kg bw per day for females). The NOAEL was 3000 ppm (equal to 278 mg/kg bw per day), the highest dose tested (Buesen et al., 2017d).

In a 90-day toxicity study in rats, metabolite DM-8007 (purity 99.7%) was administered at dietary concentrations of 0, 125, 500 or 3000 ppm (equal to 0, 7.8, 31 and 190 mg/kg bw per day for males, 0, 8.8, 36 or 215 mg/kg bw per day for females). The NOAEL was 3000 ppm (equal to 190 mg/kg bw per day), the highest dose tested (Buesen et al., 2017e).

Based on the results of experimental studies and the structural similarity to broflanilide, the Meeting concluded that metabolite DM-8007 is not of greater toxicity than broflanilide and would be covered by the health-based guidance values for the parent.

Metabolite DC-DM-8007

Metabolite DC-DM-8007 is a metabolite in rats (less than 3% of its hydroxylated and conjugated forms in faeces or bile), poultry (laying hens) and ruminants (lactating goats).

The acute oral LD_{50} of metabolite DC-DM-8007 was greater than 2000 mg/kg bw (Lee, 2016).

Metabolite DC-DM-8007 was tested in a gene mutation assay in bacteria. There was no evidence of mutagenicity (Kim, 2015b).

In a 28-day toxicity study in rats, metabolite DC-DM-8007 was administered at dietary concentrations of 0, 100, 500 or 1500 ppm (equal to 0, 11, 47 and 156 mg/kg bw per day in males, 0, 12, 44 or 145 mg/kg bw/day in females). No NOAEL could be identified due to increased extramedullary haematopoiesis in the spleen, reticulocyte counts in both sexes and increased spleen weights in females at 100 ppm (equal to 11 mg/kg bw per day), the lowest dose tested (Buesen et al., 2018).

In a 90-day toxicity study in rats, metabolite DC-DM-8007 was administered at dietary concentrations of 0, 30, 75 or 750 ppm (equal to 0, 1.9, 5.3 and 54 mg/kg bw per day in males, 0, 2.2, 5.7 and 56 mg/kg bw per day in females). The NOAEL was 30 ppm (equal to 2.2 mg/kg bw per day) based on decreased red blood cells, haematocrit, haemoglobin, increased reticulocytes and extramedullary haematopoiesis in the spleen, and increased spleen weights in females, at 75 ppm (equal to 5.7 mg/kg bw per day) (Buesen et al., 2017f).

The Meeting concluded that metabolite DC-DM-8007 has similar NOAELs to the parent compound. The reference values of the parent apply also to this metabolite.

Metabolite S(PFP-OH)-8007, (M8)

Metabolite S(PFP-OH)-8007 is a rat metabolite (less than 3% of the AD in faeces; up to 13% of total radioactivity in plasma, liver, kidney and fat) and minor plant metabolite (cabbage, tomato, Japanese radish, soya bean, rice and tea).

The acute oral LD_{50} of metabolite S(PFP-OH)-8007 was greater than 2000 mg/kg bw (Na, 2016).

Metabolite S(PFP-OH)-8007 was tested in a gene mutation assay in bacteria. There was no evidence of mutagenicity (Kim, 2015c).

In a 28-day toxicity study in rats, metabolite S(PFP-OH)-8007 was administered at dietary concentrations of 0, 300, 1000 or 3000 ppm (equal to 0, 26, 81 and 243 mg/kg bw per day for males, 0, 30, 109 and 265 mg/kg bw per day for females). The NOAEL was 300 ppm (equal to 26 mg/kg bw per day) based on increased adrenal weights and increased vacuolation in the adrenals of both sexes and increased vacuolation in the interstitial glands of the ovary at 1000 ppm (equal to 81 mg/kg bw per day) (Buesen et al., 2017g).

In a 90-day toxicity study in rats, metabolite S(PFP-OH)-8007 was administered at dietary concentrations of 0, 125, 500 or 3000 ppm (equivalent to 0, 8.3, 32, 193 mg/kg bw per day for males, 0, 9.1, 37 and 219 mg/kg bw per day for females). No NOAEL could be identified due to increased adrenal weights and increased vacuolation in the adrenals of both sexes, and increased ovary weight and vacuolation in interstitial glands of the ovary at 125 ppm (equal to 8.3 mg/kg bw per day) the lowest dose tested (Buesen et al., 2017h).

Metabolite S(PFP-OH)-8007 has a similar toxicity profile to broflanilide. After 90 days of dosing, the LOAEL of metabolite S(PFP-OH)-8007 (8.3 mg/kg bw per day) was lower than that of broflanilide (35 mg/kg bw per day). Considering the dose levels used and the severity of the effects, the Meeting concluded that metabolite S(PFP-OH)-8007 toxicity is greater than the parent compound by a factor of three.

Metabolite hippuric acid

Hippuric acid (2-benzamidoacetic acid) is a major metabolite in rats (up to 11% of AD in urine) and livestock (lactating goats).

Hippuric acid is commonly found at milligram per litre levels in human urine. The Meeting concluded that it is of no toxicological concern from the use of broflanilide.

Metabolites B-oxamic acid and B-urea

Metabolites B-oxamic acid and B-urea are found in rotational crops. These metabolites were predicted to be negative for genotoxic effects (Ames end-point) by QSAR analysis. It was concluded that metabolites B-oxamic acid and B-urea are unlikely to possess mutagenic potential. The Meeting concluded that the TTC approach should be applied using Cramer class III, 1.5 µg/kg bw per day (Urbisch & Sanches-da-Rocha, 2020).

Metabolites DC-DM-(A4-OH)-8007 and DC-DM-(A6-OH)-8007 (free and conjugate)

Free and conjugated forms of metabolites DC-DM-(A4-OH)-8007 and DC-DM-(A6-OH)-8007 are livestock metabolites (liver and kidney).

No specific toxicological data were available. The Meeting noted that metabolites DCDM(A4-OH)-8007 and DC-DM-(A6-OH)-8007 are structurally similar to the rat intermediate metabolite DC-DM-(A-OH)-8007 (M9B) (present at greater than 2% of AD in faeces), a hydroxylated form of metabolite DC-DM-8007, for which toxicity data are available.

The Meeting concluded that the toxicity of free and conjugated metabolites DC-DM-(A4-OH)-8007 and DC-DM-(A6-OH)-8007 is not greater than that of the parent compound. The reference values of the parent apply to these metabolites.

Metabolite DM-(C_2-OH)-8007 (free and conjugate)

Free and conjugated metabolite DM-(C2-OH)-8007 are livestock metabolites (liver and kidney).

No specific toxicological data were available. The Meeting noted that metabolite DM(C2OH)-8007 is structurally similar to the rat intermediate metabolite DM-(C4-OH)-8007, (C) (present at up to 15% of total radioactivity in plasma, liver and kidney), a hydroxylated form of metabolite DM-8007 for which toxicity data are available and exposure in rats was demonstrated in several toxicological studies.

The Meeting concluded that toxicity of the free and conjugated metabolite DM-(C2-OH)-8007 is not greater than that of the parent compound. The reference values of the parent apply to these metabolites.

Microbiological data

There was no information available in the public domain and no experimental data were submitted that addressed the possible impact of broflanilide residues on the human intestinal microbiome.

Human data

No information was provided on the health of workers involved in the manufacture or use of broflanilide. No information on accidental or intentional poisoning in humans was available.

The Meeting concluded that the existing database on broflanilide was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) for broflanilide of 0–0.02 mg/kg bw, based on the LOAEL of 5.9 mg/kg bw per day in the two-year toxicity and carcinogenicity study in rats, and using a safety factor of 100 for intra- and interspecies differences and an additional safety factor of three for using a LOAEL as the point of departure (POD). This ADI is supported by the 90-day rat study NOAEL of 2.0 mg/kg bw per day, and the two-generation reproductive study parental NOAEL of 2.3 mg/kg bw per day. The upper bound of this ADI provides a margin of at least 4750 relative to the LOAEL for tumours. It is considered adequately protective of effects observed at the LOAEL of 100 mg/kg bw per day in the one-year dog study.

The Meeting concluded that it was not necessary to establish an ARfD for broflanilide in view of its low acute oral toxicity and the absence of developmental toxicity and any other toxicological effects that would be likely to be elicited by a single dose.

Levels relevant to risk assessment of broflanilide

Species	Study	Effect	NOAEL	LOAEL
Mouse	78-week study of toxicity and carcinogenicity [a]	Toxicity	1500 ppm, equal to 157 mg/kg bw/day	7000 ppm, equal to 745 mg/kg bw/day
		Carcinogenicity	7000 ppm, equal to 745 mg/kg bw/day [c]	–
Rat	Acute neurotoxicity study [b]	Neurotoxicity	2000 mg/kg bw/day[c]	–
	Two-year studies of toxicity and carcinogenicity [a, d]	Toxicity	–	100 ppm, equal to 5.9 mg/kg bw/day [e]
		Carcinogenicity	300 ppm, equal to 19 mg/kg bw/day	1500 ppm, equal to 95 mg/kg bw/day
	Two-generation study of reproductive toxicity [a]	Reproductive toxicity	15 000 ppm, equal to 1147 mg/kg bw/day [c]	–
		Parental toxicity	30 ppm, equal to 2.3 mg/kg bw/day	100 ppm, equal to 7.5 mg/kg bw/day
		Offspring toxicity	300 ppm, equal to 23 mg/kg bw/day	1500 ppm, equal to 111 mg/kg bw/day
	Developmental toxicity study [b]	Maternal toxicity	1000 mg/kg bw/day [c]	–
		Embryo/fetal toxicity	1000 mg/kg bw/day [c]	–
Rabbit	Developmental toxicity study [b]	Maternal toxicity	1000 mg/kg bw/day [c]	–
		Embryo and fetal toxicity	1000 mg/kg bw/day [c]	–
Dog	13-week toxicity [f]	Toxicity	1000 mg/kg bw/day [c]	–
	One-year studies of toxicity [f]	Toxicity	–	100 mg/kg bw/day [e]
Metabolite DM-8007 (M11)				
Rat	Four-week study of toxicity [a]	Toxicity	3000 ppm, equal to 278 mg/kg bw/day [c]	–
	13-week toxicity [a]	Toxicity	3000 ppm, equal to 190 mg/kg bw/day [c]	–

[a] Dietary administration; [b] Gavage administration; [c] Highest dose tested

[d] Two or more studies combined; [e] Lowest dose tested; [f] Capsule administration

Acceptable daily intake (ADI), applies to broflanilide and DM-8007, expressed as broflanilide

0–0.02 mg/kg bw

Acute reference dose (ARfD)

Unnecessary

Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure

Critical end-points for setting guidance values for exposure to broflanilide

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid, T_{max} 1–2 hours; 14–23% of AD
Distribution	Wide; highest concentrations in fat
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	>88% of AD excreted within 48 hours, complete by 168 hours; major route via faeces
Metabolism in animals	Extensive, via hydroxylation or demethylation, and hydroxylation followed by conjugation
Toxicologically significant compounds in animals and plants	Broflanilide, DM-8007
Acute toxicity	
Rat, LD_{50}, oral	>5000 mg/kg bw
Rat, LD_{50}, dermal	>5000 mg/kg bw
Rat, LC_{50}, inhalation	>2.2 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Not irritating
Mouse and Guinea pig, dermal sensitization	Not sensitizing (LLNA, Magnussen & Kligmann)
Short-term studies of toxicity	
Target/critical effect	Adrenal gland and ovary vacuolation (mouse, rat, dog)
Lowest relevant oral NOAEL	2 mg/kg bw per day (rat)
Lowest relevant dermal NOAEL	1000 mg/kg bw per day, highest dose tested (rat)
Lowest relevant inhalation NOAEC	0.031 mg/L (rat)
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Ovary weight and vacuolization (rat)
Lowest relevant NOAEL	No NOAEL identified; LOAEL 5.9 mg/kg bw per day (rat), the lowest dose tested
Carcinogenicity	Not carcinogenic in mice; increase in Leydig cell adenomas, uterus adenocarcinomas and benign tumours of sex cord stromal origin in the ovary in rats [a]
Genotoxicity	Unlikely to be genotoxic
Reproductive toxicity	
Target/critical effect	Adrenal weight and vacuolization, ovary weight and vacuolization/decrease in pup weights (rat)
Lowest relevant parental NOAEL	2.3 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	22.6 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	1147 mg/kg bw per day, the highest dose tested (rat)
Developmental toxicity	
Target/critical effect	No effects
Lowest relevant maternal NOAEL	1000 mg/kg bw per day, the highest dose tested (rat, rabbit)
Lowest relevant embryo/fetal NOAEL	1000 mg/kg bw per day, the highest dose tested (rat, rabbit)

Neurotoxicity	
Acute neurotoxicity NOAEL	>2000 mg/kg bw, highest dose tested (rat)
Subchronic neurotoxicity NOAEL	1041 mg/kg bw per day, highest dose tested (rat)
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity	>1020 mg/kg bw per day, highest dose tested (rat)
Studies on toxicologically relevant metabolites	
Metabolite DM-8007	Acute oral LD_{50}: >2000 mg/kg bw (rat) 28-day NOAEL: 278 mg/kg bw per day, the highest dose tested (rat) 90-day NOAEL: 190 mg/kg bw per day, the highest dose tested (rat) Not genotoxic (Ames)
Microbiological data	No data available
Human data	No clinical cases or poisoning incidents have been recorded

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

Summary

	Value	Study	Safety factor
ADI	0–0.02 mg/kg bw [a]	Two-year study of toxicity and carcinogenicity (rat)	300 (100 for intra- and interspecies variation and three for using the LOAEL as the POD)
ARfD	Not necessary		

[a] Applies to broflanilide and DM-8007, expressed as broflanilide

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Dimethoate/omethoate (addendum)

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Explanation

Dimethoate is the ISO-approved common name for *O*,*O*-dimethyl-*S*-([methylcarbamoyl]methyl) phosphorodithioate-2-dimethoxyphosphinothioylsulfanyl-*N*-methylacetamide (IUPAC), with the Chemical Abstract Service number 60-51-5. dimethoate is an organophosphate insecticide, having contact and systemic action, against a broad range of insects in agriculture and also used for the control of the housefly. It acts by inhibiting acetylcholinesterase (AChE).

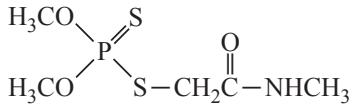
The Joint FAO/WHO Meeting on Pesticide Residues (JMPR) evaluated dimethoate for toxicological effects in 1963, 1965, 1967, 1984, 1987, 1996, 2003 and 2019. The 2019 Meeting (JMPR, 2020) established an acceptable daily intake (ADI) of 0–0.001 mg/kg body weight (bw) and an acute reference dose (ARfD) of 0.02 mg/kg bw. Omethoate (also known as dimethoxon), the oxygen analogue metabolite of dimethoate, appears to be the substance active against insects and mammals.

While considering the metabolites of dimethoate the Meeting noted the conclusion of the 1996 JMPR:

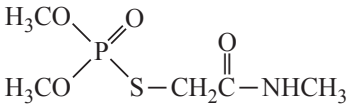
> "Omethoate has been extensively investigated for mutagenicity in vitro and in vivo. The Meeting concluded that it has clear mutagenic potential but that the weight of the evidence observed in vivo was negative; however, the positive result obtained in the mouse spot test could not be completely disregarded."

At JMPR 1996 the ADI for omethoate was withdrawn. Although a number of new studies focused on the inhibition of AChE by omethoate, no new genotoxicity studies were provided to the 2019 Meeting. Hence, the 2019 Meeting was unable to complete the assessment of omethoate with respect to its mutagenic potential, and consequently, the assessment of dimethoate. The present Meeting continued the evaluation of omethoate on the basis of data submitted in 2019, new data on the genotoxicity of omethoate and new data on other metabolites of dimethoate.

Figure 1. Chemical structures of dimethoate and omethoate



Dimethoate



Omethoate

Evaluation for acceptable daily intake

1. Omethoate

The data submitted for omethoate to the 2019 meeting were evaluated and published as part of the dimethoate monograph in the toxicological monographs resulting from that meeting (JMPR, 2021). New genotoxicity data on omethoate were provided for consideration by the 2022 Meeting, where the data submitted on omethoate in 2019 were also evaluated along with newly submitted data. This monograph addendum only reports additional comments that were offered at the 2022 meeting.

A number of studies on plant metabolites, effects on several species and on genotoxicity have been submitted and evaluated by the present meeting

As 2019 meeting could not set any ADI or ARfD for omethoate, a main metabolite of dimethoate, due to no additional genotoxicity data having been submitted, the present meeting undertook a full review of all new data submitted, as well as studies already evaluated by previous JMPR, and did so for all metabolites including omethoate.

1.1 Genotoxicity of omethoate

Omethoate was evaluated for its mutagenic and genotoxic potential in a battery of in vitro and in vivo assays.

In vitro tests to investigate mutagenic and cytogenetic effects were performed in bacterial and mammalian cells. In bacterial test systems omethoate was positive (Ames test; tester strains TA1535

and TA100 with and without metabolic activation), at the very high concentrations of 12 500 and 12 400 µg/plate, which far exceeded the recommended maximum test concentrations. (Reported only in tabular format in Table 44 of the 2019 JMPR monograph) (Herbold, 1988a; JMPR 2021)

Indications of the mutagenic potential of omethoate were noted at JMPR 2019 from the results of a hypoxanthine–guanine phosphoribosyl transferase (HPRT) assay in Chinese hamster ovary (CHO) cells, but at severely cytotoxic concentrations only and with no clear dose-dependency (Lehn, 1989).

A mouse lymphoma assay, also considered at JMPR 2019, did not reveal any effects indicative of a mutagenic potential either in the presence or absence of metabolic activation from S9 mix (rat liver supernatant fraction obtained by centrifuging at 9000 *g*) (Bootman & Rees, 1982).

As discussed at JMPR 2019, genotoxic effects were noted in an unscheduled DNA synthesis (UDS) assay in vitro in rat hepatocytes, and in a cytogenetic assay, which measured sister chromatid exchange (SCE) induction in CHO cells (Cifone, 1989).

Specific comment was made at previous JMPR evaluations of positive findings in a somatic cell assay in vivo which were noted in a mouse spot test; this indicated a potential for omethoate to induce point mutations in vivo under the described test conditions (Herbold, 1990b; see Table 1). In contrast, neither the micronucleus test in mice (Herbold, 1988b), the SCE-test in hamsters (Herbold, 1990a) nor the UDS ex vivo test in rats (Benford, 1989) revealed any indication that omethoate was an in vivo mutagenic or genotoxic agent, even when tested at clearly systemically toxic doses. In addition, there was no indication from a dominant lethal test in male mice (Herbold, 1991), that omethoate might be a germ cell mutagen.

Besides the positive result in the mouse spot test (Herbold, 1990b) no indication of omethoate being mutagenic or genotoxic in vivo was noted from any of the frequently used and very sensitive mutagenicity studies in somatic cells, which together cover several species and modes of actions. Furthermore, in the germ cell study there was no indication of omethoate being an in vivo mutagen and no clear evidence that it induced point mutations in mammalian test systems in vitro. Effects in mammalian cells, if seen at all, were observed only at severely toxic doses only. All of the above was reported at JMPR 2019.

Table 1. Results obtained with omethoate in the mouse spot test

	F1 animals without spots		F1 animals showing WMVS [a]		F1 animals showing RS [b]	
Groups and doses	**Number**	**%**	**Number**	**%**	**Number**	**%**
Vehicle control	339	97.7	6	1.7	2	0.6
Omethoate 4 mg/kg bw	334	94.6	7	2.0	12*	3.4
Omethoate 8 mg/kg bw	295	92.2	12	3.8	13*	4.1
Omethoate 16 mg/kg bw	300	87.5	24*	7.0	19*	5.5
EMU 40 mg/kg bw	284	73	22**	5.7	83**	21.3

[a] With mid-ventral spots; [b] Relevant spots; EMU: *N*-Ethyl-*N*-nitrosourea; Source: Herbold, 1990b

Significantly different from control using X^2 test: * $p \leq 0.05$, ** $p \leq 0.01$

Two in vivo studies were conducted, one to assess the potential for omethoate to induce DNA strand breaks from samples of the liver and duodenum (comet assay), and a second that assessed chromosome damage (numerical and structural) in the bone marrow.

A preliminary toxicity test was performed in rats to identify the maximum tolerated dose (MTD), defined as the highest dose that is tolerated without evidence of study-limiting toxicity, bearing in mind the duration of the study period (for example, by inducing body weight depression or haematopoietic system cytotoxicity, but not death or evidence of pain, suffering or distress necessitating humane euthanasia). To determine suitable dose levels for use in the main test, animals were administered

omethoate technical at 3 mg/kg bw per day. The dose level was selected based on the available toxicity data for the test item. No clinical signs of toxicity or body weight loss were observed. The MTD had not been achieved, therefore an additional group of two male and two female animals were administered omethoate technical at 6 mg/kg bw per day. At 6 mg/kg bw per day male animals were terminated on humane grounds on day 2 post dose. There was no evidence of misdosing. At 6 mg/kg bw per day the nature of the clinical signs observed in female animals was considered unsuitable for the main test (Barfield, 2020).

Table 2. The experimental design for preliminary toxicity study in rats

Group	Treatment	Concentration (mg/mL)	Dosage (mg/kg/day)	Number of animals	
				Male	Female
1	Omethoate technical	0.3	3	2	2
2	Vehicle [a] control	0	0	2	2
3	Omethoate technical	0.6	6	2	2

[a] Vehicle; sterile water for injection; Source: Barfield, 2020

Following dosing, the animals were examined regularly during the working day and any mortalities or clinical signs of reaction during the experiment were recorded. At the end of this observation period, surviving animals had blood samples taken prior to being killed and having their brains removed. Erythrocyte and brain cholinesterase analysis was performed on all preliminary toxicity test animals surviving until scheduled termination with the results helping to determine a suitable MTD for the main test.

Following the preliminary toxicity test, no substantial differences in toxicity were observed between sexes. Therefore, in line with current guidelines, the test was performed using males only.

The main study (Barfield, 2020) was a combined in vivo comet and micronucleus assay in which male rats (six per dose) were orally dosed with omethoate (purity 96.6%;study Batch no. D2014HRM-OMT-07-2) by gavage at 0.75, 1.5 or 3 mg/kg bw per day on three consecutive days, with the second dose administered about 24 hours after the first dose and the third dose about 21 hours after the second dose. Vehicle (sterile water) and positive control (ethyl methanesulfonate) groups were included. Animals were terminated around three hours after the last dose.

Table 3. Experimental design for genotoxicity studies of omethoate in rats

Group	Treatment	Concentration (mg/mL)	Dosage (mg/kg bw/day)	Number of animals (male)
1	Vehicle control [a]	0	0	6
2	Omethoate technical	0.075	0.75	6
3	Omethoate technical	0.15	1.5	6
4	Omethoate technical	0.3	3	6
5	EMS	20	200	3

[a] Vehicle; sterile water for injection; Source: Barfield, 2020

EMS: Ethyl methanesulfonate (comet phase positive control) dosed once only approximately three hours prior to termination at a dose volume of 10 mL/kg;

Erythrocyte (RBC) and brain cholinesterase (ChE) were evaluated at termination. DNA strand breaks were assessed by comparing group mean and median percentage tail intensities in cell suspensions prepared from liver and duodenum by single-cell gel electrophoresis (comet assay). Micronuclei in polychromatic erythrocytes were evaluated from bone marrow smears.

Marked and dose-related decreases in ChE activity in RBCs (41.7%, 66.4% and 74.3% inhibition, respectively) and the brain (41.1%, 68.5% and 85.6% inhibition, respectively) were observed in the 0.75, 1.5 and 3 mg/kg bw per day omethoate-treated groups. The degree of cholinesterase inhibition exceeded the MTD at all dose levels in this study (greater than 10%–20%)

Following omethoate exposure, no increases in micronuclei were observed at any dose level of omethoate.

In the liver, there were no statistically significant or biologically relevant increases in percentage tail intensities, and all group mean values in the omethoate-treated groups were within the laboratory′s historical control range, indicating the absence of strand breakages in the liver DNA as a result of omethoate exposure over and above that seen in controls.

In the duodenum, statistically significant increases in the percentage tail intensities were observed at all dose levels. The group percentage for the 0.75 mg/kg bw per day group was within the historical control range, however, at 1.5 and 3 mg/kg bw per day values were outside the historical control range.

Hedgehog cells were not observed in the liver but showed a dose-related increase in the duodenum. Hedgehog cells are heavily damaged cells with a specific morphology that is considered to be associated with cytotoxicity, necrosis or apoptosis. The hedgehog cells observed in the duodenum of rats administered omethoate were considered directly related to the increases seen in percentage tail intensities; both increased in a dose-related manner.

Table 4. Summary of results for genotoxicity studies of omethoate in rats

Treatment	Dose (mg/kg/day)	Number of cells scored	Group mean tail intensity (percentage) [SD][b]	Group mean of median tail intensity (percentage) [SD][b]
		Males; liver		
Vehicle	0	900	2.01 [0.5]	0.33 [0.2]
Omethoate technical	0.75	900	2.07 [0.5]	0.50 [0.2]
Omethoate technical	1.5	900	2.01 [0.5]	0.41 [0.2]
Omethoate technical	3	900	1.75 [0.2]	0.67 [0.3]*
EMS	200	450	32.98 [6.7]	31.12[c] [8.7]***+
		Males; duodenum		
Vehicle	0	900	3.55 [1.1]	0.84[c] [0.4]
Omethoate technical	0.75	900	5.84 [1.5]	3.01[c] [1.7]***
Omethoate technical	1.5	900	8.30 [1.5]	4.41[c] [1.5]***
Omethoate technical	3	900	10.05 [3.5]	6.69[c] [3.8]***
EMS	200	450	32.32 [3.2]	28.97[c] [4.3]***+

[a] Vehicle; sterile water for injection; SD: Standard Deviation Source: Barfield , 2020

[b] Occasional apparent errors of ±1% may have occurred due to rounding of values for presentation in tables;

[c] Analysis performed upon logarithmically transformed data;

EMS: Ethyl methanesulfonate (comet phase positive control) dosed once only approximately three hours prior to termination

Statistical analysis performed on median tail intensity values only;
Results of statistical analysis using the appropriate non-parametric method of analysis based on permutation (one sided probabilities);
Values of p for comparisons with control using William′s test,unless indicated otherwise (+ t-test):
* $p < 0.05$ (significant) *** $p < 0.001$ (significant) otherwise $p < 0.05$ (not significant)

Table 5. Genotoxicity study of omethoate in rats: summary of results and statistical analysis for micronucleus element (data for males)

Sampling time after third dose	Treatment	Dose (mg/kg bw per day)	Proportion of PCEs, group (percentage) [SD][c]	Group mean MPCE/4000 PCE [SD][c]	Group mean % MPCE[c]
3 hours	Vehicle [a]	0	50.9[0.9]	3.3[1.0]	0.08
3 hours	Omethoate Technical	0.75	50.8[0.7]	1.8[1.5]	0.05
3 hours	Omethoate Technical	1.5	50.1[1.2]	2.0[0.9	0.05
3 hours	Omethoate Technical	3	50.1[1.0]	2.3[1.0]	0.06
3 hours	Cyclophosphamide [b]	20	46.5*[0.6]	76.3*[15.0]	1.91

[a] Vehicle; sterile water for injection; SD: Standard Deviation Source: Barfield, 2020

[b] Positive control slides from GY05QJ; PCE: Polychromatic erythrocyte;

[c] Occasional apparent errors of ±1% may have occurred due to rounding of values for presentation in tables;

MPCE: Number of micronucleated polychromatic erythrocyte observed per 4000 PCEs examined

Results of statistical analysis using the appropriate non-parametric method of analysis based on permutation (one sided probabilities) * $p < 0.05$ (significant) otherwise $p < 0.05$ (not significant)

Therefore, the increases in percentage tail intensities were considered not be of genotoxic origin but rather related to cytotoxic effects, as supported by concurrent dose-related and significant increases in hedgehog cells. Furthermore, these findings occurred at dose levels greatly exceeding the MTD, based on the severe inhibition of RBC and brain ChE that had been demonstrated (Barfield, 2020)

`The further study conducted was to assess if increases in DNA strand breaks were still observed in the duodenum at lower omethoate concentrations, where less concurrent toxicity (that is, hedgehog cells and cholinesterase inhibition) was occurring.

Male rats were orally dosed at 0, 0.094, 0.188, 0.375 or 0.75 mg/kg bw per day in a similar fashion to the first study. Dose-related decreases in RBC ChE compared to controls occurred at 0.375 and 0.75 mg/kg bw per day (by 14.6% and 35.3%, respectively); dose-related inhibition of brain ChE was observed at all dose levels (by 6.7%, 19.7%, 29.7%, and 51.5%, respectively). A marked and statistically significant increase in percentage tail intensity was observed only at the highest dose level (0.75 mg/kg bw per day), and the value was outside the historical control range. The increase in percentage tail intensity was accompanied by a marked increase in hedgehog cells at this same dose level only. Percentage tail intensities were very low, not statistically significant and within the historical control range for the 0.094, 0.188 and 0.375 mg/kg bw per day dose groups, and these lower dose levels were accompanied by only a low level of hedgehog cells.

In conclusion, the combined results of the two in vivo studies demonstrate that omethoate does not cause structural or numerical chromosome damage in the bone marrow, nor DNA strand breaks in the liver up to and including a dose level of 3 mg/kg bw per day in male rats, at doses which cause severe inhibition of RBC and brain ChE, and therefore, significantly exceeding the MTD (0.75 mg/kg bw per day and greater). Increases in DNA strand breaks were accompanied by evidence of marked increases in cellular toxicity (hedgehog cells) and therefore considered to not be of genotoxic origin. On the basis of these two studies it can be concluded that omethoate is not a genotoxic compound (Best, 2022).

The findings of all mutagenicity studies considered are summarized below in Table 6.

Table 6. Summary of genotoxicity studies on omethoate

End-point	Test object	Concentration	Purity	Results	Reference
		In vitro			
Bacterial reverse mutation assay (Ames test)	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537;	20–12 500 µg/plate (±S9)	96%	Positive (+/– S9)	Herbold, 1988a
Sister chromatid exchange	Chinese hamster ovary cells	25–3000 µg/mL (–S9) 166.7–5000 µg/mL (+S9)	96%	Positive (+/– S9)	Taalman, 1988
Gene mutation	L5178Y mouse lymphoma cells (TK)	500–2500 µg/mL (–S9) 500–5000 µg/mL (+S9)	96.9%	Negative (+/– S9)	Bootman & Rees, 1982
Forward mutation assay in mammalian cells (HPRT test)	Chinese hamster ovary cells	500–6000 µg/mL (–S9) 3000–6000 µg/mL (+S9)	97.4%	Positive (+/– S9)	Lehn, 1989
Unscheduled DNA synthesis in mammalian cells	Rat hepatocytes	25.6–1030 µg/mL	97.4%	Positive	Cifone, 1989
		In vivo			
Micronucleus test	NMRI mouse, male and female (oral gavage)	22.5 mg/kg bw	96%	Negative	Herbold, 1988b
Sister chromatid exchange test	Chinese hamsters	0, 5, 10 and 20 mg/kg bw	96.7%	Negative	Herbold, 1990a
Mouse spot test*	Mouse somatic cells	0, 4, 8 and 16 mg/kg bw	96.7%	Positive	Herbold, 1990b
Unscheduled DNA synthesis	Rat hepatocytes	0, 3, 10 and 30 mg/kg bw	96.6%	Negative	Benford, 1989
Dominant lethal test	NMRI mice (oral administration)	0, 10 and 20 mg/kg bw	96.9%	Negative	Herbold, 1991
Comet assay	Male rat (liver and duodenum)	0.75, 1.5 and 3mg/kg	96.6%	Negative	Barfield, 2020
Micronucleus assay	Male rats	0.094, 0.188, 0.375 and 0.75 mg/kg bw	96.6%	Negative	Best, 2022

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*

1.2 Studies on cholinesterase activity

To compare the effects of omethoate when administered as an acute gavage dose at the time of peak effect on cholinesterase inhibition, male and female postnatal day (PND) 11 neonatal pups and male and female adult rats (Crl:CD(SD) were administered a single dose of 0 (vehicle), 0.1, 0.3, 0.6 or 0.9 mg/kg bw omethoate (purity 96.5%). Red blood cell and brain ChE activities were measured at the predetermined time-of-peak effect (following dose administration, three hours for the adults and four hours for the pups).

All male and female PND 11 pups and adult rats survived until scheduled sacrifice, and there were no adverse clinical signs observed after dose administration. Therefore the no-observed-adverse-effect level (NOAEL) for systemic toxicity was considered to be greater than 0.9 mg/kg bw in male and female adult and neonatal rats. For adult male and female rats, RBC ChE activity was reduced (with statistical significance) in the 0.3, 0.6 and 0.9 mg/kg bw dose groups compared with values for the vehicle control group. In PND 11 male and female pups, statistically significant reductions in RBC ChE activity were seen in the 0.3, 0.6 and 0.9 mg/kg bw dose groups compared with the vehicle control group values. No effect on RBC cholinesterase activity was observed in the male, female adult or neonatal rats at 0.1 mg/kg bw when compared with the vehicle controls. For adult rats, brain ChE activity was reduced

compared with the vehicle control group to a statistically significant extent in all dose groups for males, and in the 0.3, 0.6 and 0.9 mg/kg bw dose groups for the females. In PND 11 pups, brain ChE activity was reduced (with statistical significance) in all dose groups for males and females compared with the vehicle control group values. The statistically significant decreases observed in brain ChE activity of adult male rats and the male and female pups at 0.1 mg/kg bw were not considered to be biologically important because all values were below 10% (between 5.4% and 7.4%) of the vehicle control group values. Estimates of a benchmark dose (BMD) for adults and PND 11 pups were compared and the statistical significance of the ratio of BMDs tested. The relative sensitivity of adults compared to PND 11 pups can be estimated from the ratio between the BMDs for adults and that for pups. The ratio of adult to PND 11 pup BMDs for both brain and RBC ChE activity ranged from 1.07 to 1.32, and these ratios were not statistically different from unity, indicating that the pup and adult responses were similar. For RBC ChE, the ratio can be estimated using either the BMD_{10} or the BMD_{20}, while for the brain the ratio was estimated using the BMD_{10}. The ratios estimated for RBC cholinesterase activity were: 1.26 (BMD_{10} for the male rats), 1.08 (BMD_{10} for the female rats), 1.32 (BMD_{20} for the male rats), and 1.07 (BMD_{20} for the female rats). The ratio estimates for brain cholinesterase activity were 1.13 for the male rats and 1.25 for the female rats. The BMDs for PND 11 pups and adults were not statistically different for either brain or RBC ChE inhibition. Therefore, these data indicate that a relative sensitivity factor is not necessary for omethoate (Barnett, 2012).

Table 7. Omethoate inhibition of RBC cholinesterase activity in adult rats (15 per group)

Group	Dose level (mg/kg bw)	Mean ChE activity (U/mL) ± SD	Percentage decrease compared with controls
Males			
1	0 [a]	1.220 ± 0.219	-
2	0.1	1.148 ± 0.174	5.9%
3	0.3	0.992 ± 0.138**	18.7%
4	0.6	0.811 ± 0.135**	33.5%
5	0.9	0.772 ± 0.118**	36.7%
Females			
1	0 [a]	1.233 ± 0.113	-
2	0.1	1.180 ± 0.095	4.3%
3	0.3	1.020 ± 0.137**	17.3%
4	0.6	0.811 ± 0.114**	34.2%
5	0.9	0.807 ± 0.127 **	34.5%

[a] Control; deionized water (reverse osmosis) only; Source: Barnett, 2012

** Significantly different from the vehicle control substance group value at $p \leq 0.01$

Table 8. Omethoate inhibition of brain cholinesterase activity in adult rats (15 per group)

Group	Dose level (mg/kg bw)	Mean ChE activity (U/mL) ± SD	Percentage decrease compared with controls
Males			
1	0 [a]	14.675 ± 0.935	-
2	0.1	13.585 ± 0.462 **	7.4%
3	0.3	12.141 ± 0.600 **	17.3%
4	0.6	10.746 ± 0.707 **	26.8%
5	0.9	10.154 ± 0.846 **	30.8%

Group	Dose level (mg/kg bw)	Mean ChE activity (U/mL) ± SD	Percentage decrease compared with controls
Females			
1	0 [a]	14.495 ± 0.591	-
2	0.1	14.117 ± 0.512	2.6%
3	0.3	12.666 ± 0.710 **	12.6%
4	0.6	11.080 ± 0.782 **	23.6%
5	0.9	10.454 ± 0.716 **	27.9%

[a] Control; deionized water (reverse osmosis) only; Source: Barnett, 2012

** Significantly different from the vehicle control substance group value at $p \leq 0.01$

Table 9. Omethoate inhibition of RBC cholinesterase activity in PND 11 pups

Group	Dose level (mg/kg bw)	Mean ChE activity (U/mL) ± SD [N]	Percentage decrease compared with controls
Male pups			
1	0 [a]	1.514 ± 0.218 [14]	-
2	0.1	1.453 ± 0.175 [15]	4.0%
3	0.3	1.183 ± 0.135 [15]**	21.9%
4	0.6	0.874 ± 0.168 [14]**	42.3%
5	0.9	0.774 ± 0.197 [15]**	48.9%
Female pups			
1	0 [a]	1.554 ± 0.182 [15]	-
2	0.1	1.478 ± 0.189 [14]	4.9%
3	0.3	1.273 ± 0.198 [15]**	18.1%
4	0.6	0.972 ± 0.199 [15]**	37.5%
5	0.9	0.830 ± 0.204 [15]**	46.6%

** Significantly different from the vehicle control substance group value at $p \leq 0.01$; Source: Barnett, 2012

[a] Control; deionized water (reverse osmosis) only; *N* : The number of rats evaluated for cholinesterase activity

Table 10. Omethoate inhibition of brain cholinesterase activity in PND 11 pups

Group	Dose level (mg/kg bw)	Mean ChE activity (U/mL) ± SD [N]	Percentage decrease compared with controls
Male pups			
1	0 [a]	6.614 ± 0.454 [15]	-
2	0.1 1	6.156 ± 0.389 [15]*	6.9%
3	0.3 1	5.283 ± 0.328 [15]**	20.1%
4	0.6 1	4.489 ± 0.804 [15]**	32.1%
5	0.9 1	3.729 ± 0.691 [15]**	43.6%
Female pups			
1	0 [a]	6.570 ± 0.329 [15]	-
2	0.1	6.213 ± 0.354 [15]*	5.4%
3	0.3	5.391 ± 0.268 [15]**	17.9%
4	0.6	4.480 ± 0.599 [15]**	31.8%
5	0.9	3.707 ± 0.506 [15]**	43.6%

** Significantly different from the vehicle control substance group value at $p \leq 0.01$; Source: Barnett, 2012

[a] Control; deionized water (reverse osmosis) only; *N* : The number of rats evaluated for cholinesterase activity

Table 11. BMD results for RBC cholinesterase activity in adults and pups after a single, oral, gavage dose of omethoate

Age	Sex	*A* (unit/mL)	P_B	BMD_{10} (mg/kg bw)	BMD_{20} (mg/kg bw)
Adults	Male	1.230	0.5	0.133	0.304
	Female	1.251	0.5	0.149	0.316
Pups	Male	1.553	0.3	0.106	0.231
	Female	1.573	0.1	0.138	0.295

$BMD_{10/20}$: Lower confidence limit on the benchmark dose for a 10%/20% response Source: Barnett, 2012

A: Defines the cholinesterase inhibition without exposure;

P_B: Defines the asymptotic limit for cholinesterase inhibition at a high dose

Table 12. BMD results for brain cholinesterase activity in adults and pups after a single, oral, gavage dose of omethoate

Age	Sex	*A* (unit/mL)	P_B	BMD_{10} (mg/kg bw)
Adults	Male	14.615	0.6	0.163
	Female	14.665	0.6	0.200
Pups	Male	6.612	0.3	0.144
	Female	6.593	0.1	0.160

A: Defines the cholinesterase inhibition without exposure; Source: Barnett, 2012

P_B: Defines the asymptotic limit for cholinesterase inhibition at a high dose;

BMD_{10}: Lower confidence limit on the benchmark dose for a 10% response

Table 13. Omethoate inhibition of cholinesterase; BMD estimates and derived adult:pup ratios

Cholinesterase compartment	BMD	Gender	Pups	Adults	Ratio adult:pup [CI]
Red blood cells	BMD_{10} (mg/kg bw)	Males	0.106	0.133	1.26 [0.90–1.7]
		Females	0.138	0.149	1.08 [0.83–1.4]
	BMD_{20} (mg/kg bw)	Males	0.231	0.304	1.32 [0.93–1.8]
		Females	0.295	0.316	1.07 [0.84–1.4]
Brain	BMD_{10} (mg/kg bw)	Males	0.144	0.163	1.13 [0.93–1.4]
		Females	0.160	0.200	1.25 [1.0–1.5]

CI: 95th percentile confidence intervals for ratio of adult to pup BMDs; Source: Barnett, 2012

The BMD_{10} and BMD_{20} for ChE inhibition in RBCs and the BMD_{10} for brain Che inhibition in adults and pups suggest a slightly higher sensitivity in pups. On these bases, also observing that RBC ChE is slightly more sensitive to inhibition than brain cholinesterase inhibition, the BMD_{20} for erythrocyte cholinesterase inhibition in male pups (0.231 mg/kg bw) can be used as a point of departure (POD) for derivation of a ARfD.

The study (Barnett, 2012) was GLP compliant and has a QA statement attached; the conclusion of the Meeting was partially modified from that reached at 2019 JMPR.

1.3 Microbiological aspects

The impact of dimethoate residues on the human intestinal microbiome was evaluated through a decision-tree approach, adopted by the sixty-sixth meeting of the JECFA Committee (JECFA, 2006) for food-producing animal drugs, which complies with VICH GL36(R)2, and can also be used for pesticides.

The human intestinal microbiome is considered an additional target organ (Baquero & Nombela, 2012), in which changes in the composition and function of intestinal microbes (microbiota dysbiosis) has been associated with diseases ranging from localized gastroenterologic disorders to neurologic, respiratory, metabolic, hepatic and cardiovascular illnesses (Lynch & Pedersen, 2016). Thus, as one more toxicological target of concern, sponsors of products submitted for evaluation will

need to address the effects of residues on the human intestinal microbiome, for two relevant end-points of concern; the disruption of the colonization barrier and an increase in bacterial resistance. A product, or its metabolite, might not be an antimicrobial but could still produce disruption and/or increase the population of resistant bacteria, to the extent that an microbiological ADI (mADI) and/or microbiological ARfD (mARfD) need be calculated.

The VICH GL36(R) discusses test systems that can be used to address this toxicological end-point of concern, considering the complexity of the human intestinal microbiome. This approach can be used to determine if a mADI would be necessary, based on an evaluation of whether product residues reaching the human colon are microbiologically active. It entails answering three questions to determine the need for establishing a mADI. First, determine if the product residues, and/or its metabolites are microbiologically active against representatives of the human intestinal microbiota; second, if the product residues enter the human colon; and third, if the residues entering the human colon remain microbiologically active. If the answer to any of these questions is "no" then there is no need to calculate a mADI, and the assessment need not be completed. However, if a mADI needs to be calculated, two end-points of concern for human health are considered for the assessment: disruption of the colonization barrier of the human intestinal microbiome and increases in populations of resistant bacteria in the human intestinal microbiome.

A publication by Yang et al., (2019), described a study to evaluate the effect of three common pesticides, (amitraz, chlorpyrifos and dimethoate) one perspective of which was the midgut bacterial communities of adult worker bees (*Apis cerana* and *mellifera*). Although other end-points of concern were tested for all three pesticides, the effect on bee midgut bacterial communities was not tested for dimethoate.

Two other publications available in the public domain (Dai et al., 2018; Wu, et al., 2022) describe the use of 45 mg/L dimethoate as a positive control, to determine the effect of other pesticides on the gut microbiota of bees, but no specific details regarding dimethoate's impact on the gut microbiota was included.

A publication from Wu et al., (2022), described an in vitro study to determine the effect of chlorothalonil on the survival, weight and gut microbiota of immature bees. In this study, 45 mg/L of dimethoate was used as positive control for larval survival testing, but not for the experiments related to gut bacterial community composition and diversity.

Since no other information was provided, and the information available in the public domain was insufficient, it was not possible for the Committee to determine if there is a need for the calculation of a mADI for dimethoate. This conclusion also applies to the need for a microbiological acute reference dose (mARfD).

1.4 Observations in humans

One case of suicide with omethoate was reported. An 18-year-old apprentice gardener had ingested an unknown amount of omethoate. His body was found in his room lying in the storage space under his bed. An autopsy first showed multiple superficial incisions in the skin of his wrists, furthermore haemorrhagic pulmonary oedema, dilation of the right cardiac ventricle and oedema of the brain. The gastric mucosa was swollen and a dark brownish colour. An intense, chemical-like smell arose from the corpse and organs. Toxicological analysis revealed omethoate in cardiac blood (208 µg/mL), urine (225 µg/mL) and bile (524 µg/mL), in the liver (341 µg/ml) and kidneys (505 µg/ml). In the gastric content the level was 48 223 µg/ml. The amount of the active AChE in peripheral blood serum was reduced to less than 0.2% of the normal level. The author stated that to best of their knowledge no case of a fatal suicide by ingestion of omethoate had been reported in literature (Pavlic, 2002).

Review of literature

Levels of AChE and neurotoxic esterase have been measured in brain autopsy material. In tissue from a fatal human poisoning and from hens given four to eight times the unprotected LD_{50}, the tissue's AChE activity was strongly inhibited, but neurotoxic esterase activity (also known as neuropathy target esterase or NTE) remained uninhibited. The findings correlate with the inhibitory power of omethoate against these enzymes in vitro. It was concluded that omethoate has negligible potential to cause delayed neuropathy (Lotti et al.,1981).

2. Other metabolites of dimethoate/omethoate

The toxicity and anticholinesterase activity of dimethoate, omethoate and four plant metabolites of dimethoate (O-desmethyl omethoate, O-desmethyl omethoate carboxylic acid, O-desmethyl-N-desmethyl omethoate and O-desmethyl isodimethoate) were investigated in two studies performed in 2000.

Study 1

Groups of male CD rats were gavaged with a single dose of test material in distilled water as detailed in Table 14 and observed for 14 days. Fasting blood samples were taken from the orbital sinus 2.5 and 24 hours after dosing, and erythrocyte ChE activities measured using an automated procedure based on the method of Ellman et al., (1961). Gross necropsy was performed on all animals.

No deaths occurred in any group. No clinical signs of toxicity were observed during the course of the study that were related to treatment.

Table 14. Toxicity of dimethoate metabolites; study design and percentage of ChE inhibition compared to predose values

Group	Compound	Purity	Dose level (mg/kg bw)	ChE inhibition 2.5 h post dose (%)	*p* value [a]	ChE inhibition 24 h post dose (%)	*p* value [b]
1	Dimethoate	99.5 %	30	53%	0.0001	40	0.0021
2	Omethoate	96.3 %	5	74%	0.0000 [c]	34	0.0009
3	Metabolite I, O-desmethyl omethoate	98.6 %	30	19%	0.0799	16	0.1393
4	Metabolite II, O-desmethyl omethoate carboxylic acid	89.5 %	30	25%	0.0220	21	0.0512
5	Metabolite III, O-desmethyl-N-desmethyl omethoate	96.4 %	30	–2%	0.8647	9	0.3627
6	Metabolite IV , O-desmethyl isodimethoate	98.2 %	30	28%	0.0133	20	0.0698

ChE: Cholinesterase; Source: Brennan, 2001

[a] Statistical analysis performed on differences between predose and a 2.5 hours post dose; [b] 24 hours post dose.

[c] *p* value of 0.000000000806568, reported as 0.0000;

The results of this study indicate that omethoate is a considerably more potent ChE inhibitor than dimethoate. Findings also indicate that *O*-demethylation of omethoate markedly reduces anticholinesterase activity. Activity is further reduced (or abolished) by *N*-demethylation. Metabolite O-desmethyl omethoate (Met I) and its carboxylic acid derivative (Met II) were shown to have similar anticholinesterase activity. The O-demethylation of isodimethoate was similarly shown to markedly reduce anticholinesterase activity. The metabolites O-desmethyl omethoate carboxylic acid, O-desmethyl-N-desmethyl omethoate, and O-desmethyl isodimethoate seemed to have a markedly lower (or even zero) potential to inhibit cholinesterase compared to dimethoate, or omethoate as well.

The study was GLP compliant and a QA statement was attached (Brennan, 2001).

Study 2

Another study was performed to identify the single dose effect of dimethoate and the plant metabolites O-desmethyl omethoate carboxylic acid (Met XX) and des-O-methyl isodimethoate (Met XII) that cause approximately 50 percent inhibition of RBC and/or brain ChE activity in the rat. This was done to identify the time of peak effect on ChE activity after a single oral dose of each test substance, as a prerequisite for subsequent studies.

Sixty-eight male and 68 female Crl:CD(SD) rats (60 days old on receipt) were assigned to 13 dosage groups, eight rats per sex in the control group, and five rats per sex in each treatment group, as shown in Table 15.

Table 15. Dosing scheme for time to peak effect study in rats

Test article	Dosage group	Number of rats per sex	Dosage (mg/kg)	Concentration (mg/mL)	Dosage volume (mL/kg)
Control	1	8	0	0	5
Dimethoate, technical	2	5	5	1	5
	3	5	10	2	5
	4	5	20	4	5
	5	5	30	6	5
Met XX O-Desmethyl omethoate carboxylic acid, disodium salt	6	5	10	2	5
	7	5	20	4	5
	8	5	30	6	5
	9	5	40	8	5
Met XII O-Desmethyl isodimethoate, sodium salt	10	5	10	2	5
	11	5	20	4	5
	12	5	30	6	5
	13	5	40	8	5

Source: Lawson & Barr, 1987

Animals received single oral doses by gavage Control animals received vehicle only. The dose volume was 5 mL/kg for all groups.

Animals were checked for viability twice daily and for clinical symptoms and general appearance once daily from the predose period until sacrifice. Body weights were recorded prior to dose administration. Whole blood samples (2–3 mL) were collected into EDTA-coated tubes from the inferior vena cava of the anaesthetized animals at 2.5 hours post dose. After blood sample collection, the brains were excised and weights recorded. Blood samples were stored mixed, on cold packs and the brains were stored in saline on iced water until assayed. After sample collection, the animals were discarded without further evaluation.

Analysis of RBC and brain ChE levels was performed within two hours of sample collection according to the modified Ellman procedure (Ellman, et al., 1961; Lawson & Barr, 1987).

No mortality occurred during this study. Three male (one at 10 mg/kg bw and two at 20 mg/kg bw) and three female rats (two at 10 mg/kg bw and one at 20 mg/kg bw) dosed with dimethoate were observed with miosis prior to sacrifice. No other clinical signs were observed.

In the rats administered dimethoate, test substance-related inhibition of brain ChE was observed at 10, 20 and 30 mg/kg bw in males and in all treated groups of female rats. Erythrocyte ChE activity was inhibited at all dose levels, in both sexes.

For male rats the inhibition of brain ChE activity ranged from 10.8% to 71.6% of control, while inhibition of RBC ChE ranged from 27.8% to 73.0% . Whilst at dose levels of 5 and 10 mg/kg bw the degree of ChE inhibition was higher in RBCs than in the brain there was no such difference at higher dose levels. In female rats brain and RBC ChE activity was inhibited to much the same extent, with inhibitions ranging from 26.1% to 66.5%.

For the two plant metabolites, Met XX and Met XII, no cholinesterase inhibition was observed even at 40 mg/kg bw, the highest dose level tested this study.

Table 16. Brain and RBC cholinesterase activity levels and percent inhibition at 2.5 hours after dosing in males

		Brain ChE		RBC ChE	
Test compound	Dose (mg/kg bw)	Mean ChE activity (U/g) ± SD [*N*]	Inhibition [%][a]	Mean ChE activity (U/g) ± SD [*N*]	Inhibition [%][a]
Control	0	13.407 ± 1.224 [8]	-	1.139 ± 0.083 [8]	-
Dimethoate	5	11.953 ± 0.500 [4][b]	10.8	0.822 ± 0.164 [5]	27.8
	10	7.703 ± 1.822 [5]	42.5	0.470 ± 0.105 [5]	58.7
	20	3.802 ± 0.439 [5]	71.6	0.354 ± 0.015 [3][b]	68.9
	30	4.513 ± 0.111 [5]	66.3	0.308 ± 0.048 [4][b]	73.0
Met XX O-Desmethyl omethoate carboxylic acid)	10	14.623 ± 0.546 [5]	0 [–9.1]	1.172 ± 0.137 [5]	0 [–2.9]
	20	15.031 ± 0.261 [5]	0 [–12.1]	1.242 ± 0.198 [5]	0 [–9.0]
	30	15.133 ± 0.705 [5]	0 [–12.9]	1.411 ± 0.090 [5]	0 [–23.9]
	40	14.264 ± 0.673 [4][b]	0 [–6.4]	1.140 ± 0.014 [5]	0 [–0.1]
Met XII Des-O-methyl isodimethoate, sodium salt	10	19.856 ± 1.047 [5]	0 [–48.1]	1.226 ± 0.225 [5]	0 [–7.6]
	20	14.559 ±1.234 [5]	0 [–8.6]	1.054 ± 0.158 [5]	7.5
	30	13.458 ± 0.875 [5]	0 [–0.4]	1.129 ± 0.085 [5]	0.9
	40	13.982 ± 1.460 [5]	0 [–4.3]	1.052 ± 0.341 [5]	7.6

N: Number of adult rats evaluated for cholinesterase levels; SD: Standard deviation; Source: Barnett, 2009a

[a] Inhibition expressed as percentage change compared to controls;

[b] Samples were excluded from summarization as sample results did not meet the acceptability criteria

Table 17. Brain and RBC cholinesterase activity levels and percent inhibition at 2.5 hours after dosing in females

		Brain ChE		RBC ChE	
Test compound	Dose (mg/kg bw)	Mean ChE activity (U/g) ± SD [*N*]	Inhibition [%][a]	Mean ChE activity (U/g) ± SD [*N*]	Inhibition [%][a]
Control	0	12.964 ± 0.736 [7][b]	–	1.236 ± 0.132 [8]	–
Dimethoate	5	9.583 ± 0.863 [5]	26.1	0.812 ± 0.156 [5]	34.3
	10	5.190 ± 1.452 [5]	60.0	0.472 ± 0.131 [4]	61.8
	20	4.399 ±0.646 [5]	66.1	0.450 ± 0.023 [4]	63.6
	30	4.512 ± 0.502 [5]	65.2	0.414 ± 0.037 [4]	66.5
Met XX O-Desmethyl omethoate carboxylic acid)	10	15.508 ± 1.784 [5]	0 [–19.6]	1.301 ± 0.063 [5]	0 [–5.3]
	20	15.143 ± 2.611 [5]	0 [–16.8]	1.449 ± 0.103 [5]	0 [–17.2]
	30	15.281 ± 1.119 [5]	0 [–17.9]	1.303 ± 0.082 [5]	0 [–5.4]
	40	13.979 ± 0.697 [5]	0 [–7.8]	1.283 ± 0.147 [5]	0 [–3.8]
Met XII Des-O-methyl isodimethoate, sodium salt	10	16.146 ± 1.449 [5]	0 [–24.5]	1.311 ± 0.270 [5]	0 [–6.1]
	20	13.682 ± 0.912 [5]	0 [–5.5]	1.119 ± 0.148 [5]	9.5
	30	13.033 ± 0.989 [5]	0 [–0.5]	1.180 ± 0.229 [5]	4.5
	40	13.171 ± 1.359 [5]	0 [–1.6]	1.385 ± 0.175 [5]	0 [–12.1]

: Number of adult rats evaluated for cholinesterase levels SD: Standard deviation; Source: Barnett, 2009a

[a] Inhibition expressed as percentage change compared to controls;

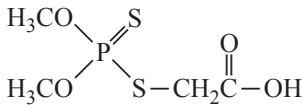
[b] Samples were excluded from summarization as sample results did not meet the acceptability criteria

Treatment with either of the plant metabolites, Met XII and Met XX, did not cause ChE inhibition up to 40 mg/kg bw, the highest dose level tested this study. The study was not GLP compliant (Barnett, 2009a).

2.1 Metabolite III (dimethoate carboxylic acid)

This compound is an important metabolite in food animals (goat, hen). It is also a major rat metabolite, therefore, its toxicity would be covered by that of the parent, dimethoate

Figure 2. Chemical structure of Met III (dimethoate carboxylic acid)



(a) Acute toxicity

Table 18. Summary of acute toxicity study of Met III, dimethoate carboxylic acid

Species	Strain	Sex	Route	Purity	Result	Reference
Rat	Crl:CD(SD)	Female	Oral	97.1%	$LD_{50} > 2000$	Barnett, 2013a

LD_{50}: Median lethal dose

(b) Acute cholinesterase studies

Study 1

A dose range-finding study in adult Crl:CD(SD) rats allocated 8 animals/sex per group with body weights in the range 309–340 g for males and 210–250 g for females, both aged 62 days at the start of the study. These were administered, once by oral gavage, the test substance (Met III, purity 97.1%; Lot/Batch no:924-BSe-53B) at 1000 mg/kg bw (dose calculation adjusted for purity) or the vehicle control (reverse osmosis deionized water). The dose volume was 5 mL/kg bw, and doses were based on body weights recorded prior to dose administration. Viability, clinical signs, body weights, and cholinesterase activity were evaluated in this study. On day 1 of study male and female rats were anaesthetized under isoflurane/oxygen prior to blood sampling at approximately 2.5 hours post dose (the estimated time of peak effect for dimethoate). Following blood collection from the vena cava, the rats were euthanized by an injection of sodium pentobarbital into the inferior vena cava. The brain from each rat was excised and weighed. Red blood cell and brain samples were analyzed for ChE activity at the testing facility. All rats were discarded without further evaluation. There were no mortalities or test substance-related adverse clinical signs. Body weights were comparable among the male and female rats on the day of dose administration. Brain and RBC ChE values in both the male and female rats at 1000 mg/kg bw were comparable to vehicle control group values, and there were no statistically significant differences.

There was no indication of test substance-related inhibition of ChE activity in either brain or RBC samples taken 2.5 hours after dose administration, from male or female rats dosed with 1000 mg/kg bw of Met III (Barnett, 2013b).

Study 2

A study was undertaken with the objective of measuring RBC and brain ChE activity in Crl:CD(SD) adult rats at various time points after a single oral administration of Met III (dimethoate carboxylic acid, purity 97.1%; Lot/Batch no.924-BSe-53B). Control groups consisted of 10 rats of each sex and dosed groups 20 rats of each sex. Male rats weighed 318–357 g each, female rats 219–265 g each, and all were 63 days old. Dose calculation was adjusted for purity and rats were administered 1000 mg/kg bw of the test substance, or the vehicle control, deionized water, once by oral gavage.

Viability, clinical signs, body weight, and cholinesterase activity were evaluated in this study.

On day 1 of study male and female rats were anaesthetized under isoflurane/oxygen prior to blood sampling from five rats/sex per time point at approximately four and 16 hours post dose for the vehicle controls, and 4, 8, 12 and 16 hours after dose administration for the 1000 mg/kg bw dosed group. Following blood collection from the vena cava, the rats were euthanized by an injection of sodium pentobarbital into the inferior vena cava. The brain from each rat was excised and weighed. Red blood cell and brain samples were analyzed for cholinesterase activity at the testing facility. All rats were discarded without further evaluation.

There were no mortalities or test substance-related adverse clinical signs. On the day of dose administration body weights within the male group were comparable, as were those within the female group.

The brain and RBC ChE values in both male and female rats at 1000 mg/kg bw were comparable to the vehicle control group values at 4, 8, 12 and 16 hours post dose, and there were no statistically significant differences.

In neither males nor females was there any indication at 4, 8, 12 or 16 hours post dose of brain or RBC ChE activity being inhibited by Met III (dimethoate carboxylic acid) when dosed at 1000 mg/kg bw (Barnett, 2013c).

(c) Short-term studies of toxicity

Oral administration

Rat

A study was undertaken with the objective of determining the effects of 14 days of repeated oral gavage doses of Met III (dimethoate carboxylic acid, purity 97.5%; Lot/Batch No. D1707-ET-06-A) on red RBC and brain ChE activity. The dose calculation was adjusted for purity, and the test item administered by repeated oral gavage to forty Crl:CD(SD) rats, aged 52 days. Each dose group consisted of five males and five females, males body weight in the range 257–282 g, females 176–202 g. Groups received Met III at 0, 250, 500 or 1000 mg/kg bw per day for 14 consecutive days.

The study also provided information on the tolerability of the test item following repeated dosing. Information gained in this study was used as the basis for dose level selection for longer term repeat-dose studies in the rat.

The test item or vehicle control substance was administered to the appropriate rats once daily by oral gavage on days 1 to 14 of the study . Doses were adjusted based on the most recently recorded body weight and the start of dose administration began at approximately the same time each day.

Whole blood samples (up to 3 mL) were collected at ca 2.5 hours post dose (timing began with the gavage of the rat on day 14 and ended with blood collection).

Checks for viability were made at least twice daily. Rats were observed for clinical signs and general appearance prior to dose administration, and ca 2.5 hours after dose administration. Body weights were recorded once daily during the dose period (prior to dose administration). Food consumption was recorded daily during the dose period and prior to euthanasia (food left value).

The rats that died or were euthanized prior to scheduled termination were examined for cause of death or condition as soon as possible after the observation was made. The lungs, trachea and oesophagus were perfused. The rats were examined for gross lesions and subjected to a gross necropsy of the thoracic, abdominal and pelvic viscera.

On dosing day 14 all surviving rats were anaesthetized under isoflurane/oxygen approximately 2.5 hours post dose, and following blood collection from the inferior vena cava, euthanized by an injection of sodium pentobarbital (390 mg/mL) into the inferior vena cava. The rats were examined for gross lesions and were subjected to a gross necropsy of the thoracic, abdominal and pelvic viscera. The brain was excised, weighed and evaluated for cholinesterase activity; all other tissues were discarded.

After repeated doses of Met III, there were reductions in body weight and/or body weight losses observed in the 500 and 1000 mg/kg bw per day dose groups in both male and females. Adverse clinical signs were observed in the 500 and 1000 mg/kg bw per day female rats and the 1000 mg/kg bw per day male rats. From the 1000 mg/kg bw per day dose groups, three male and two female rats had to be euthanized due to adverse clinical signs, and an additional female rat from this group was found dead. A slight but statistically significant decrease (c 20% lower than concurrent control values) in brain ChE activity was observed in male and female rats dosed at 1000 mg/kg bw per day.

Table 19. Repeat-dose, 14-day study of the effect of Met III (dimethoate carboxylic acid) on RBC cholinesterase activity

Group	Dose level (mg/kg bw)	Mean ChE activity (U/mL) ± SD [*N*]	Percent difference compared with controls
Male rats			
1	0 (vehicle control)	1.168 ± 0.388 [5]	-
2	250	1.138 ± 0.181 [5]	–2.6%
3	500	1.281 ± 0.114 [5]	+9.7%
4	1000	1.183 ± 0.196 [2] [a]	+1.3%
Female rats			
1	0 (vehicle control)	1.392 ± 0.186 [5]	-
2	250	1.329 ± 0.103 [5]	–4.5%
3	500	1.309 ± 0.047 [4] [b]	–6.0%
4	1000	1.202 ± 0.127 [2] [a]	–13.6%

N: The number of rats evaluated for cholinesterase activity; Source: Barnett, 2015a

SD: Standard deviation;

[a] Excludes rats that were euthanized due to adverse clinical observations;

[b] Excludes a rat that did not have a sample analyzed

Erythrocyte ChE activities in both the male and female rats, at doses up to and including 1000 mg/kg bw per day were comparable to vehicle control group values; no statistically significant differences were noted.

Table 20. Repeat-dose, 14-day study of the effect of Met III (dimethoate carboxylic acid) on brain cholinesterase activity

Group	Dose level (mg/kg bw)	Mean ChE activity (U/g) ± SD [*N*]	Percent difference compared with controls
Male rats			
1	0 (vehicle control)	14.704 ± 0.555 [5]	-
2	250	13.793 ± 0.483* [5]	–6.2%
3	500	14.352 ± 0.829 [5]	–2.4%
4	1000	11.903 ± 0.057** [2] [a]	–19.0%
Female rats			
1	0 (vehicle control)	14.981 ± 1.220 [5]	-
2	250	13.667 ± 0.924 [5]	–8.7%
3	500	13.360 ± 0.889* [5]	–10.8%
4	1000	11.813 ± 0.305** [2] [a]	–21.1%

N: The number of rats evaluated for cholinesterase activity; Source: Barnett, 2015a

SD: Standard deviation;

[a] Excludes rats that were euthanized due to adverse clinical observations;

[b] Excludes a rat that did not have a sample analyzed;

Statistically significant difference from the control group value: * $p \leq 0.05$, ** $p \leq 0.01$

Based on this study it was considered that a dose level of less than 1000 mg/kg bw per day should be employed as the high dose in subsequent repeat-dose studies, and values of 250 mg/kg bw per day and below should be used for repeat-dose studies where the establishment of a NOEL were required.

The study is not GLP compliant (Barnett, 2015a).

Met III (dimethoate carboxylic acid, purity 97.5%; Lot/Batch No. D1707-ET-06-A) was administered to adult male and female Crl:CD(SD) rats, with 10 animals of each sex per group, males of body weight 224–253 g, females 161–189 g, all aged 52 days. Dosing was by oral gavage for 28 consecutive days at doses of 0 (vehicle control), 45, 90, 225 or 450 mg/kg bw per day (groups 1 to 5, respectively). Doses were adjusted for purity and at each dosing based on the most recently recorded body weight; start of dosing began at approximately the same time each day.

The following parameters and end-points were evaluated in this study: viability, clinical signs, food consumption, body weight, body weight change, functional observational battery (FOB) testing, detailed clinical observations, motor activity evaluation, ophthalmic examinations, clinical pathology parameters (haematology, coagulation, clinical chemistry), gross necropsy observations, organ weights, brain and RBC ChE activity, histology and histopathological evaluations.

All male and female rats in the dose groups up to and including 225 mg/kg bw per day survived until scheduled euthanasia. In the 450 mg/kg bw per day dose group there was one male and three females that were either found dead or electively euthanized due to adverse clinical observations. These deaths were considered to be related to the test substance. Common clinical signs in these rats included effects on respiration (for example gasping, rales, dyspnoea), and there were additional test substance-related clinical signs noted prior to death, for instance mild or moderate dehydration, hunched posture, pale ears and/or extremities. Additional clinical signs that were attributed to the test substance included rales and slight excess salivation in females at 225 mg/kg bw per day and above and in males at 450 mg/kg bw per day, as well as dyspnea and dehydration (mild or moderate) in females at 450 mg/kg bw per day.

Body weight, body weight gain and food consumption were unaffected by doses up to and including 450 mg/kg bw per day in both males and females. There were no test substance-related differences in FOB evaluations, motor activity assessments or ophthalmological evaluations conducted during the fourth week of dosing. There were no noteworthy gross necropsy observations at the completion of the dosing period.

No test substance-related differences were observed in any haematology or coagulation parameter in males or females at doses up to and including 450 mg/kg bw per day.

Though there were some minor changes in clinical chemistry parameters that were attributed to the test substances at 225 mg/kg bw per day and above, these changes are not consistent with respect to dose response and not large enough to be considered adverse.

In female rats at 450 mg/kg bw per day, there was a statistically significant increase of 10% in absolute and relative (to brain weight) weight of the liver, and paired kidneys (11%) compared to controls.

Treatment-related microscopic alterations seen in males and females at 450 mg/kg bw per day were limited to gastritis, and this was more common in the male rats. However, the possibility of non-specific stress resulting from increased levels of acetylcholine within the brain cannot be ruled out. Whether or not this is the case, gastritis is not considered to represent a manifestation of direct toxicity to the stomach, but rather a secondary manifestation related to increases in the level of acetylcholine, possibly resulting from stress. Minimal degrees of gastritis are not considered to be of biologic significance.

In the female rats at doses of 225 and 450 mg/kg bw per day there was a statistically significant decrease in RBC ChE activity compared to controls. There was also a statistically significant decrease in brain ChE activity observed in the male rats at 450 mg/kg bw per day.

Table 21. Results of a 28-day study into effects of Met III (dimethoate carboxylic acid) on cholinesterase activity in rats

		RBC cholinesterase activity		Brain cholinesterase activity	
Group	Dose level (mg/kg bw)	Mean(U/mL) ± SD [*N*]	Difference [a]	Mean(U/g) ± SD [*N*]	Difference [a]
	Male rats				
1	0 (vehicle control)	1.236 ± 0.095 [10]	-	13.440 ± 0.852 [10]	-
2	45	1.217 ± 0.143 [10]	–1.5%	12.763 ± 1.030 [10]	–5.0%
3	90	1.275 ± 0.097 [10]	+3.2%	12.910 ± 1.192 [9b]	–3.9%
4	225	1.210 ± 0.110 [10]	–2.1%	12.586 ± 1.009 [10]	–6.4%
5	450	1.199 ± 0.154 [9] [b]	–3.0%	11.613 ± 1.756** [9] [b]	–13.6%
	Female rats				
1	0 (vehicle control)	1.515 ± 0.191 [10]	-	13.224 ± 0.901 [10]	-
2	45	1.560 ± 0.257 [10]	+3.0%	14.117 ± 1.427 [10]	+6.8%
3	90	1.508 ± 0.109 [10]	–0.5%	14.690 ± 0.779** [10]	+11.1%
4	225	1.270 ± 0.155** [10]	–16.2%	12.865 ± 0.453 [10]	–2.7%
5	450	1.241 ± 0.094** [7] [b]	–18.1%	12.940 ± 0.718 [7] [b]	–2.1%

N: Number of rats evaluated for cholinesterase activity; RBC: Red blood cell Source: Barnett, 2016a

[a] Percentage change compared with concurrent vehicle controls;

[b] Excludes rats that were found dead or euthanized due to adverse clinical observations.

Statistically significant difference from the control group value: * $p \leq 0.05$, ** $p \leq 0.01$

In the female rats at doses of 250 and 450mg/kg bw per day, there were slight, but statistically significant decreases ($p \leq 0.01$) in RBC ChE activity in comparison with the vehicle control group.

The NOAEL was 90 mg/kg bw per day based on selected clinical signs (for example, rales and salivation), a slight reduction in RBC ChE activity (females only and not to a degree considered toxicologically relevant) and a marginally lower globulin level ,at 225 mg/kg bw per day. (Barnett, 2016a)

(d) Genotoxicity

Dimethoate Met III was assayed for mutational potential in four histidine-requiring strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537) and tester strain of *Escherichia coli* strain WP2 uvrA (Schreib, 2013a) and for the ability to induce mutation at the *tk* locus (5-trifluorothymidine resistance) in mouse lymphoma cells (Keigh-Shevlin, 2015a), and in an in vitro micronucleus assay using duplicate human lymphocyte cultures (Watters, 2015a). It was also assayed in vivo for its potential to induce micronuclei in polychromatic erythrocytes (PCE) of the bone marrow of treated rats, and/or to induce DNA damage in the liver or duodenum.

While Met III did not prove to be mutagenic in the bacterial reverse mutation assay, it induced dose-related and statistically significant increases in micronuclei in the absence of S9 metabolic activation (rat liver supernatant fraction obtained by centrifuging at 9000 g) following 24 hours incubation. It also induced mutation at the *tk* locus of L5178Y mouse lymphoma cells when tested up to toxic concentrations in the absence of S-9 activation with a 24-hour incubation. At the highest three dose-levels (500, 550 and 600 µg/mL) of the eight dose-levels evaluated, the proportions of small mutant colonies at these concentrations were 60%, 56% and 68% respectively, compared to a vehicle control value of 45%. These data were suggestive of a clastogenic effect.

To address the effects seen in vitro, Met III was also assessed in a combined study in vivo for its potential to induce micronuclei in polychromatic erythrocytes of the bone marrow of treated rats, and/or to induce DNA damage in the liver or duodenum. The results obtained indicated that there were no statistically significant increases in micronucleus frequency or mean tail intensity and tail moment

values in the duodenum for any of the groups receiving Met III, compared to the concurrent vehicle control groups. In the liver, there was a small but statistically significant increase in tail intensity at the highest dose-level (2000 mg/kg bw per day), with a significant linear trend. However, all animals fell within the 95% reference ranges of the historical control data. In a second experiment the findings observed in the previous experiment were examined by comet analysis of the liver only, and negative results were obtained. The Meeting further noted that the duodenum is more relevant as a target tissue, since the in vitro clastogenic effects were only observed in the absence of S9 metabolic activation.

The meeting concluded that Met III (dimethoate carboxylic acid) is unlikely to be genotoxic.

Table 22. Summary of genotoxicity studies on Met III (dimethoate carboxylic acid)

End-point	Test object	Concentration	Purity	Results	Reference
		In vitro			
Bacterial gene mutation assay	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537; *Escherichia coli* strain WP2uvrA; Plate incorporation and preincubation assay; ± S9 mix	31.6, 100, 316, 1000, 2500 and 5000 µg/plate	97.1%	Negative [a]	Schreib, 2013a
Forward mutation assay in mammalian cells (HPRT test)	*tk* locus of L5178Y mouse lymphoma cells	Experiment 1: Nine concentrations, 125–2163 µg/mL; ± S9 Experiment 2 Twelve concentrations, 100–1000 µg/mL; –S9 (24 hours incubation); Nine conc. ranging from 150 to 2163 µg/mL; +S9 (3 h incubation),	97.5%	Negative[b] after 3 h incubation with and without S9 Positive;[b] predominantly small colony increase, after 24 h incubation without S9, at higher concentration only	Keig-Shevlin, 2015a
Unscheduled DNA synthesis in mammalian cells	Human lymphocytes	0, 10, 50, 100 and 200 µg/mL	97.5%	Negative [c] following 3 h incubation ± S9 Positive following 24 h incubation without S9, at higher concentration only	Watters, 2015a
		In vivo			
Micronucleus induction	Male rat (test article, vehicle and positive control were given as three administrations, at ca 0, 24 and 45.5 h; All animals were sampled at 48 h (isolated fumeral bone marrow)	0,500,1000,1500 & 2000mg/kg bw per day	97.5%	Negative [d]	Beevers, 2015

End-point	Test object	Concentration	Purity	Results	Reference
DNA breakage (comet assay)	Male rat (test article, vehicle and positive control were given as three administrations, at ca 0, 24 and 45.5 h; All animals were sampled at 48 h	0, 500, 1000, 1500 and 2000 mg/kg bw per day	97.5%	Negative [d]	Beevers, 2015

S9: 9000 × *g* supernatant fraction from rat liver homogenate; h: Hours;

[a] Study compliant with the current OECD TG 471; [b] Study compliant with the current OECD TG 490;

[c] Study compliant with the current OECD TG 487;

[d] The study (combined rat bone marrow micronucleus test and liver comet assay) meets the requirements of the current OECD TG 489 (Adopted 29 July 2016).

(OECD TG: Organisation for Economic Co-operation and Development test guideline)

(e) Reproductive and developmental toxicity

A study was undertaken with the objective of detecting adverse effects of Met III (dimethoate carboxylic acid, purity 97.5%; Lot/Batch No. D1707-ET-06-A) on Crl:CD(SD) male and female rats, before cohabitation, through mating and implantation, and on development of the offspring consequent to exposure of the female from implantation until postpartum day 21. The animals were administered Met III (dose calculation adjusted for purity) by oral gavage, at doses of 0, 10, 50, 100 or 300 mg/kg bw per day. The experimental design is shown in Table 23.

Table 23. The effect of Met III (dimethoate carboxylic acid) on RBC cholinesterase activity; experimental design

Group	Dose material	Number of rats		Dose level (mg/kg bw/day)	Dose concentration (mg/mL)	Dose volume (mL/kg)
		Male	Female			
1	Vehicle control	10	10	0	0	10
2	Met III	10	10	10	1	10
3	Met III	10	10	50	5	10
4	Met III	10	10	100	10	10
5	Met III	10	10	300	30	10

Source: Barnett, 2016b

Males were given the test article or vehicle control once daily by oral gavage beginning 28 days before cohabitation with females, during cohabitation and continuing until the day before euthanasia. Males were given 66 doses (25 rats) or 70 doses (25 rats) of the test article or vehicle control. Doses were adjusted based on the most recently recorded body weight and administered at approximately the same time each day. The first day of dosing was designated day 1 of the study.

Females were given the test article or vehicle control once daily by oral gavage beginning 15 days before cohabitation with males and continuing until postpartum day (PPD) 21 in the case of females that delivered a litter, or gestation day (GD) 24 where rats did not deliver a litter. Lactating females that survived until scheduled euthanasia were given 56 (one rat), 57 (two rats), 58 (seven rats), 59 (eight rats), 60 (17rats), 61 (nine rats) and 66 (one rat) doses of the test article or vehicle control. Any dam in the process of parturition was not given the test substance and/or the vehicle control substance formulation until the following day.

Pups from the F1 generation were not directly given the test or vehicle control substance formulations, but were possibly exposed to the test or vehicle control substance formulations during maternal gestation (in utero exposure) and via maternal milk during the lactation period.

The following parameters and end-points were evaluated in this study: viability, clinical signs, food consumption, body weight, reproductive capacity, maternal behaviour, natural delivery observations, gross necropsy observations, organ weights, brain and RBC cholinesterase activity, histology and histopathological evaluations.

All male rats survived until scheduled euthanasia at doses up to and including 300 mg/kg bw per day. One female in the 300 mg/kg bw per day dose group was found dead on day 6, and based on the necropsy findings and microscopic results it was determined that this death was due to an intubation error. Another female in the 50 mg/kg bw per day dose group was euthanized on LD 1 due to issues during delivery. This death was not considered to be test substance-related.

In the 300 mg/kg bw per day group there was a statistically significant increase in the number of males observed with slight excess salivation. There was also a statistically significant increase in the number of females in the 300 mg/kg bw per day group observed with slight excess salivation during the gestation and lactation periods. Moderate excess salivation was observed in two females during the gestation period and one female during the lactation period. In the 300 mg/kg bw per day dose group rales also occurred in two females, one each in the premating and gestation periods.

Body weights and food consumption were unaffected by the test substance during the dosing period in the males and during the premating, gestation and lactation periods in the female at doses up to and including 300 mg/kg bw per day.

Observations of the estrous cycling before cohabitation (mean estrus stages per 14 days, rats with six or more consecutive days in diestrus and rats with six or more consecutive days of estrus)were unaffected by the test substance at doses up to 300 mg/kg bw per day. All mating and fertility parameters in males and females were unaffected by the test substance up to 300 mg/kg bw per day.

There were no notable test substance-related necropsy observations in the male or female rats.

In 300 mg/kg bw per day dose group females there were statistically significant increases in the liver weights, both absolute and relative to brain weight.

Pregnancy occurred in 9, 9, 10, 10 and 8 mated females in the 0, 10, 50, 100 and 300 mg/kg bw per day dose groups, respectively: a total of 9 (100%), 9 (100%), 10 (100%), 10 (100%) and 8 (100%) of the respective pregnant dams delivered litters.

Pup body weights per litter were lower (some with statistical significance) in the 300 mg/kg bw per day group at all time points during the postpartum period. However, litter size was larger in the 300 mg/kg bw per day dose group compared with the concurrent controls. The differences observed were considered slight and were within historical control ranges for rats of this strain. This observation was therefore considered to be of limited toxicological significance.

No clinical observations in the F1 generation pups were attributable to maternal doses of the test substance, as high as 300 mg/kg bw per day. All pups that were found dead or were euthanized and necropsied on PND 21 appeared normal at the time of necropsy.

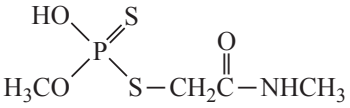
No biologically relevant differences in RBC or brain ChE activity were observed in either sex at any dose.

In view of the above, the NOAEL for general toxicity was 100 mg/kg bw per day in the male and female rats, based on increased in incidence of clinical signs in male and female rats and an increase in liver weights in the females at 300 mg/kg bw per day.

The reproductive NOAEL was 300 mg/kg bw per day, the highest dose tested, as there were no test substance-related changes in estrous cycling in the female, nor effects on mating and fertility in the males or females. The NOAEL for offspring toxicity was also 300 mg/kg bw per day, based on the absence of toxicologically significant effects on relevant parameters (Barnett, 2016b).

2.2 Metabolite X (O-Desmethyl dimethoate)

Figure 3. Chemical structure of Met X (O-desmethyl dimethoate)



(a) Cholinesterase study

The objective of this study was to measure red blood cell and brain cholinesterase activity at various time points after a single oral administration of the sodium salt of Met X, (O-desmethyl dimethoate Na salt) to adult Crl:CD(SD) rats. Male and female rats, aged 52 days (body weights, male 221–255 g, female 162–197 g) were administered 1000 mg/kg bw of the test substance, (purity 41%; Batch/Lot no. D1707-ET-03-A2) or the vehicle control (deionized water). The dose calculation was adjusted for both purity and molecular mass due to sodium. Doses were based on body weights recorded prior to dose administration. Rats were dosed once by oral gavage after which RBC and brain ChE activity was measured at various time points. The dose volume was 10 mL/kg.

Viability, clinical signs, body weight, gross necropsy observations, organ (brain) weight and brain and RBC ChE activity were evaluated for all animals.

On day 1 of study male and female rats were administered the test or vehicle control substance and anaesthetized under isoflurane/oxygen prior to blood sampling from five rats per sex per time point at approximately 4 and 16 hours post dose for the vehicle control rats and 4, 8, 12 and 16 hours after dose administration for the 1000 mg/kg bw dose group. The study was extended to evaluate the ChE inhibition levels at two hours post dose. Following blood collection from the vena cava the rats were euthanized by an injection of sodium pentobarbital into the inferior vena cava. The brain from each rat was excised and weighed, and split in half. The RBC and brain samples were analysed for ChE activity as soon as possible. All rats were examined for gross lesions, and were subjected to a gross necropsy of the thoracic, abdominal, and pelvic viscera.

There were no mortalities or adverse clinical signs, and no noteworthy test substance-related necropsy observations. On the day of dose administration body weights within the male group were comparable, as were those within the female group.

Brain and RBC ChE values in both the male and female rats at 1000 mg/kg bw were comparable to the vehicle control group values at 2, 4, 8, 12 and 16 hours following dose administration, and there were no statistically significant or biologically relevant changes.

In conclusion, there was no indication of test substance-related inhibition of RBC or brain cholinesterase activity in male or female rats dosed with 1000 mg/kg bw of Met X sodium salt (O-desmethyl dimethoate Na salt) at any time point up to 16 hours post dose (Barnett, 2015b).

(b) Genotoxicity

Met X (O-desmethyl dimethoate) was assayed for mutational potential in five histidine-requiring strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537 and TA102; McGarry, 2014a) and for the ability to induce mutation at the *tk* locus (5-trifluorothymidine resistance) in mouse lymphoma cells (Massip, 2014) and in an in vitro micronucleus assay using duplicate human lymphocyte cultures (Watters, 2014a).

The results obtained did not show any genotoxic potential in any of the assays. A summary of these studies is shown in Table 24. Based on the results of these studies the Meeting concluded that Met X (O-desmethyl dimethoate) is unlikely to be genotoxic.

Table 24. Summary of genotoxicity studies with Met X (O-desmethyl dimethoate)

End-point	Test object	Methodology/concentration	Purity	Results	Reference
		In vitro			
Bacterial gene mutation	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102;	All treatments with S9 were further modified by the inclusion of a pre-incubation step Experiment 1: 5, 16, 50, 160, 500, 1600 and 5000 µg/plate; ± S9 Experiment 2: 160, 300, 625, 1250, 2500 and 5000 µg/plate; ± S9	91.6%	Negative [a]	McGarry, 2014a
Mammalian gene mutation	*tk* locus of L5178Y mouse lymphoma cells	Experiment 1: 0, 500, 750,1000, 1500, 2000 and 2153 µg/min ± S9 Experiment 2: 0, 50, 100, 200, 400, 500, 600, 700, 800 and 900 µg/mL; ± S9 3 hours incubation	91.6%	Negative [b]	Massip, 2014
Micronucleus induction	Human lymphocytes	Tested up to 2153 µg/mL in absence and presence of S9.	91.6%	Negative [c]	Watters, 2014a

S9: 9000 x g supernatant fraction from rat liver homogenate

OECD TG: Organisation for Economic Co-operation and Development Guideline;

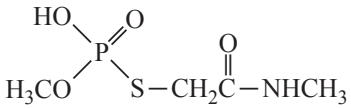
a Study compliant with the current OECD TG 471;

b Study compliant with the current OECD TG 490;

c Study compliant with the current OECD TG 487;

2.3 Metabolite XI (O-desmethyl omethoate)

Figure 4. Chemical structure of Met XI (O-desmethyl omethoate)



(a) Acute toxicity

Oral administration

The aim of this study was to examine the potential toxicity of a single oral gavage administration to adult rats of the sodium salt of Met XI, (O-desmethyl omethoate, Na-salt) in aqueous solution. The test article (purity 41.4%; Batch/Lot No D1707-ET-12-A) was administered to male and female Crl:CD(SD) rats 61 days of age (body weight for males, 259–265 g, for females 217–222 g) once by oral gavage at a dose level of 1000 mg/kg bw. The dose volume was 10 mL/kg bw, and doses were based on body weights recorded prior to dose administration.

Viability, clinical signs, and body weight were evaluated in this study.

On day 8 of the study animals were anaesthetized via an intravenous injection of sodium pentobarbital, then exsanguinated and carcasses discarded without further evaluation.

The acute oral dose of 1000 mg/kg bw of Met XI sodium salt was well tolerated by male and female rats. There were no test substance-related effects on clinical observations or body weights. Based on the results of this study, a dose level of 1000 mg/kg bw was considered tolerable for subsequent single dose studies in the rat.

The study was not GLP-compliant (Barnett, 2015c).

(b) Cholinesterase studies

Single administration

In an oral (gavage) dose range-finding acute cholinesterase study the sodium salt of Met XI, in aqueous solution, was administered to adult (52 days old) Crl:CD(SD) rats, 52 days of age, males of body weight 254–293 g, females 182–218 g. The test article (Met XI, purity 41.4%; Batch/Lot no. D1707-ET-12-A) was administered to the rats, five of each sex per group, at 0 (vehicle control, deionized water) or 1000 mg/kg bw, with a dose volume of 10 mL/kg. Doses were based on body weights recorded prior to dose administration.

Viability, clinical signs, body weight, organ (brain) weight, gross necropsy observations and RBC and brain ChE activity were measured.

On day 1 of the study male and female rats were anaesthetized under isoflurane/oxygen prior to blood sampling at approximately 2.5 hours post dose (the estimated time of peak effect for dimethoate). Following blood collection from the vena cava, the rats were euthanized by an injection of sodium pentobarbital into the inferior vena cava. The brain from each rat was excised, weighed, and cut into halves. Red blood cell and half-brain samples were analyzed for ChE activity as soon as possible. The rats were examined for gross lesions and subjected to a gross necropsy of the thoracic, abdominal, and pelvic viscera.

There were no mortalities or test substance-related adverse clinical signs. No gross lesions were identified at necropsy in the male or female rats. On the day of dose administration body weights within the male group were comparable, as were those within the female group.

The RBC and brain ChE values in both the male and female rats at 1000 mg/kg bw were comparable to the vehicle control group values, and there were no statistically significant changes.

In conclusion, at 2.5 hours after dosing there was no indication of test substance-related inhibition of RBC or brain ChE activity in male or female rats dosed with 1000 mg/kg bw of Met XI sodium salt in aqueous solution. The dose level of 1000 mg/kg bw was therefore considered appropriate for use in subsequent acute cholinesterase activity studies in the rat (Barnett, 2015d).

To determine the time of peak cholinesterase inhibition, male and female Crl:CD(SD) rats of age 52 days were used, the control group of 10 rats of each sex and dosed group 20 of each sex. Body weight was males, 263–306 g, females, 176–206 g. Rats received either vehicle alone (deionized water), or Met XI sodium salt (O-desmethyl omethoate Na salt, purity 41.4%; Batch/Lot no. D1707-ET-12-A) once by oral gavage. The dose administered to the rats was 1000 mg/kg bw, and the dose volume 10 mL/kg bw. Doses were based on body weights recorded prior to dose administration. Red blood cell and brain ChE activity was measured at various time points after administration.

Viability, clinical signs, body weight, gross necropsy observations, organ (brain) weights and RBC and brain ChE activities were measured.

On day 1 of the study, following dose administration, five rats/sex per time point were anaesthetized with isoflurane/oxygen prior to blood sampling. Time points were approximately 4 and 16 hours post dose for the vehicle control rats and 4, 8, 12 and 16 hours after dosing for the 1000 mg/kg bw group. Following blood collection from the vena cava, the rats were euthanized by an injection of sodium pentobarbital into the inferior vena cava. The brain from each rat was excised, weighed, and split in half. Brain and RBC samples were analysed for ChE activity as soon as possible. The rats were examined for gross lesions and were subjected to a gross necropsy of the thoracic, abdominal and pelvic viscera.

There were no mortalities, test substance-related adverse clinical signs or noteworthy observations at necropsy. On the day of dose administration body weights within the male group were comparable, as were those within the female group.

At 4, 8, 12 and 16 hours following dosing the RBC and brain ChE values in both the males and females at 1000 mg/kg bw were comparable to the vehicle control values, and there were no statistically significant changes.

In conclusion, there was no indication of test substance-related inhibition of RBC or brain ChE activity in male or female rats dosed with 1000 mg/kg bw of Met XI sodium salt in aqueous solution at any time point up to 16 hours after dose administration.

The study was GLP compliant (Barnett, 2015e).

Repeated administration

Study 1

In a repeat-dose, oral gavage, cholinesterase study, the sodium salt of Met XI, in aqueous solution, was administered to adult (52 days old) Crl:CD(SD) rats, males of body weight 244–270 g, females 176–207 g. The test article (O-desmethyl omethoate, Na-salt; purity 41.4%; Batch/Lot no. D1707-ET-12-A) was administered once daily to groups of five rats of each sex per dose level at 0, 100, 450 or 750 mg/kg bw per day and a dose volume of 10 mL/kg bw on days 1–14 of the study. The objective for this study was to determine effects on RBC and brain cholinesterase activity in and to provide information on the tolerability of the test item with repeated dosing.

The viability, clinical signs, body weights, food consumption, gross necropsy observations, brain and liver weights and cholinesterase activity were measured.

On day 14 all rats were anaesthetized with isoflurane/oxygen approximately 2.5 hours post-dose and following blood collection from the inferior vena cava, were euthanized by an injection of sodium pentobarbital into the inferior vena cava. The rats were examined for gross lesions, and were subjected to a gross necropsy of the thoracic, abdominal and pelvic viscera. The brain was excised, weighed, and split in half, the liver was weighed, then all other tissues were discarded. Red blood cell and half brain samples were analyzed for ChE activity.

All male and female rats survived until scheduled euthanasia. In the female rats at 450 and 750 mg/kg bw per day, mild dehydration was noted during the second week of dosing. Reductions in body weight gain were noted in the male and female rats at and above 450 mg/kg bw per day. Food consumption values were also reduced in both sexes at 750 mg/kg bw per day. Terminal body weights were slightly reduced in both sexes at 750 mg/kg bw per day. Liver and brain weights were unaffected at all dose levels. In the male and female rats at doses up to and including 750 mg/kg bw per day, there were no statistically significant or biologically important differences observed in RBC or brain ChE activity compared with controls at the time of euthanasia.

Based on this study it was concluded that a dose level of 450 mg/kg bw per day should be employed as the high dose level for subsequent repeat-dose studies. The NOAEL was 100 mg/kg bw per day based on the dehydration and reduction in body weight gain at 450 mg/kg bw per day. (Barnett, 2015f).

Study 2

In a repeat-dose, oral gavage, cholinesterase study, the sodium salt of Met XI, in aqueous solution, was administered to adult (51 days old) Crl:CD(SD) rats, males of body weight 179–208 g, females 150–180 g. The test article (O-desmethyl omethoate, Na-salt; purity 41.4%; Batch/Lot no. D1707-ET-12-A) was administered once daily to groups of 10 rats of each sex per dose level at 0, 15, 50, 100 or 450 mg/kg bw per day and a dose volume of 10 mL/kg bw on days 1–28 of the study The objective of this study was to determine the effects of repeated dose administration of Met XI sodium salt in aqueous solution on RBC and brain ChE activity over a period of 28 consecutive days to Crl:CD(SD) adult rats.

Viability, clinical signs, food consumption, body weight, body weight changes, FOB testing, motor activity evaluation, ophthalmic examinations, clinical pathology parameters (haematology, coagulation, clinical chemistry), gross necropsy observations, organ weights, RBC and brain cholinesterase activity, histology and histopathological evaluations were recorded.

Ophthalmological examinations and FOB testing were undertaken during the last week of study. On day 28 all rats were anaesthetized with isoflurane/oxygen approximately 2.5 hours post dose and following blood collection from the inferior vena cava, were euthanized by an injection of sodium pentobarbital. The rats were examined for gross lesions and subjected to a gross necropsy of the

thoracic, abdominal and pelvic viscera. Selected organs were weighed and preserved for microscopic assessment. Blood samples were processed for subsequent analysis of haematology, coagulation and clinical chemistry parameters. Red blood cell and half-brain samples were analysed for cholinesterase activity.

All male and female rats from all groups survived until scheduled euthanasia. There were no test substance-related clinical signs or detailed clinical signs in male or female rats at any dose.

Effects related to Met XI were noted in male rats at 450 mg/kg bw per day at intervals during the study; these included reductions (often statistically significant) in body weight, body weight gain and food consumption. In female rats there were no test substance-related effects on body weight, body weight gain or food consumption at any dose.

No test substance-related differences were apparent from FOB evaluations, motor activity assessments or ophthalmological evaluations conducted during the fourth week of dosing.

In females at 450 mg/kg bw per day there were slight reductions in erythrocyte counts, haemoglobin (Hb) and haematocrit (Ht). In addition, group mean reticulocyte counts were moderately increased and RBC distribution width was consequently marginally higher in the females at 450 mg/kg bw per day than in controls. A similar pattern of findings was absent, or at least less apparent, in males at 450 mg/kg bw per day.

In the males at 450 mg/kg bw per day, there was a statistically significant decrease in terminal body weight. There were no test substance-related changes in organ weights in males at any dose. In females the absolute and relative weights of the spleen and of the nongravid uterus (with cervix and oviduct) were increased at 450 mg/kg/day.

There were no noteworthy gross necropsy observations at the completion of the dose period and nor treatment-related microscopic observations in either sex at any dose.

There were no test substance-related effects on RBC or brain ChE activity in males or females at any dose.

In conclusion, Met XI was administered to male and female rats via oral gavage for 28 consecutive days at doses of 0, 15, 50, 100 or 450 mg/kg bw. In the males at 450 mg/kg bw per day there were reductions in body weight, body weight gain and food consumption. In addition, there were changes in haematology and organ weights in females at 450 mg/kg bw per day.

In view of the above, the NOAEL was 100 mg/kg bw per day based on reductions in body weight, body weight gain and food consumption in male rats, and changes in haematology and organ weights in females at 450 mg/kg bw per day (Barnett, 2016c).

(c) Genotoxicity

The genotoxic potential of Met XI was examined in five histidine-requiring strains (TA98, TA100, TA1535, TA1537 and TA102) of *Salmonella typhimurium* (McGarry, 2014b), by its ability to induce mutation at the *tk* locus (5-trifluorothymidine resistance) in mouse lymphoma cells (Massip 2015a), and in an in vitro micronucleus assay using duplicate human lymphocyte cultures (Watters, 2014b).

The results obtained and shown below in Table 25 did not reveal any genotoxic potential in any of the assays. The Meeting concluded that Met XI (O-desmethyl omethoate) is unlikely to be genotoxic.

Table 25. Summary of genotoxicity studies of Met XI (O-desmethyl omethoate)

End-point	Test object	Methodology/concentration	Purity	Results	Reference
		In vitro			
Bacterial gene mutation	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102; Plate incorporation and preincubation assay; ± S9 mix	All treatments with S9 were further modified by the inclusion of a pre-incubation step Experiment 1: 0, 5, 16, 50, 160, 500, 1600 and 5000 µg/plate Experiment 2: 0, 16, 50, 160, 500, 1600 and 5000 µg/plate (±S9)	98.1%	Negative [a]	McGarry, 2014b
Mammalian gene mutation	*tk* locus of L5178Y mouse lymphoma cells	Experiment 1: 0, 400, 800, 1200, 1600, 1800 and 1982 µg/mL ± S9, (3 hours incubation) Experiment 2: 0, 300,600,900,1200, 1500, 1800, and 1982 µg/mL ± S9, (24 hours incubation)	98.1%	Negative [b]	Massip, 2015a
Micronucleus induction	Human lymphocytes	Tested up to 1982 µg/mL ± S9	98.1%	Negative [c]	Watters, 2014b

S9: 9000 × *g* supernatant fraction from rat liver homogenate;

[a] Study compliant with the current OECD TG 471;

[b] Study compliant with the current OECD TG 490;

[c] Study compliant with the current OECD TG 487;

(OECD TG: Organisation for Economic Co-operation and Development test guideline)

(d) Reproductive and developmental toxicity

The objective of this study was to detect adverse effects of Met XI on Crl:CD(SD) male and female rats before cohabitation, through mating and implantation, and on development of the offspring consequent to exposure of the female from implantation until postpartum day 21. The animals were administered the sodium salt of Met XI in aqueous solution (purity 41.4%; Batch/Lot no. D1707-ET-12-A). Doses, adjusted for purity, were given by oral gavage at 0, 15, 100 or 450 mg/kg bw per day. Males were given the test or vehicle control substance once daily beginning 28 days before cohabitation with females, during cohabitation and continuing until the day before euthanasia. Females were given the test or vehicle control substance once daily beginning 15 days before cohabitation with males and continuing until PPD 21.

Viability, clinical signs, food consumption, body weight, body weight changes, reproductive capacity, maternal behaviour, natural delivery observations, gross necropsy observations, organ weights, RBC and brain ChE activity, histology and histopathological evaluations were recorded.

On the day of scheduled euthanasia, F0 generation rats were anaesthetized with isoflurane/oxygen approximately 2.5 hours post dose and following blood collection from the inferior vena cava, were euthanized by an injection of sodium pentobarbital. The rats were examined for gross lesions, and were subjected to a gross necropsy of the thoracic, abdominal and pelvic viscera. Selected organs were weighed and preserved for microscopic assessment. Red blood cell and half brain samples were analyzed for ChE activity.

Pups were euthanized on PPD 21 by an intraperitoneal injection of sodium pentobarbital followed by exsanguination and examined for gross lesions. Carcasses were discarded without further evaluation.

There were no test substance-related mortalities or clinical signs observed in males or females at doses up to and including 450 mg/kg bw per day.

Although not statistically significant, an initial reduction in body weight gain was observed in males in the 450 mg/kg bw per day at all intervals until day 18 of the period before cohabitation. Thereafter body weight gain was comparable among the dose groups for the remainder of the dose period. Mean body weights within the group were only very slightly reduced compared to control with the differences being too small to be considered toxicologically relevant.

In females at 100 mg/kg bw per day a statistically significant reduction in body weight gain was observed on study days 4–8, and body weight gain overall was reduced during the period before cohabitation. Among females at 450 mg/kg bw per day, body weight gain was depressed (with statistical significance in some cases) at all premating intervals beginning on study days 4–8, and continuing through the entire period before cohabitation, study days 1–15. There were no toxicologically relevant effects on maternal body weight or body weight gain during the gestation and lactation periods at doses up to and including 450 mg/kg bw per day.

Food consumption values were unaffected by the test substance in the males and females at doses up to and including 450 mg/kg bw per day.

Premating estrous cycle was unaffected by doses of the test article as high as 450 mg/kg bw per day. All mating and fertility parameters in males and females were unaffected by doses of the test substance up to and including 450 mg/kg bw per day.

Pregnancy occurred in 10, 10, 10 and 9 mated females in the 0, 15, 100 and 450 mg/kg bw per day dose groups, respectively; all pregnant dams delivered litters.

There were no effects on litter size, viability of pups and pup body weight, and no clinical observations among the F1 generation pups that were attributable to maternal doses of the test substance up to and including 450 mg/kg bw per day. All pups that were found dead or were euthanized and necropsied on PPD 21 appeared normal at necropsy.

There were no noteworthy test substance-related necropsy observations in male or female rats. Nor were there any test substance-related effects on organ weights recorded for male or female rats at doses up to and including 450 mg/kg bw per day. Terminal body weight in both sexes was unaffected by treatment at all dose levels.

No treatment-related microscopic alterations were noted during histopathological evaluations. All stages of spermatogenesis were apparent within sections of testis from the males receiving 450 mg/kg bw per day and no treatment-related microscopic alterations were present in any of the ovarian sections.

No biologically relevant differences in RBC or brain ChE activity were observed in either sex at any dose.

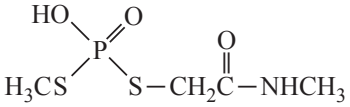
In view of the results of this study, the NOAEL for general toxicity was 450 mg/kg bw per day, the highest dose tested. Although an initial depression in body weight gain was observed in males and females during the period prior to cohabitation this was not considered to be biologically relevant because this trend did not continue through the remainder of the dose period, and the overall difference in body weights compared to control was minimal (between –0.5% and –4%).

The reproductive NOAEL was 450 mg/kg bw per day, the highest dose tested, as there were no test substance-related changes in estrous cycling in the female and no effects on mating and fertility in males or females. The NOAEL for viability and growth in the offspring was also 450 mg/kg bw per day, the highest dose tested (Barnett, 2016d).

2.4 Metabolite XII (des-O-methyl isodimethoate)

This is a minor metabolite occurring when dimethoate is sprayed on agricultural crop.

Figure 5. Chemical structure of Met XII (des-O-methyl isodimethoate)



(a) Acute toxicity

Oral administration

Study 1

A study was performed to identify the acute single dose toxicity of dimethoate and of the plant metabolites O-desmethyl omethoate carboxylic acid (Met XX) and des-O-methyl isodimethoate (Met XII) that caused ca 50% inhibition of RBC and/or brain ChE activity in rats. This was as a prerequisite for subsequent studies to identify the time to peak anticholinesterase effect after a single oral dose of each test substance.

Male and female Crl:CD(SD) rats, 68 of sex, 60 days old on receipt, were assigned to treatment groups and received single oral doses, control animals receiving vehicle only. The doses were based on body weight recorded prior to administration. The dose volume was 5 mL/kg bw for all groups. Dose levels were as follows: dimethoate, 5, 10, 20 or 30 mg/kg bw; Met XX, 10, 20, 30 or 40 mg/kg bw; Met XII, 10, 20, 30 or 40 mg/kg bw.

No mortality occurred throughout this study.

Three male (one at 10 mg/kg bw and two at 20 mg/kg bw) and three female rats (two at 10 mg/kg bw and one at 20 mg/kg bw) dosed with dimethoate were observed with miosis prior to sacrifice. No additional clinical signs were observed.

Among rats administered dimethoate, test substance-related inhibition of brain ChE was observed at 10, 20 and 30 mg/kg bw in males and in all treated female rats. Red blood cell ChE activity was inhibited at all dose levels, in both sexes. In male rats inhibition of the brain ChE activity ranged from 10.8% to 71.6% of control values, while inhibition of RBC ChE activity ranged from 27.8% to 73.0% of control. While at the lower dose levels (5 and 10 mg/kg bw) the percentage inhibition is higher for RBC ChE than brain ChE, no such difference was apparent at the higher dose levels. In female rats RBC and brain ChE activity was inhibited to the same extent, with inhibitions ranging from 26.1% to 66.5%.

For the two plant metabolites XX and XII, no ChE inhibition was observed, even at 40 mg/kg bw, the highest dose level tested in this study.

In the view of the absence of RBC or brain ChE inhibition attributable to the plant metabolites Met XX (O-desmethyl omethoate carboxylic acid), and Met XII (des-O-methyl isodimethoate) the performance of a second dose range-finding study was considered necessary.

The study was not GLP-compliant (Barnett, 2009a).

Study 2

Another study was performed to determine the tolerability of the plant metabolites O-desmethyl omethoate carboxylic acid (Met XX) and des-O-methyl isodimethoate (Met XII) at a dose level of 1000 mg/kg bw (part A), and as a second step, to evaluate the corresponding RBC and brain ChE activity levels (part B). Subsequently the RBC and brain ChE levels were tested at various time points (1, 2.5, 4 and 8 hours post dose) to identify the potential time of peak effect (the study extension).

In study part A (tolerability test) 12 rats were assigned to two dose groups of three rats/sex per group and dosed with 1000 mg/kg bw Met XX (purity 95%; Batch/Lot no. 861-IA-138-2), or 1000 mg/kg bw Met XII (purity 85.7%; Batch/Lot unknown). In the case of Met XX groups body weights were 305.7 ± 17.0 g for males, 219.0 ± 9.2 g for females: in the case of Met XII body weights were 301.3 ± 10.5 g for males, 219.3 ± 6.5 g for females. The animals were observed for viability twice daily and for clinical

symptoms and general appearance prior to test substance administration. A check for clinical signs and detailed clinical observations were performed every 30 minutes for the first four hours post-dose, then hourly until eight hours post-dose and finally prior to sacrifice at 24 hours post dose. Body weights were recorded weekly prior to dosage and approximately 24 hours after dosing.

In study part B (effects on ChE at the maximum tolerable dose) 18 rats were assigned to three dose groups each of three male and three female animals and dosed as follows: 0 mg/kg bw (vehicle control), 1000 mg/kg bw Met XX or 1000 mg/kg bw Met XII. The animals were observed for viability twice daily and for clinical symptoms and general appearance weekly during the acclimation period as well as prior to test substance administration. Detailed clinical observations were performed prior to sacrifice at 2.5 hours post dose. Body weights were recorded weekly during acclimation and prior to test substance administration.

In the study extension (investigation of time of peak effect) 60 male and 60 female rats were assigned to three dose groups (20 rats per sex per group) and dosed with 0 mg/kg bw (vehicle control), 1000 mg/kg bw Met XX or 1000 mg/kg bw Met XII. The animals were observed for viability twice daily and for clinical symptoms and general appearance weekly during the acclimation period as well as prior to test substance administration. Detailed clinical observations were performed prior to sacrifice. Body weights were recorded weekly during acclimation and prior to test substance administration.

Based on the data obtained under the conditions of this study, there was no statistically significant or biologically relevant RBC or brain ChE inhibition in male or female rats after receiving an acute oral dose of 1000 mg/kg bw of the dimethoate plant metabolites O-desmethyl omethoate carboxylic acid (Met XX) or des-O-methyl isodimethoate (Met XII) at any time point investigated.

The study was not GLP-compliant (Barnett, 2009b).

(b) Cholinesterase studies

Oral (gavage) repeat-dose cholinesterase study

Study 1

This study was conducted to determine the effect of 14 days of repeated oral gavage dosing with the sodium salt of Met XII in aqueous solution (des-O-methyl isodimethoate, Na-salt, purity 38.9%; Batch/Lot no. D1707-ET-11-A) on RBC and brain ChE activity. Groups of five male and five female Crl:CD(SD) adult rats were used, 52 days old and weighing 269–305 g for males, 182–209 g for females. The groups were administered the test article once daily by oral gavage on days 1–14 of the study at dose levels of 0, 125, 250 or 500 mg/kg bw per day. Doses were adjusted based on the most recently recorded body weight and the initiation of dose administration began at approximately the same time each day. The study also provided information on the tolerability of the test item following repeated dosing. Information gained in this study was used as the basis for dose level selection in longer term repeat-dose studies in the rat.

Viability, clinical signs, body weight, food consumption, gross necropsy observations, brain weight and ChE activity were recorded.

On study day 14 all rats were anaesthetized with isoflurane/oxygen approximately 2.5 hours post dose and, following blood collection from the inferior vena cava, were euthanized by an injection of sodium pentobarbital. The rats were examined for gross lesions, and were subjected to a gross necropsy of the thoracic, abdominal and pelvic viscera. The brain was excised, weighed, and split in half; all other tissues were discarded. Red blood cell and half brain samples were analysed for ChE activity.

All male and female rats survived until scheduled euthanasia. After two weeks of repeated dosing with Met XII sodium salt, there were no adverse clinical signs or effects on body weight gain or food consumption up to and including 500 mg/kg bw per day. There was also no indication of test substance-related inhibition of RBC or brain ChE activity in male or female rats at any dose level.

Based on this study, it was concluded that a dose level greater than 500 mg/kg bw per day should be employed as the high dose level in subsequent repeat-dose studies.

The study was not GLP-compliant (Barnett, 2015g)

Study 2

Another repeat-dose oral gavage study, employed groups of 10 male and 10 female adult Crl:CD(SD) rats, 52 day old, with body weights 229–259 g for males, 162–181 g for females. Rats were administered the sodium salt of Met XII in aqueous solution (des-O-methyl isodimethoate, Na-salt, purity37.5%; Batch/Lot no. D1707-ET-11-A) once daily on study days 1–28 at dose levels of 0, 250, 500, 750 or 1000/850 mg/kg bw per day, and a dose volume of 10 mL/kg bw. Doses were adjusted based on the most recently recorded body weights and the initiation of dose administration began approximately the same time each day.

Due to the adverse clinical observations and/or mortality, dosing of all surviving rats assigned to the 1000 mg/kg bw per day group was stopped for one day starting on day 11 to 13 (females) or 13 to 15 (males). Starting on days 12 to 14 (females) or 14 to 16 (males) the dose level was reduced to 850 mg/kg bw per day.

The following parameters and end-points were evaluated during this study: viability, clinical signs, food consumption, body weight, body weight changes, FOB, , motor activity evaluation, ophthalmic examinations, clinical pathology parameters (haematology, coagulation, clinical chemistry), gross necropsy observations, organ weights, RBC and brain ChE activity, histology and histopathological signs.

Ophthalmological examinations and FOB testing were undertaken during the last week of study. On day 28 all rats were anaesthetized with isoflurane/oxygen approximately 2.5 hours post dose and following blood collection from the inferior vena cava, were euthanized by an injection of sodium pentobarbital. The rats were examined for gross lesions, and were subjected to a gross necropsy of the thoracic, abdominal and pelvic viscera. Selected organs were weighed and preserved for microscopic assessment. Blood samples were processed for subsequent analysis of haematology, coagulation and clinical chemistry parameters. Red blood cell and half-brain samples were analyzed for ChE activity.

Prior to the reduction in top dose an increase in mortality was observed in both male and female rats. Two male rats and two female rats were euthanized due to adverse clinical signs, and two male rats and one female rat were found dead. For one of the male rats found dead and one of the prematurely sacrificed females the cause of death was concluded to be related to an intubation error and not test substance-related. The remaining deaths were considered to be test substance-related.

Common clinical signs in the rats that were either found dead or humanely euthanized included effects on gait (for example ataxia, decreased motor activity, splayed front and/or hind limbs, impaired righting reflex), and additional adverse clinical signs (including prostration, mild to severe dehydration, hyperpnea, thin body condition). In addition, one surviving male rat at 1000/850 mg/kg bw per day was observed with test substance-related findings consisting of urine-stained abdominal fur and an ungroomed coat.

Mean body weights were depressed and body weight gains reduced (with statistical significance in some cases) in male rats at 500 (by 29%), 750 (by 34%) and 1000/850 mg/kg bw per day (by 40%) beginning on day 8 and continuing through the remainder of the dosing period. In female rats at 500, 750 and 1000/850 mg/kg bw per day there was a reduction or statistically significant reduction in body weight gain on days 15 to 22. There was also a slight reduction in body weight gain observed during the first week of dose administration, and during the overall dose period in the female rats at 1000/850 mg/kg bw per day. Mean food consumption of female rats at 1000/850 mg/kg bw per day, was also reduced compared with controls during the first week of dose administration.

There were no test substance-related adverse observations apparent from ophthalmologic examinations, FOB evaluations or motor activity evaluations performed during the study.

Among female rats receiving 500, 750 and 1000/850 mg/kg bw per day and for male rats at 1000/850 mg/kg bw per day, there was an increase in animals observed with a dark red and/or large spleen at the time of scheduled necropsy. In the male rats at 1000/850 mg/kg bw per day, there were also two rats observed with a mass on the left cauda of the epididymis.

Haematology and coagulation measurements revealed decreases in erythrocyte counts, Hb and Ht levels in male and female rats at all dose levels. An increase in reticulocyte counts and RBC distribution width was also observed in all dose groups in male and female rats. Key haematological findings are shown below in Table 26.

Table 26. 28-day repeat-dose oral toxicity study in rats; summary of haematology results

		Dose (mg/kg bw per day)									
		Males					Females				
Parameter		**0**	**250**	**500**	**750**	**1000/850**	**0**	**250**	**500**	**750**	**1000/850**
Erythrocytes (10^6/µL)	Mean	7.68	7.00*	6.65*	6.50*	6.34*	7.33	6.27*	5.93*	5.73*	5.67*
	SD	0.290	0.337	0.324	0.333	0.409	0.336	0.206	0.301	0.329	0.194
	N	10	10	10	10	6	10	10	9	10	7
	%[a]	-	–8.9	–13.4	–15.4	–17.4	-	–14.4	–19.1	–21.8	–22.6
Haemoglobin (g/dL)	Mean	14.9	13.4*	12.9*	12.8*	12.7*	14.1	12.2*	12.1*	11.8*	11.9*
	SD	0.60	0.41	0.51	0.70	0.60	0.40	0.29	0.59	0.58	0.40
	N	10	10	10	10	6	10	10	9	10	7
	%[a]	-	–10.0	–13.2	–14.2	–14.5	-	–13.5	–14.4	–16.3	–16.1
Haematocrit (%)	Mean	44.8	41.2*	40.0*	39.5*	39.3*	41.6	36.8*	36.8*	35.9*	35.5*
	SD	1.94	1.40	1.45	2.09	2.28	0.93	0.95	1.51	1.85	1.61
	N	10	10	10	10	6	10	10	9	10	7
	%[a]	-	–8.1	–10.7	–11.9	–12.3	-	–11.7	–11.7	–13.7	–14.7

SD: Standard deviation; *N*: Number of rats sampled; Source: Barnett, 2016e

[a] Percentage change compared to concurrent control value;

Statistically significant difference from the control group value: * $p \leq 0.05$

With respect to clinical chemistry parameters, an increase in potassium levels was observed in males at 1000/850 mg/kg bw per day. There was also an increase in the albumin : globulin (A : G) ratio in females at 1000/850 mg/kg bw per day.

Depressed terminal body weights in relation to controls were observed in male rats at 500, 750 and 1000/850 mg/kg bw per day. Absolute and relative spleen weights were increased at all doses in both male and females. In addition, in male rats absolute and relative prostate weights were decreased in all dose groups, absolute and relative brain weights were increased at 500, 750 and 1000/850 mg/kg bw per day, and the absolute weight of the seminal vesicles with fluid and the absolute and relative weight of the adrenals were decreased at 750 and 1000/850 mg/kg bw per day. There was also a decrease in the absolute and relative thymus weights in the male rats at 1000/850 mg/kg bw per day.

Treatment-related microscopic alterations to the spleen and brain were noted in male and female rats. Splenic congestion was present in rats in all dose groups and was considered to most likely represent a sequela of treatment-related macrocytic anaemia. Brain lesions were limited to male and female rats in the 1000/850 mg/kg bw per day group, primarily restricted to the lateral aspect of the striatum (caudate-putamen) and included vacuolation of the neuropil, neuronal degeneration, microglial cell activation and reactive astrocytosis.

There was no indication in any group of test substance-related inhibition of RBC or brain or ChE activity in male or female rats after repeated exposure to the test substance.

In conclusion, 28-days of repeated oral administration of Met XII sodium salt resulted in changes in haematology, clinical chemistry parameters and organ weights at doses of 250 mg/kg bw per day and above. Body weights and/or body weight changes were depressed at 500, 750 and 1000/850 mg/kg bw per day in male and female rats. Mortality, adverse clinical signs, a reduction in food consumption (female rats) and histopathological alterations were observed at the highest dose of 1000/850 mg/kg bw per day. Based on the results of this study, the NOAEL for general toxicity was less than 250 mg/kg bw per day.

After the experimental phase had ended, a repeat analysis of the test substance performed by the sponsor confirmed that degradation had occurred in refrigerated storage prior to shipment to the testing facility and that the test substance used for preparation of dose formulations during the last three weeks of dosing was slightly degraded (purity 1.4% lower than targeted) in comparison with the material used

during the first week of dosing and for the earlier two-week dose range-finding study. It could not be ruled out that the observed toxicity in this 28-day repeat-dose study was impacted by toxic breakdown products formed within the Met XII test substance during storage (Barnett, 2016e).

(b) Genotoxicity

Metabolite XII (des-O-methyl isodimethoate) was assayed for mutational potential in four histidine-requiring strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537), and the tester strain WP2 uvrA of *Escherichia coli* (Schreib, 2013a), by its ability to induce mutation at the *tk* locus (5-trifluorothymidine resistance) in mouse lymphoma cells (Massip, 2015b), and in an in vitro micronucleus assay using duplicate human lymphocyte cultures (Watters, 2014c).

The results obtained (see Table 27) did not reveal any genotoxic potential in any of the assays. The Meeting concluded that Met XII is unlikely to be genotoxic.

Table 27. Summary of genotoxicity studies of Met XII (des-O-methyl isodimethoate)

End-point	Test object	Concentration	Purity	Results	Reference
		In vitro			
Bacterial gene mutation	*Salmonella typhimurium* strains TA98, TA100, TA1537 and *E. coli* WP2 uvrA ± S9 mix	Experiment 1: (plate incorporation assay) 31.6, 100, 316, 1000, 2500 and 5000 µg/plate Experiment 2: (pre-incubation assay) 31.6, 100, 316, 1000, 2500 and 5000 µg/plate	84.2%	Negative [a]	Schreib, 2013b
Mammalian gene mutation	*tk* locus of L5178Y mouse lymphoma cells	Experiment 1: 0, 400, 800, 1000, 1500,2000, 2143 µg/mL ± S9 (3 hours incubation) Experiment 2: 0, 400, 800, 1000, 1200, 1400,1600, 1800 and 2000 µg/mL –S9, (24 hours incubation) 0, 300, 600, 900, 1200, 1500, 1800 and 2143 µg/mL +S9, (24 hours incubation)	84.2%	Negative [b]	Massip, 2015b
Micronucleus induction	Human lymphocytes	0, 800,1200, 1750, & 2143 µg/mL ± S9	84.2%	Negative [c]	Watters, 2014c

S9: 9000 × *g* supernatant fraction from rat liver homogenate;

[a] Study compliant with the current OECD TG 471;

[b] Study compliant with the current OECD TG 490;

[c] Study compliant with the current OECD TG 487;

(OECD TG: Organisation for Economic Co-operation and Development test guideline)

(d) Reproductive and developmental toxicity

A study was conducted with the objective of detecting adverse effects of Met XII on Crl:CD(SD) male and female rats before cohabitation, through mating and implantation, and on the development of offspring consequent to exposure of the female from implantation until PPD 21. Rats were administered the sodium salt of Met XII in aqueous solution (purity 45.2%: Batch/Lot no. P1707HRM-DMT-91-X) with the dose calculation adjusted for purity, via oral gavage at 0, 15, 50, 100 or 450 mg/kg bw per day.

Males were given the test or vehicle control once daily beginning 28 days before cohabitation with females, during cohabitation and continuing until the day before euthanasia. Females were given the test or vehicle control substance once daily by oral gavage beginning 15 days before cohabitation

with males and continuing until PPD 21.

Viability, clinical signs, food consumption, body weight, body weight changes, reproductive capacity, maternal behaviour, natural delivery observations, clinical pathology parameters (haematology, coagulation and clinical chemistry), gross necropsy observations, organ weights, RBC and brain ChE activity, histology and histopathological findings were recorded.

On the day of scheduled euthanasia, F0 generation rats were anaesthetized with isoflurane/oxygen approximately 2.5 hours post dose and following blood collection from the inferior vena cava, were euthanized by an injection of sodium pentobarbital. The rats were examined for gross lesions and subjected to a gross necropsy of the thoracic, abdominal and pelvic viscera. Selected organs were weighed and preserved for microscopic assessment. Blood samples were processed for subsequent analysis of haematology, coagulation and clinical chemistry parameters. Red blood cell and half-brain samples were analyzed for ChE activity. Pups were euthanized on PPD 21 and examined for gross lesions.

No test substance-related mortalities nor test article-related adverse clinical signs were observed in either male or female rats at doses up to and including 450 mg/kg bw per day.

Body weight, body weight gains and food consumption were unaffected in both male and female rats at doses up to an including 450 mg/kg bw per day.

A slight decrease in body weight gain during the premating period in females at 450 mg/kg bw per day was not considered to be treatment-related and was of limited toxicological significance.

Estrous cycling prior to cohabitation and all mating and fertility parameters in the males and females were unaffected at any of the doses administered.

Pregnancy occurred in 11, 10, 11, 12 and 12 mated females in the 0, 15, 50, 100 and 450 mg/kg bw per day groups, respectively; a total of 11 (100%), 10 (91%), 11 (100%), 12 (100%) and 12 (100%) of the respective pregnant dams delivered litters.

There were no effects on litter size, viability of pups or pup body weight, and no clinical observations in the F1 pups that were attributable to maternal doses of the test substance up to the highest dose tested.

Terminal body weights were not affected by the test substance in either sex at any dose level. Absolute and relative spleen weights were increased in males and females at 450 mg/kg bw per day.

There was an increase in the number of male rats presenting with dark red and/or large spleens at terminal necropsy at and above 50 mg/kg bw per day, and in the females at 450 mg/kg bw per day. The number of female rats observed with a small thymus was also increased at and above 50 mg/kg bw per day.

In both males and females changes were observed in haematology parameters, consisting of reductions in red cell mass (by 6% in males, 18% in females) and increases in reticulocytes (by 55% in males, 154% in females) at 450 mg/kg bw per day on study day 14. At scheduled euthanasia this increase in reticulocytes was apparent at 100 mg/kg bw per day (males only) and 450 mg/kg bw per day (males and females).

Splenic congestion and increased amounts of pigment within the reticuloendothelial cells were noted in males and females at and above 50 mg/kg bw per day. These histological changes were considered to most likely have resulted from increased red blood cell removal (and destruction) by the spleen. All stages of spermatogenesis were apparent within sections of testis from the 450 mg/kg bw per day group males and no treatment-related microscopic alterations were present in any of the ovarian sections.

No biologically relevant differences in the RBC or brain ChE activity were observed in either sex at any dose.

In view of the above it was concluded that when Met XII was administered to male and female rats via gavage before cohabitation, through mating and implantation, and development of the offspring consequent to exposure of the female from implantation through PPD 21 at doses of 0, 15, 50, 100 and 450 mg/kg bw per day, treatment at and above 50 mg/kg bw per day in males and 450 mg/kg bw per day in females was associated with effects on the spleen, specifically, reddening and/or increase in spleen size, histopathological findings of splenic congestion and increased amounts of pigment within

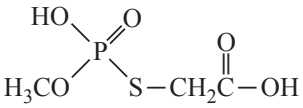
the reticuloendothelial cells, and in addition increased spleen weights in both sexes at 450 mg/kg bw per day. The number of females observed with a small thymus was also increased at and above 50 mg/kg bw per day. At 100 (males only) and 450 mg/kg bw per day (males and females) changes were observed in haematology parameters (reductions in red cell mass and increases in reticulocytes).

Based on the results of this study, the NOAEL for general toxicity was 15 mg/kg bw per day in the male and female rats based on the effects on the spleen (reddening and/or increased size) and histopathological findings of splenic congestion at 50mg/kg bw per day. The reproductive NOAEL was 450 mg/kg bw per day , the highest dose tested, as there were no test substance-related changes in estrous cycling, mating or fertility. The NOAEL for offspring toxicity was also 450 mg/kg bw per day, the highest dose tested (Barnett, 2016f).

2.5 Metabolite XX (O-desmethyl omethoate carboxylic acid)

This is a minor metabolite occurring when dimethoate is sprayed on agricultural crops.

Figure 6. Chemical structure of Met XX (O-desmethyl omethoate carboxylic acid)



(a) Acute toxicity

Oral administration

Table 28. Summary of acute toxicity study (fixed dose method) on Met XX

Species	Strain	Sex	Route	Purity	Result	Reference
Rat [a]	Sprague Dawley CD strain	Female	Oral (gavage)	98.3%	300< LD_{50}<2000	Barnett, 2009c

LD_{50}: Median lethal dose

[a] The animal treated at a dose level of 2000 mg/kg bw and one animal treated at a dose level of 300 mg/kg bw were found dead one day after dosing

An acute oral toxicity study was conducted adopting the fixed dose method.

Hunched posture and ataxia were noted four hours after dosing in two animals treated at 300 mg/kg bw. Three animals treated at a dose level of 300 mg/kg bw appeared normal throughout the observation period and the remaining surviving animals appeared normal again one day after dosing.

All surviving animals showed expected gains in body weight.

Abnormalities noted at necropsy of the animals that died during the study consisted of haemorrhagic or abnormally red lungs, dark liver, dark kidneys and epithelial sloughing of the gastric mucosa. No abnormalities were noted at necropsy of the remaining animals treated at 300 mg/kg bw that were necropsied at the end of the observation period.

The LD_{50} of the test material in the rat was estimated to be greater than 300 mg/kg bw but less than 2000 mg/kg bw (Barnett, 2009c).

(b) Cholinesterase study

This study was designed to provide information on toxicity, indicate target organs and provide an estimate of NOAEL that could be used for establishing safety criteria for human exposure. It evaluated the effects of repeated oral doses of the disodium salt of Met XX, in aqueous solution (O-desmethyl omethoate carboxylic acid Na salt) on male and female Crl:CD(SD) rats and in particular measured ChE activity in brain and RBCs in dosed rats.

Metabolite XX (purity 30.6%; Batch/Lot no. D1707-ET-27-A) was administered via oral gavage to groups of 10 male and 10 female rats (body weights 216–269 g for males, 162–192 g for females)

aged 52 days, for 28 consecutive days at doses of 0, 25, 100, 400/300 mg/kg bw per day (groups 1–4 respectively). As the high dose of 400 mg/kg bw per day resulted in mortality, adverse clinical signs and reductions in body weight gain and food consumption during the first week of dose administration, it was reduced to 300 mg/kg bw per day on study day 6 or 7 for the female rats and 8 or 9 for the male rats .

The following parameters and end-points were evaluated in this study: viability, clinical signs, food consumption, body weight, body weight changes, FOB testing, detailed clinical observations, motor activity, ophthalmic status, clinical pathology parameters (haematology, coagulation, clinical chemistry), gross necropsy observations, organ weights, RBC and brain ChE activity, histology and histopathological findings.

Prior to the reduction in top dose to 300 mg/kg bw per day, one male and two females rats were found dead. These deaths were considered to be test substance-related based on the microscopic lesions observed in the brain, heart, lungs or kidneys of these rats. All other male and female rats survived until scheduled euthanasia.

In the 400/300 mg/kg bw per day group there was a statistically significant increase in the number of females observed with mild or moderate dehydration. The females at 400/300 mg/kg bw per day observed with dehydration were also seen to exhibit a hunched posture, ataxia, decreased motor activity and/or ptosis.

Rats in the high dose group demonstrated a reduction in body weight gain and food consumption during the first week of dosing (400 mg/kg bw per day during this period). Once the dose was reduced to 300 mg/kg bw per day a rapid recovery was observed and the body weight gains and food consumption values then remained comparable across the dose groups for the remainder of the dose period.

There were no test substance-related differences in FOB evaluations, motor activity assessments or ophthalmological evaluations conducted during the fourth week of dosing.

At the end of the dose period, no test substance-related changes in haematology, clinical chemistry or coagulation parameters were observed at any dose level.

There were no test substance-related effects on terminal body or organ weights in males or females at any dose level. There were also no noteworthy test substance-related macroscopic observations at necropsy examination.

A variety of spontaneous (not treatment-related) background histological alterations were observed during this study. Lesions considered treatment-related were limited to four rats assigned to the 400/300 mg/kg bw per day dose group (three of these rats died while receiving the initial higher dose and one rat that had survived to scheduled euthanasia). The findings suggest that the test substance-related lesions were probably initiated during the administration of 400 mg/kg bw per day. As a result, the pathologist considered that the replacement high dose of 300 mg/kg bw per day was likely to represent the NOAEL for the rat when administered under the conditions of the study.

No biologically relevant differences in RBC or brain ChE activity were observed in either sex at any dose.

Based on the results of this study, the no-observed-effect level (NOEL) for general toxicity was 100 mg/kg bw per day, based on effects equivocally attributable to the test article at 300 mg/kg bw per day (Barnett, 2016g).

(c) Genotoxicity

Met XX (O-desmethyl omethoate carboxylic acid) was assayed for mutational potential in five histidine-requiring strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537 and TA102) and *Escherichia coli* WP2 uvrA (McGarry, 2014c), for the ability to induce mutation at the *tk* locus (5-trifluorothymidine resistance) in mouse lymphoma cells (Keig-Shevlin, 2015b) and in an in vitro micronucleus assay using duplicate human lymphocyte cultures prepared from the pooled blood of two female donors in a single experiment (Watters, 2015b).

The results obtained did not show any genotoxic potential in any of the assays. The Meeting concluded that Met XX (O-desmethyl omethoate carboxylic acid) is unlikely to be genotoxic.

Table 29. Summary of genotoxicity studies with Met XX

End-point	Test object	Concentration	Purity	Results	Reference
		In vitro			
Bacterial gene mutation	*Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 *Escherichia coli* WP2 uvrA	Experiment 1: (plate incorporation) 31.6, 100, 316, 1000, 2500 and 5000 µg/plate; ± S9 Experiment 2: (pre-incubation assay) 31.6, 100, 316, 1000, 2500 and 5000 µg/plate; ± S9	92.8%	Negative [a]	McGarry, 2014c
Mammalian gene mutation	*tk* locus of L5178Y mouse lymphoma cells	Experiment 1: 0, 100, 400, 1000, 1300, 1600 and 1852 µg/mL ± S9 (3 hours incubation) Experiment 2: 0, 600, 900, 1200, 1500, 1600, 1700 and 1852 µg/mL –S9, (24 hours incubation) 0, 300, 600, 900, 1200, 1600 and 1852 µg/ml +S9, (24 hours incubation)	98.3%	Negative [b]	Keig-Shevlin, 2015b
Micronucleus induction	Human lymphocytes	Tested up to 1852 µg/mL; ± S9	98.3%	Negative [c]	Watters, 2014b

S9: 9000 × *g* supernatant fraction from rat liver homogenate;

[a] Study compliant with the current OECD TG 471;

[b] Study compliant with the current OECD TG 490;

[c] Study compliant with the current OECD TG 487;

(OECD TG: Organisation for Economic Co-operation and Development test guideline)

(d) Reproductive and developmental toxicity

A study was undertaken with the objective of detecting adverse effects due to Met XX in Crl:CD(SD) male and female rats before cohabitation, through mating and implantation, and on the development of offspring consequent to exposure of females from implantation until PPD 21. Rats were administered the sodium salt of Met XX in aqueous solution (O-desmethyl omethoate carboxylic acid Na salt, purity 30.6%; Batch/Lot no. D1707-ET-27-A) by oral gavage at doses, adjusted for purity, of 0, 25, 100 and 300 mg/kg bw per day.

Male rats were given the test article or vehicle control once daily by beginning 28 days before cohabitation with females, during cohabitation and continuing until the day before euthanasia. Males were given 63 (20 rats) or 64 (20 rats) administrations. Doses were adjusted based on the most recently recorded body weight and administered at approximately the same time each day. The first day of dosing was designated day 1.

Female rats were given the test or vehicle control once daily beginning 15 days before cohabitation with males and continuing PPD 20 (LD 20 for rats that delivered a litter) or GD 24 (rats that did not deliver a litter). Lactating females that survived until scheduled euthanasia were given 56 (two rats), 58 (six rats), 59 (eight rats), 60 (four rats), 61 (three rats) and 62 (four rats) administrations. Any dam in the process of parturition was not given the test substance or the vehicle control formulation until the following work day.

Pups of the F1 generation were not dosed directly, but were possibly exposed to the test or vehicle control formulations during maternal gestation (in utero exposure) or via maternal milk during the lactation period.

Viability, clinical signs, food consumption, body weight, reproductive capacity, maternal behaviour, natural delivery observations, clinical pathology parameters (haematology, coagulation and clinical chemistry), gross necropsy observations, organ weights, RBC and brain ChE activity, histology and histopathological evaluations were recorded.

All male rats survived until scheduled euthanasia at doses up to and including 300 mg/kg bw per day.

Prior to termination of females in the 300 mg/kg bw per day group, there was an increase in mortality observed in on GDs 21 or 22. There were five females from the 300 mg/kg bw per day group that were found dead, and an additional female that was euthanized due to adverse clinical signs. This euthanized female was observed to be prostrate and showed laboured breathing prior to euthanasia on GD 21. At necropsy this female was found to have an oedematous pancreas. No test substance-related adverse clinical signs were observed in the five females that were subsequently found dead, however, four of the five females were observed to have numerous black areas on the mucosal surface of the stomach. One female in the 100 mg/kg bw per day dose group that was euthanized due to adverse clinical signs on GD 20. This female displayed chromodacryorrhea, pale extremities, decreased motor activity, chromorhinorrhea, severe dehydration, hunched posture, coldness to the touch and piloerection on the day of euthanasia. Necropsy revealed this rat to have large adrenal glands, a small thymus, pale liver (all lobes) and red fluid in the uterus. The deaths observed in the 300 mg/kg bw per day dose groups were considered to be test substance-related. Another female at 100 mg/kg bw per day showed signs of acute ill health (including chromodacryorrhea, pale extremities, decreased motor activity, chromorhinorrhea, severe dehydration, hunched posture, coldness to the touch and piloerection),and was therefore euthanized and subjected to pathological examination. Mild foci of peracute hepatocellular necrosis, enlarged cortical cells within the zona fasciculata of the adrenal gland and acute degeneration of lymphoid cells within the thymus were reported. It was noted that the adrenal and thymic changes present in this female rat was most suggestive of reactions to acute stress. The hepatocellular necrosis present in this rat is of uncertain pathogenesis, but was peracute in nature so may also represent a response to haemodynamic changes associated with stress. In addition, it was noted that foci of hepatocellular necrosis are occasionally encountered as nonspecific lesions in control group rats. One female in the 25 mg/kg bw per day group was found dead on study day 7 and was observed with chromodacryorrhea, pale extremities and hyperpnea prior to being found dead. At necropsy this rat was revealed to have a perforation in the oesophagus, cloudy red fluid and tan granular material in the thoracic cavity and an oedematous thymus. Based on the clinical and necropsy observations, this death was concluded to be related to an intubation error. All other female rats survived until scheduled euthanasia.

In the 100 mg/kg bw per day group, one female rat exhibited negative signs on GD 20 (the day of euthanasia) and was subsequently euthanized. These clinical signs included chromodacryorrhea, pale extremities, decreased motor activity, chromorhinorrhea, severe dehydration, hunched posture, coldness to the touch and piloerection. The cause of demise for this rat was considered most likely to be unrelated to an effect of the test substance. In the 300 mg/kg bw per day dose group, there was an increase in the number of males observed with urine-stained abdominal fur.

At doses up to and including 300 mg/kg bw per day, body weight, body weight gains and food consumption were unaffected in male rats, and there was no effect in the female rats during the premating or gestation periods. During the lactation period there were no test substance-related effects on maternal body weight, body weight gains or food consumption at dose levels up to and including 100 mg/kg bw per day.

Estrous cycling observations prior to cohabitation (mean estrous stages per 14 days, rats with six or more consecutive days in diestrus and rats with six or more consecutive days of estrus) were unaffected by doses of the test substance up to and including 300 mg/kg bw per day. All mating and fertility parameters in males and females were unaffected by doses of the test substance up to and including 300 mg/kg bw per day.

Pregnancy occurred in 10, 8, 10 and 9 mated females in the 0, 25, 100 and 300 mg/kg bw per day groups, respectively. Six pregnant dams in the 300 mg/kg bw per day group were either found dead or euthanized before scheduled termination, as previously described. All surviving dams delivered a litter.

There was a slight reduction in the total number of pups delivered in the two litters that were delivered by 300 mg/kg bw per day dams; mean value of 7.5 pups per litter, compared with 13.2 pups per litter in the concurrent control group. One pup from the 300 mg/kg bw per day group was missing and presumed cannibalized on PND 1. Due to the small number of females remaining in the high dose group, it was decided that the group was of diminished use in evaluating postnatal parameters and therefore the two surviving dams and their litters were subject to early termination on LDs 1 and 2.

There was a statistically significant increase in the number of females in the 300 mg/kg bw per day group exhibiting numerous black areas on the mucosal surface of the stomach. Additionally one 300 mg/kg bw female presented with an oedematous pancreas at necropsy. As a result of these findings, there was a statistically significant reduction in the number of females at 300 mg/kg bw per day that appeared normal at scheduled euthanasia. There were no test substance-related necropsy findings observed in the male rats.

Marginally higher blood chloride levels were apparent in females at 300 mg/kg bw per day when compared with concurrent controls. The observation was considered likely to be related to the presence of sodium in the dosing formulation and not a direct effect of the free metabolite. A statistically significant increase in bile acid level was observed in male rats at 300 mg/kg bw per day at scheduled euthanasia. No additional test substance-related changes in haematology, clinical chemistry or coagulation parameters were observed at any dose.

No biologically relevant differences in RBC or brain ChE activity were observed in male rats at doses up to and including 300 mg/kg bw per day and in female rats at doses up to and including 100 mg/kg bw per day; scheduled samples for females dosed at 300 mg/kg bw per day were not evaluated.

No treatment-related microscopic alterations were seen in the tissue sections evaluated in this study, but a number of the female rats (including all 10 females in the 300 mg/kg bw per day group) had either died or were euthanized prior to the end of the study. Only a limited number of tissues (brain, ovaries and tissues in which gross changes were observed at necropsy) were examined from these females, but these tissues showed changes suggesting nonspecific stress. This stress may have been centrally mediated as a result of the test substance, but no lesions were observed in brain sections stained with haemotoxylin and eosin. No treatment-related microscopic alterations were present in any of the tissues from male rats examined in this study. All stages of spermatogenesis were apparent within sections of testis from the 300 mg/kg bw per day males and no treatment-related microscopic alterations were observed in any of the ovarian sections.

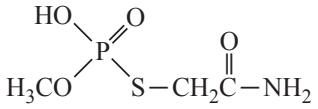
In view of the results of this study, the NOAEL for general toxicity was 100 mg/kg bw per day. At the LOAEL of 300 mg/kg bw per day, findings in the males were of uncertain relationship to any effect of the test substance and of minor toxicological significance, for instance an increase in urine-stained abdominal fur during the in-life portion of the study as well as a marginal increase in blood chloride concentration. By contrast, in the female rats at 300 mg/kg bw per day, there was an increase in mortality during late-stage gestation resulting in the necessary termination of this group. At necropsy there was an increase in the number of females in this group observed to have numerous black areas on the mucosal surface of the stomach. When the histopathological evaluation of these lesions was performed, they appeared suggestive of non-specific stress that may have been centrally mediated as a result of the test substance.

The reproductive NOAEL in this study was 300 mg/kg bw per day, the highest dose tested, as there were no test substance-related changes in estrous cycling in the female, nor effect on mating and fertility in the males or females. The NOAEL for offspring toxicity was 100 mg/kg bw per day (Barnett, 2016h).

2.6 Metabolite XIII (O-desmethyl-N-desmethyl omethoate)

This is a significant metabolite occurring when dimethoate is sprayed on agricultural crops.

Figure 6. Chemical structure of Met XXIII (O-desmethyl-N-desmethyl omethoate)



(a) Acute toxicity

Oral acute tolerability study

A study was undertaken to determine potential toxicity of Met XXIII following a single oral administration of its, sodium salt in aqueous solution to Crl:CD(SD) rats. Met XXIII (O-desmethyl-N-desmethyl omethoate Na salt, purity 40.3%; Batch /Lot no. D1707-ET-31-A) was administered to six rats (three of each sex) once by oral gavage at a dose of 1000 mg/kg bw.

Viability was checked at least twice daily. Rats were observed for clinical observations and general appearance once during the acclimation period, immediately prior to dose administration, between one and two hours after dose administration, and then daily during the post dose period (eight days). Body weight was recorded once during the acclimation period, on the day of dose administration and daily during the post dose period. Food consumption was not measured, however food was monitored and replenished as necessary to maintain the health and well-being of the animals.

One male rat dosed at 1000 mg/kg bw died on day 5 of the observation period. The rat was examined for the cause of death as soon as possible after the observation was made. The lungs, trachea and oesophagus were perfused and no perforations noted. The rat was examined for gross lesions and subjected to a gross necropsy of the thoracic, abdominal and pelvic viscera. All tissues were discarded. A dose level of 1000 mg/kg bw of Met XXIII was generally well tolerated. There were no clinical signs of toxicity or effects on body weight noted during the course of the study.

Based on the results of this study, a dose level of 1000 mg/kg bw was considered tolerable for subsequent single-dose studies in the rat.

The study was not GLP-compliant. (Barnett, 2014).

(b) Cholinesterase studies

Study 1

The objective of this study was to determine the effect of an acute dose administration of the sodium salt of Met XXIII in aqueous solution RBC and brain ChE activity in Crl:CD(SD) adult rats The test substance (purity 40.3%; Batch /Lot no. D1707-ET-31-A) at a dose of 1000 mg/kg bw was given orally by gavage to 10 male and 10 female adult rats as a single dose. A vehicle control was also included in the study.

Approximately 2.5 hours post dose, all rats were anaesthetized and blood collected from the inferior vena cava. Rats were examined for gross lesions, and subjected to gross necropsy of the thoracic, abdominal and pelvic viscera. Brains were excised, weighed and the left half was evaluated for ChE activity; all other tissues were discarded.

Red blood cell ChE values 2.5 hours after administration were slightly reduced in both male and female rats of the 1000 mg/kg bw groups compared to controls; reduction was by 13.9% in males and 19.1% in females, achieving statistical significance in females. By contrast there was no indication of test substance-related inhibition of brain ChE activity in male or female rats dosed with 1000 mg/kg bw of Met XXIII. The dose level of 1000 mg/kg bw was therefore considered to be an appropriate dose level for use in subsequent acute ChE activity studies in the rat. The study was GLP-compliant (Barnett, 2015h).

Study 2

A study was carried out to measure RBC and brain ChE activity at various time points after a single oral administration of the sodium salt of Met XXIII in aqueous solution to adult Crl:CD(SD) rats. Animals were administered the test article (purity 40.3%; Batch /Lot no. D1707-ET-31-A) at a dose level of 1000 mg/kg bw. As a control group five rats of each sex per time point, (just 4 and 16 hours post dose) were utilized for cholinesterase control evaluations. Treatment groups (1000 mg/kg bw) consisted of five rats of each sex per time point, sampled at four time points (1, 4, 8 and 16 hours post dose) for cholinesterase evaluations

Viability, clinical signs, body weight, brain weight, RBC and brain ChE activity were evaluated.

There were no effects on survival or body weight during the study.

The mean RBC ChE values in female rats at 1000 mg/kg bw were slightly lower or statistically significantly lower ($p \leq 0.05$ to $p \leq 0.01$). Values were lower when compared with concurrent controls by 22.2% at one hour, by 19.4% at four hours, by 22.9% at eight hours, and by 19.6% at 16 hours post dose. In male rats at 1000 mg/kg bw, the RBC ChE values were comparable to control values at all time points, and there were no statistically significant differences. Brain ChE values in both the males and females at 1000 mg/kg bw were comparable to their control values at all time points, and there were no statistically significant differences. Despite the AChE effects in females that are outlined above, values in all treated animals were within historical control ranges, with a single, atypically high value for RBC cholinesterase activity noted in a concurrent control animal at 4 and 16 hours post dose. There was no obvious temporal relationship across the time points measured. The differences in group mean values between concurrent control and treated values at these time points is therefore of uncertain relationship to any effect of the test item and was considered of equivocal toxicological significance.

The study was GLP-compliant (Barnett, 2015i).

Study 3

Another study was undertaken to determine the effect of an acute dose of the sodium salt of Met XXIII, in aqueous solution on RBC and brain ChE activity in adult Crl:CD(SD) rats. The test article (purity 40.3%; Batch /Lot no. D1707-ET-31-A) was administered via oral gavage, as a single dose, to five groups of rats, each consisting of 10 males and 10 females, at dose levels of 0, 250, 500, 750 or 1000 mg/kg bw.

Viability, clinical signs, body weight, brain weight, RBC and brain ChE activities were recorded. Whole blood samples (3.0 mL) were collected approximately one hour after dose administration for females, or approximately 2.5 hours after dose administration for males (timing began with gavage and ended with blood collection).

There were no effects on survival, body weight or macroscopic necropsy observations.

Values of RBC ChE in females were lower than in comparable controls (statistically significant at $p \leq 0.05$ to $p \leq 0.01$) at all dose levels up to and including 1000 mg/kg bw. In male rats, RBC ChE activity was lower (sometimes with statistical significance at $p = 0.01$ or smaller) at the 750 and 1000 mg/kg bw dose levels as compared to controls. Observed differences between treated group means and vehicle controls was small (less than 20%), there was no apparent dose–effect relationship observed with either sex, and the values observed were within expected ranges for rats of this strain and age. Therefore the differences between the treated values and the concurrent control values were not considered to be treatment-related. A summary of results from this study is shown below in Table 30.

Table 30. Single dose study of the effect of Met XXIII on RBC cholinesterase activity in rats

	Group	Dose level (mg/kg bw)	Mean ChE activity (U/mL) ± SD [*N*]	Percentage decrease compared with controls
Male adult rats				
2.5 hours	1	0	1.396 ± 0.155 [10]	-
	2	250	1.363 ± 0.085 [10]	2.4%
	3	500	1.386 ± 0.199 [10]	0.7%
	4	750	1.156 ± 0.101 [10]**	17.2%
	5	1000	1.254 ± 0.185 [10]	10.2%
Female adult rats				
1 hour	1	0	1.497 ± 0.119 [10]	-
	2	250	1.358 ± 0.132 [10]*	9.3%
	3	500	1.326 ± 0.101 [10]*	11.4%
	4	750	1.284 ± 0.170 [10]**	14.2%
	5	1000	1.301 ± 0.154 [10]**	13.1%

N: The number of rats evaluated for cholinesterase activity; Source: Barnett, 2015j

SD: Standard deviation;

Statistically significant difference from the control group value: * $p \leq 0.05$, ** $p \leq 0.01$

In male and female rats at doses up to and including 1000 mg/kg bw there were no statistically significant or biologically important differences in brain ChE activity when compared with concurrent control values.

The NOAEL was 1000 mg/kg bw, the highest dose tested.

The study was GLP-compliant (Barnett, 2015j).

Oral repeat-dose cholinesterase study

A study was conducted to evaluate and characterize the general toxicity of Met XXIII, including measuring of RBC and brain ChE activity in rats, using repeat-dose oral administrations. Male and female Crl:CD(SD) rats,were administered Met XXIII (purity 40.3%; Batch /Lot no. D1707-ET-31-A) via oral gavage for 28 consecutive days at doses of 0, 10, 50, 100 and 450 mg/kg bw per day (Groups 1–5, respectively).

The following parameters and end-points were evaluated in this study: viability, clinical signs, food consumption, body weight, body weight changes, FOB, detailed clinical observations, motor activity evaluation, ophthalmic status, clinical pathology parameters (haematology, coagulation, clinical chemistry), gross necropsy observations, organ weights, RBC and brain ChE activity, histology and histopathological findings.

All rats in all dose groups survived until scheduled euthanasia. There were no test substance-related clinical signs or detailed clinical signs in male or female rats at any dose level. In addition, there were no test substance-related effects on body weight, body weight gain, or food consumption in male or female rats at any dose. There were no test substance-related differences in FOB evaluations, motor activity assessments or ophthalmological evaluations conducted during the fourth week of dosing. At the end of the dose period, no test substance-related changes in coagulation parameters were observed at any dose level. There were no test substance-related effects on terminal body or organ weights in males or females at any dose level. No test substance-related macroscopic observations were noted at necropsy.

Test substance-related changes in haematology parameters included a slightly lower (statistically significant) erythrocyte concentration and Ht in the males and females and slightly lower Hb levels in females, all at 450 mg/kg bw per day. Reticulocyte concentrations were slightly higher in males and females (statistically significant in females only) at 450 mg/kg bw per day, and statistically significant increases in chloride levels in males and females at 450 mg/kg bw per day.

There were statistically significant reductions in RBC ChE activity at 450 mg/kg bw per day compared to concurrent controls, by 21% in males and 35% in females. There was considered to be no test substance-related changes in brain cholinesterase activity in either sex at any dose level.

In view of the above, the NOAEL was 100 mg/kg bw per day based on changes in haematology, clinical chemistry and reductions in RBC cholinesterase activity observed in both males and females at 450 mg/kg bw per day (Barnett, 2016i).

(c) Genotoxicity

Met XXIII (O-desmethyl-N-desmethyl omethoate) was assayed for mutational potential in five histidine-requiring strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537 and TA102) (McGarry, 2015), for the ability to induce mutation at the *tk* locus (5-trifluorothymidine resistance) in mouse lymphoma cells (Keig-Shevlin, 2015c) and in an in vitro micronucleus assay using duplicate human lymphocyte cultures (Watters, 2014d). The results revealed no genotoxic potential. The Meeting concluded that Met XXIII (O-desmethyl-N-desmethyl omethoate) is unlikely to be genotoxic.

Table 31. Summary of genotoxicity studies of dimethoate Met XXIII

End-point	Test object	Concentration	Purity	Results	Reference
		In vitro			
Bacterial gene mutation	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 ± S9 mix	Experiment 1: 0, 5, 16, 50, 160, 500, 1600 and 5000 µg/plate Experiment 2: 0, 160, 300, 625, 1250, 2500 and 5000 µg/plate	99.0%	Negative [a]	McGarry, 2015
Mammalian gene mutation	*tk* locus of L5178Y mouse lymphoma cells	Experiment 1: 0, 400, 800, 1000, 1500, 1700 and 1842 µg/mL; ± S9 Experiment 2:, 0, 400, 800, 1000, 1500,1700 and 1842 µg/mL –S9, (24 hours incubation) 0, 400, 800, 1000, 1500,1700 and 1842 µg/mL +S9, (3 hours incubation)	99.0%	Negative [b]	Keig-Shevlin, 2015c
Micronucleus induction	Human lymphocytes	Tested up to 1842 µg/mL; ± S9	99.0%	Negative [c]	Watters, 2014d

S9: 9000 × *g* supernatant fraction from rat liver homogenate;

[a] Study compliant with the current OECD TG 471;

[b] Study compliant with the current OECD TG 490;

[c] Study compliant with the current OECD TG 487;

(OECD TG: Organisation for Economic Co-operation and Development test guideline)

(d) Oral reproduction and developmental toxicity

A study was undertaken with the objective of detecting adverse effects in Crl:CD(SD) male and female rats administered Met XXIII before cohabitation, through mating and implantation, and on the development of the offspring consequent to exposure of the female from implantation until PPD 21.Rats were administered, by oral gavage, the sodium salt of Met XXIII in aqueous solution (purity 40.3%; Batch /Lot no. D1707-ET-31-A) at doses of 0, 10, 25, 75 and 450 mg/kg bw per day, the doses being adjusted for purity. Males were dosed once daily beginning 28 days before cohabitation with females, during cohabitation and continuing until the day before euthanasia. The first day of dosing was designated day 1. Females were dosed once daily by oral gavage beginning 15 days before cohabitation with males,

and continuing until PPD 21 (LD 21). Lactating females that survived until scheduled euthanasia were given 57 (nine rats), 58 (10 rats), 59 (11 rats), 60 (13 rats), 61 (six rats) and 68 (one rat) doses of the test article or vehicle control. Any dam in the process of parturition was not dosed until the following day.

In the case of males and females doses were adjusted based on the most recently recorded body weight and administered at approximately the same time each day.

F1 generation pups were not dosed directly, but were possibly exposed to the test or vehicle control formulations during maternal gestation (in utero exposure) and/or via maternal milk during the lactation period.

The following parameters and end-points were evaluated in this study: viability, clinical signs, food consumption, body weight, reproductive capacity, maternal behaviour, natural delivery observations, gross necropsy observations, organ weights, RBC and brain ChE activity, clinical pathology parameters (haematology, coagulation, clinical chemistry), histology and histopathological findings.

All male and female rats survived until scheduled euthanasia at doses up to and including 450 mg/kg bw per day. No test substance-related clinical signs were observed in males or females at doses up to and including 450 mg/kg bw per day.

Body weights and food consumption were unaffected by the test substance during the dose period in the males and during the premating, gestation and lactation periods in the females at doses up to and including 450 mg/kg bw per day.

Estrous cycling prior to cohabitation (mean estrous stages per 14 days, rats with six or more consecutive days in diestrus and rats with six or more consecutive days of estrus) was unaffected by doses of the test substance as high as 450 mg/kg bw per day. All mating and fertility parameters in the males and females were unaffected up to the highest dose tested.

All male and female rats appeared normal at necropsy. Nor were there any test substance-related effects on organ weights in male or female rats at doses up to and including 450 mg/kg bw per day.

Pregnancy occurred in females at all doses and all of these pregnant dams delivered litters.

There were no test substance-related effects on pup body weight and no clinical observations in the F1 generation pups. There were no test substance-related effects to be seen in the pups that were found dead or were euthanized and necropsied on PPD 21 at the time of necropsy.

At the end of the dose period no test substance-related changes in coagulation parameters were observed in either sex at any dose level. There was a test substance-related reduction (statistically significant) in erythrocyte concentration in the females at 450 mg/kg bw per day and a statistically significant increase in chloride level in males at 450 mg/kg bw per day.

There were statistically significant reductions in the RBC ChE activity compared with controls (by 20.2% and 34.8% for males and females respectively) and brain ChE activity (by 18.8% and 17.6% of controls for males and females respectively) at 450 mg/kg bw per day.

No treatment-related microscopic alterations were seen in the tissue sections evaluated for this study. All stages of spermatogenesis were apparent within sections of testis from the 450 mg/kg bw per day group males and no treatment-related microscopic alterations were present in any of the ovarian sections.

In view of the above the NOAEL for general toxicity was 75 mg/kg bw per day based on a reduction in the mean number of erythrocytes in females and an increase in chloride levels in males, reductions in RBC and brain ChE activity in both males and females, at 450 mg/kg bw per day. The reproductive NOAEL was 450 mg/kg bw per day, the highest dose tested as there were no test substance-related changes in estrous cycling nor effects on mating and fertility in males or females. The NOAEL for offspring toxicity was also 450 mg/kg bw per day, the highest dose tested (Barnett, 2016j).

Comments

Biochemical aspects of omethoate

In the rat, omethoate was rapidly and more than 98% absorbed and rapidly excreted largely unchanged (85–96%) via the urine. The pattern of excretion and metabolism was similar for oral and intravenous dosing. Retention within organs and tissues after 48 hours was very low.

The main metabolic pathway of omethoate in the rat consisted of hydrolysis of the thiophosphoric acid structure to yield the desmethylated metabolite, or the sulfur-containing side chain, which is then *S*-methylated, followed by sulfoxidation to form the sulfinyl metabolite. The main radioactive compound in urine was the parent omethoate, and the major metabolites were *N*-methyl-2-(methylsulfonyl)-acetamide and *O*-desmethyl omethoate (Hoshino, 1990).

Toxicological data for omethoate

The median lethal dose (LD_{50}) in rats was 22–28 mg/kg bw via the oral route (Flucke, 1978; Krötlinger, 1989a), via the dermal route 145–232 mg/kg bw (Krötlinger, 1989b), and the median lethal concentration (LC_{50}) was 0.287 mg/L air by inhalation (Pauluhn, 1989).

In both short- and long-term studies the main toxic effect in all tested species was the inhibition of AChE and consequent clinical signs. Clinical signs generally occurred at doses higher than those causing critical (greater than 20%) inhibition of erythrocyte and/or brain AChE.

In four short-term studies in rats, the overall NOAEL was 0.08 mg/kg bw per day based on inhibition of erythrocyte AChE, with an overall LOAEL of 0.16 mg/kg bw per day. There were two 28-day studies, the first employing dietary concentrations of 0, 0.2, 0.4, 0.8, 1.6 or 8 ppm (equivalent to 0, 0.02, 0.04, 0.08, 0.16 and 0.8 mg/kg bw per day), the second, range-finding, study using dietary concentrations of 0, 2.5 or 15 ppm (equal to 0, 0.23 and 1.24 mg/kg bw per day for males, 0, 0.26 and 1.40 mg/kg bw per day for females) (Fogleman & Levinskas, 1963; Loser, 1968a). A 90-day dietary toxicity study employed concentrations of 0, 0.5, 1, 2 or 4 ppm (equal to 0, 0.04, 0.08, 0.17 and 0.34 mg/kg bw per day for males, 0, 0.05, 0.10, 0.19 and 0.36 mg/kg bw per day for females) (Loser, 1968b). In addition, a 32-week drinking water toxicity study administering concentrations of 0, 0.1 or 0.3 ppm (equal to 0, 0.0093 or 0.0271 mg/kg bw per day for males, 0, 0.0109 or 0.0322 mg/kg bw per day for females) (Schladt, 1994) was taken into account by the Meeting.

In two 90-day studies, dogs were administered omethoate in the diet at 0, 0.4, 0.8 or 1.6 ppm (equal to 0, 0.016, 0.032 and 0.063 mg/kg bw per day for males, 0, 017, 0.034 and 0.069 mg/kg bw per day for females), or 0 or 0.0125 mg/kg bw per day by gavage (Ruf & Mager, 1991; Hutchison et al., 1968). A one-year gavage study of omethoate in dogs employed doses of 0, 0.025, 0.125 or 0.625 mg/kg bw per day (Hoffmann & Schilde, 1984). From these the Meeting identified an overall NOAEL for oral toxicity of 0.063 mg/kg bw per day based on inhibition of erythrocyte and brain AChE, with an overall LOAEL of 0.125 mg/kg bw per day.

In a 24-month chronic toxicity/carcinogenicity study in mice treated with omethoate in drinking water at concentrations of 0, 0.5, 4 or 32 ppm (equal to 0, 0.10, 0.82 and 6.48 mg/kg bw per day for males, 0, 0.11, 0.80 and 6.61 mg/kg bw per day for females), the NOAEL for toxicity could not be identified due to an inhibition of erythrocyte AChE activity slightly above the threshold value of 20% at the lowest dose tested. The NOAEL for carcinogenicity in mice was 32 ppm (equal to 6.48 mg/kg bw per day), the highest dose tested (Schladt, 2001).

In a 24-month combined chronic toxicity/carcinogenicity study in rats, omethoate was administered through drinking water at concentrations of 0, 0.5, 4 or 32 ppm (equal to 0, 0.04, 0.30 and 2.92 mg/kg bw for males, 0, 0.05, 0.44 and 3.93 mg/kg bw per day for females). The NOAEL for toxicity was 0.5 ppm (equal to 0.04 mg/kg bw per day) based on inhibition of AChE activities at the LOAEL of 4 ppm (equal to 0.30 mg/kg bw per day). The NOAEL for carcinogenicity was 32 ppm (equal to 2.92 mg/kg bw per day), the highest dose tested (Schladt, 1995).

The Meeting concluded that omethoate is not carcinogenic in mice or rats.

Omethoate was tested for genotoxicity in an adequate range of in vitro and in vivo assays. It gave a positive/equivocal response in a number of in vitro tests, including a bacterial reverse mutation assay (Herbold, 1988a), sister chromatid exchange (SCE; Taalman, 1988), forward mutation assay in mammalian cells (HPRT test; Lehn, 1989) and unscheduled DNA synthesis in mammalian cells (Cifone, 1989). Positive responses were only obtained at either high or severely cytotoxic concentrations and with no clear dose-dependency. Omethoate gave negative responses in vivo in the micronucleus (Herbold, 1988b), SCE (Herbold, 1990a) and unscheduled DNA synthesis tests (Benford, 1989).

In studies previously evaluated by the Meeting, positive findings in a somatic cell assay in vivo were noted for the mouse spot test, indicating a potential for omethoate to induce point mutations in vivo. However, the Meeting noted that the doses used were well above those causing AChE inhibition (Herbold, 1990b).

A combination of two newly submitted, more recent in vivo studies (comet and combined comet/micronucleus assay) showed that neither strand breaks nor chromosomal damage occurred at noncytotoxic doses of omethoate (Barfield, 2020; Best, 2022).

The Meeting concluded that omethoate is unlikely to be genotoxic in vivo.

In view of the lack of genotoxicity in vivo and the absence of carcinogenicity in mice and rats, the Meeting concluded that omethoate is unlikely to pose a carcinogenic risk to humans at levels occurring in the diet.

In a two-generation reproduction study in rats, omethoate was administered via drinking water at doses of 0, 0.5, 3.0 or 18 ppm (equal to 0, 0.08, 0.57 and 3.16 mg/kg bw per day for males, 0, 0.12, 0.72 and 4.35 mg/kg bw per day for females). The NOAEL for parental toxicity was 0.5 ppm (equal to 0.08 mg/kg bw per day) based on inhibition of AChE activity at 3 ppm (equal to 0.57 mg/kg bw per day). The NOAEL for reproductive toxicity was 3 ppm (equal to 0.57 mg/kg bw per day) based on reduced fertility and impairment of reproductive performance in the parental generation females at 18 ppm (equal to 3.16 mg/kg bw per day). The NOAEL for offspring toxicity was 3 ppm (equal to 0.57 mg/kg bw per day) based on depressed body weights, retarded body weight gains, and inhibition of brain AChE in pups at 18 ppm (equal to 3.16 mg/kg bw per day) (Dotti et al. 1992).

In a developmental study employing doses of 0, 0.3, 1.0 or 3.0 mg/kg bw per day of omethoate administered by gavage to pregnant rats from GDs 6 to 15, the NOAEL for both maternal and developmental toxicity was 1.0 mg/kg bw per day based on tremors, depressed food consumption, reduction in body weight gain and mortality in dams, depression in placental weights and a reduction in the mean fetal weight in the highest dose group of 3.0 mg/kg bw per day (Holzum, 1990a).

In a developmental toxicity study in rabbits, omethoate was administered by gavage at doses of 0, 0.2, 1.0 or 5.0 mg/kg bw per day. The NOAEL for both maternal and developmental toxicity was 0.2 mg/kg bw per day based on inhibition of erythrocyte and brain AChE and increased malformations (arthrogryposis, epignathus, which were probably secondary to AChE inhibition) at 1 mg/kg bw per day (Holzum, 1990b).

In another developmental toxicity study in rabbits, omethoate was administered at doses of 0, 0.20, 1.0 or 4.0 mg/kg bw per day. The maternal NOAEL was 0.20 mg/kg bw per day based on reduction in red blood cell AChE activity at 1.0 mg/kg bw per day. The developmental NOAEL was 1.0 mg/kg bw per day based on reductions in gravid uterine weights, fetal body weights and delayed skeletal ossification occurring at 4.0 mg/kg bw per day (Barnett, 2015).

The Meeting concluded that omethoate is not teratogenic at doses that do not substantially inhibit cholinesterase.

In an acute neurotoxicity study in the rat, with administration of omethoate by gavage at dose levels of 0, 0.2, 0.25, 0.35 or 5 mg/kg bw the NOAEL was 0.25 mg/kg bw, based on changes in respiration, impairment of co-ordination, effects on pupil reflex and inhibition of brain AChE activity at 0.35 mg/kg bw (Mellert et al., 2003).

In a delayed polyneuropathy study in hens gavaged with a single dose of 140 mg/kg bw of omethoate there was no behavioural or histopathological evidence of delayed neurotoxicity. There was no measurement of cholinesterase activity or neuropathy target esterase (NTE) inhibition as would be

expected in a more recent study (Bomann & Sykes, 1993). However, enzyme studies with human and hen autopsy tissue suggested that omethoate does not cause delayed neuropathy in humans. No inhibition of NTE was found in humans or hens at four times the LD_{50} (Lotti et al., 1981).

The Meeting concluded that omethoate is neurotoxic but does not cause delayed polyneuropathy.

A comparative cholinesterase assay in neonatal pups and adult rats after acute oral dosing with omethoate at 0.1, 0.3, 0.6 or 0.9 mg/kg bw did not show significant differences in AChE inhibition due to age or sex. A point of departure (POD) was determined at 0.2 mg/kg bw (BMD_{20}, rounded to one significant figure) for inhibition of erythrocyte AChE in pups, as the erythrocyte enzyme was slightly more sensitive than the brain enzyme (Barnett, 2012).

Toxicological data on metabolites other than omethoate

Apart from omethoate, a number of plant metabolites and animal metabolites have been identified, including:

- *O*-desmethyl-*N*-desmethyl omethoate (Met XXIII)
- *O*-desmethyl-isodimethoate (Met XII)
- desmethyl dimethoate (Met X)
- *O*-desmethyl omethoate (Met XI)
- *O*-desmethyl omethoate carboxylic acid (Met XX)
- *O*,*O*-dimethyl phosphonic acid (Met XVII), and
- dimethoate carboxylic acid (Met III).

Newly submitted ChE studies and reproduction screening studies were conducted on dimethoate plant metabolites Met III, Met X, Met XI, Met XII, Met XX and Met XXIII. These studies showed that the metabolites possessed a very low potency for AChE inhibition.

New genotoxicity studies were submitted for in vitro gene mutation in bacterial and mammalian cells and micronucleus induction in human lymphocytes: with the exception of Met III all studies were negative. With Met III positive results were seen for in vitro mammalian cell gene mutation and micronucleus tests, but results were negative in appropriate follow-up in vivo studies.

The Meeting concluded that these metabolites were of no toxicological relevance compared to dimethoate.

QSAR analysis for *O*,*O*-dimethyl phosphonic acid (XVII) did not give any genotoxicity alert. Hence TTC Cramer class III (0.0015 mg/kg bw per day) should be applied to this metabolite as it does not contain a structure consistent with an AChE inhibitor.

O-desmethyl-isodimethoate (Met XII) and O-desmethyl omethoate carboxylic acid (Met XX)

Metabolites *O*-desmethyl-isodimethoate and *O*-desmethyl omethoate carboxylic acid are weaker AChE inhibitors than dimethoate and can be considered covered by the ADI and ARfD for dimethoate.

Microbiological data

There was not sufficient information available in the public domain and no experimental data were submitted to enable assessment of the possible impact of dimethoate residues on the human intestinal microbiome.

Human data

There was no information on the effect of omethoate on humans.

The Meeting concluded that the existing database on omethoate was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

Toxicological evaluation

The Meeting established an ADI of 0–0.0004 mg/kg bw for omethoate, separate to that for dimethoate, on the basis of a NOAEL of 0.04 mg/kg bw per day for inhibition of AChE activities in a two-year dietary toxicity/carcinogenicity study in rat. A safety factor of 100 was applied. This value is considered sufficiently protective for the slight AChE inhibition observed in red blood cells in the two-year dietary study in mice at 0.5 ppm (equal to 0.1 mg/kg bw per day).

The Meeting established an ARfD for omethoate of 0.002 mg/kg bw, separate to that for dimethoate, on the basis of the BMD_{20} of 0.2 mg/kg bw from an acute comparative cholinesterase assay for red blood cell and brain AChE inhibition in adult rats and pups. A safety factor of 100 was applied.

The ADI and ARfD for omethoate are 2.5-fold and 10-fold respectively lower than those established for dimethoate.

Levels relevant to risk assessment of omethoate

Species	Study	Effect	NOAEL	LOAEL
Mouse	Two-year study on toxicity and carcinogenicity [f]	Toxicity	-	0.5 ppm, equal to 0.10 mg/kg bw/day [e]
		Carcinogenicity	32 ppm, equal to 6.48 mg/kg bw/day [c]	-
Rat	Two 28-day, one 90-day dietary and one 32-week drinking water studies of toxicity [a,b]	Toxicity	0.08 mg/kg bw/day	0.16 mg/kg bw/day
	Two-year study of toxicity and carcinogenicity [f]	Toxicity	0.5 ppm, equal to 0.04 mg/kg bw/day [e]	4 ppm, equal to 0.30 mg/kg bw/day
		Carcinogenicity	32 ppm, equal to 2.92 mg/kg bw/day [c]	-
	Two-generation study of reproductive toxicity [f]	Reproductive toxicity	3 ppm, equal to 0.57 mg/kg bw/day	18 ppm, equal to 3.16 mg/kg bw/day
		Parental toxicity	0.5 ppm, equal to 0.08 mg/kg bw/day	3 ppm, equal to 0.57 mg/kg bw/day
		Offspring toxicity	3 ppm, equal to 0.57 mg/kg bw/day	18 ppm, equal to 3.16 mg/kg bw/day
	Developmental toxicity study [d]	Maternal toxicity	1 mg/kg bw/day	3 mg/kg bw/day
		Embryo/fetal toxicity	1 mg/kg bw/day	3 mg/kg bw/day
	Acute neurotoxicity study	ChE inhibition, clinical signs	0.25 mg/kg bw/day	0.35 mg/kg bw/day
	Acute comparative cholinesterase assay	ChE inhibition, pups and adults	0.2 mg/kg bw (POD from BMD analysis) [g]	-
Rabbit	Developmental toxicity study [b,d]	Maternal toxicity	0.2 mg/kg bw/day	1 mg/kg bw/day
		Embryo/fetal toxicity	0.2 mg/kg bw/day	1 mg/kg bw/day
Dog	90-day gavage, 90-day dietary and one-year gavage study [a,b,d]	Toxicity	0.063 mg/kg bw/day	0.125 mg/kg bw/day

[a] Dietary administration; [b] Two or more studies combined; [c] Highest dose tested [d] Gavage administration; [e] Lowest dose tested [f] Administration through drinking water; [g] Methodology described in Section 1.2, above

Acceptable daily intake(ADI) applies to omethoate,

0–0.0004 mg/kg bw

Acute reference dose (ARfD) applies to omethoate,

0.002 mg/kg bw

Information that would be useful for the continued evaluation of the compound

Results from further epidemiological, occupational health and other such observational studies of human exposure.

Critical end-points for setting guidance values for exposure for omethoate

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapidly and completely absorbed (≥98%)
Dermal absorption	No data
Distribution	Widely distributed
Potential for accumulation	None
Rate and extent of excretion	Excretion is rapid: >80% of the dose excreted in urine within 24 hours
Metabolism in animals	Limited
Toxicologically significant compounds in animals and plants	Omethoate
Acute toxicity	
Rat, LD_{50}, oral	22–28 mg/kg bw
Rat, LD_{50}, dermal	145–232 mg/kg bw
Rat, LC_{50}, inhalation	0.287 mg/L
Short-term studies of toxicity	
Target/critical effect	Inhibition of acetylcholinesterase
Lowest relevant oral NOAEL	0.063 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	2.5 mg/kg bw per day (rabbit)
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Inhibition of RBC and brain acetylcholinesterase (rat, mouse)
Lowest relevant NOAEL	0.04 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic in mice or rats
Genotoxicity	Unlikely to be genotoxic in vivo
Reproductive toxicity	
Target/critical effect	Reduced fertility and impairment of reproductive performance
Lowest relevant parental NOAEL	0.8 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	0.57 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	0.57 mg/kg bw per day (rat)
Developmental toxicity	
Target/critical effect	Increase in malformations (arthrogryposis,epignathus), probably secondary to acetylcholinesterase inhibition (rabbit)
Lowest relevant maternal NOAEL	0.2 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	0.2 mg/kg bw per day (rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	0.25 mg/kg bw (rat)
Acute comparative cholinesterase assay, BMD_{20}	0.2 mg/kg bw (rat)

Summary

	Value	Study	Safety factor
ADI	0–0.0004 mg/kg bw	Two-year chronic toxicity and carcinogenicity studies (rat)	100
ARfD	0.002 mg/kg bw	Acute comparative cholinesterase assay (rat)	100

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Fluazaindolizine

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Explanation

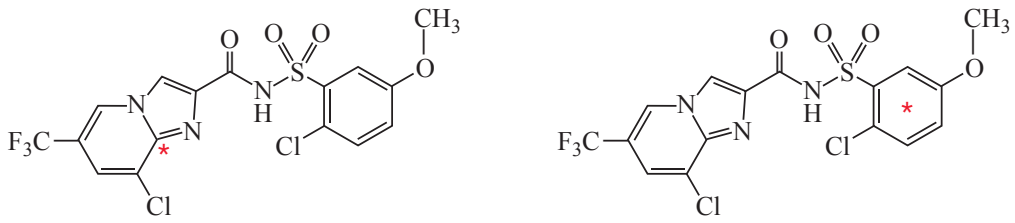
Fluazaindolizine is the ISO-approved common name for 8-chloro-*N*-[(2-chloro-5-methoxyphenyl) sulfonyl)]-6-(trifluoromethyl)-imidazol[1,2-a]pyridine-2-carboxamide (IUPAC), Chemical Abstracts Service number 1254304-22-7. This compound is a sulfonamide of the imidazopyridine class. The substance is a nematicide for application in various crops including cucumbers, tomatoes and carrots. Its mode of action (MOA) in target organisms has not been sufficiently elucidated as yet (Lahm et al., 2017; Oka, 2020).

Fluazaindolizine has not previously been evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and was reviewed by the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR). All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with current test guidelines, unless otherwise specified. Additional information that would complement the toxicological studies is scarce from public scientific literature, as is to be expected for such a new compound.

Evaluation for acceptable daily intake

1 Biochemical aspects

Figure 1. Chemical structure of fluazaindolizine with positions of radiolabelling indicated



[*Imidazo(1,2-a)pyridine-2*-^{14}C]fluazaindolizine

[*Phenyl-U*-^{14}C]fluazaindolizine

* indicates position of ^{14}C label

1.1 Absorption, distribution and excretion

(a) Oral route, rat

In a pilot study, kinetics and metabolic fate of fluazaindolizine was studied in a single experiment in a small group of Sprague Dawley [Crl:CD(SD)] rats. The test compound (purity 99.7%), ^{14}C-radiolabelled either in the imidazole(1,2-*a*)pyridine or phenyl moiety (see Fig. 1), was administered as a single dose of 10 mg/kg bw by oral gavage to two animals per sex and label. The vehicle was 0.1% Tween 80 in 0.5% methylcellulose solution and the dosing volume 10 mL/kg body weight (bw). Radiochemical purity of both test item preparations was analyzed by means of high-performance liquid chromatography (HPLC) and was found to be 100% for [*imidazo(1,2-a)pyridine-2*-^{14}C]fluazaindolizine and 99.7% in the case of [*phenyl(U)*-^{14}C]-fluazaindolizine.

The rats were placed singly in metabowls to collect urine and faeces over the whole post-observation period of 168 hours, after which the rats were humanely killed. Collection of expired air was confined to the first 48 hours post dosing. Radioactivity was determined in excreta, cage wash and exhaled air and, following termination, also in blood and plasma (obtained from a terminal whole-blood sample), liver, kidneys, renal fat, spleen, gastrointestinal (GI) tract and remaining carcass; this was done to obtain information on elimination and mass balance. In addition, metabolism was studied by examination of urine, faeces and cage wash with a combined approach using several methods such as comparative chromatography with reference standards, precise mass measurement and/or diagnostic liquid chromatography–mass spectrometry (LC/MS) fragmentation.

No adverse reactions were observed in the treated animals. With both radiolabels, elimination of the administered radioactivity (AR) was quantitative and took place mainly within the first 48 to 72 hours

post dosing. Following administration of phenyl-labelled fluazaindolizine, total excretion of radioactivity after 48 hours amounted to 95% in males and 96% in females. Where the imidazopyridine-labelled fluazaindolizine had been given, 93% AR was recovered with males and 97% with females, within 72 hours. Exhalation was negligible. Both faeces and urine contributed nearly equal amounts to total excretion in female rats if the cage wash (which showed a similar metabolite pattern particularly to urine in females) is added to give total urine, whereas in males faeces was the major excretion route. An overview on elimination parameters and mass balance is provided in Table 1.

In blood, plasma or in the few organs and tissues under examination, the remaining radioactive residue levels at termination were very low. The highest concentrations, found in the liver or plasma, were around 0.02 µg equiv./g liver tissue or 0.02 µg equiv./mL plasma.

Table 1. Elimination of radioactivity (rounded means) in the pilot ADME study with fluazaindolizine in the rat following administration of 10 mg/kg bw to two rats per sex and label

	Sex and radiolabel position			
	Imidazopyridine		Phenyl	
Matrix	**Males**	**Females**	**Males**	**Females**
Urine (0–168 hours)	25	46	36	46
Faeces (0–168 hours)	58	49	57	46
Cage wash	13	5	6	8
Total recovery	96	100	99	100

Source: Punler & Green, 2016a

The most abundant component in urine, faeces or cage wash was always the unchanged parent, accounting for 69–81% of the detected material in the various matrices but with figures slightly differing for sex and radiolabel. A number of metabolites were identified but represented mostly less than 2% of the AR. Metabolites occurring at higher concentrations were IN-QEK31 (up to 6.6 % of administered dose (AD) in males, or 5.6% in females, all excreta considered) and IN-A5760 or its sulfate conjugate (4.4% of AD in total in males and 5.9% in females; detected in urine and cage wash).

The main metabolic pathways comprised hydroxylation of the phenyl ring, *O*-demethylation and hydrolysis of the amide bond in the intermediate IN-REG72 to give IN-QEK31 or IN-A5760, and sulfate conjugation of the latter. Hydrolysis was also presumed but not directly demonstrated. (Punler & Green, 2016a)

The main study employed the same radiolabels: [*imidazo(1,2-a)pyridine-2*-^{14}C]fluazaindolizine (IP label) and [*phenyl(U)*-^{14}C]fluazaindolizine (Ph label). The radiochemical purity of both was greater than 99%. Kinetics, excretion and mass balance, tissue distribution and metabolism of fluazaindolizine were investigated in male and female Sprague Dawley [Crl:CD(SD)] rats which received the test compound as a single low (10 mg/kg bw) or high (200 mg/kg bw) dose. Experiments on bile duct-cannulated rats were also included. In addition, a low dose of unlabelled fluazaindolizine was administered to rats of both sexes for 14 subsequent days, followed by a single low dose of the phenyl-labelled compound.

For all experiments, the test substance was dissolved in 0.1% Tween 80 in 0.5% methylcellulose and this was administered by oral gavage at a dose volume of approximately 10 mL/kg bw. The rats scheduled for excreta analysis were kept singly in all-glass metabowls, whereas those for toxicokinetic study were housed in pairs of the same sex in grid-bottomed cages.

The complex study design consisted of 17 Phases with 42 study groups in total, and part of this is shown in Table 2 for the Ph label (Phases 9–17). Exactly the same research program was used for the IP label (Phases 1-8), with the only exception that no repeat-dose trial was conducted. Apart from the experiments in bile duct-cannulated rats (in which only the GI tract, its contents and the residual carcass were retained), the following organs and tissues or samples were taken: adrenals, bladder, blood, bone and bone marrow, brain, carcass, GI tract and contents, heart, kidneys, liver, lungs, muscle, ovaries, pancreas, pituitary, skin, spleen, testes, thymus, thyroid and uterus. Samples from excreta and bile were pooled within 24-hour intervals. For identification and characterization of metabolites in excreta, liver,

kidneys, and plasma, a combination of chromatography, accurate mass measurement, isotope pattern and diagnostic fragmentation using LC/MS was applied.

Table 2. Overview of the design of the ADME study with [phenyl[U]-^{14}C]-fluazaindolizine in the rat

Experimental phase, all with two sex-divided groups	Dose (mg/kg bw)	Number and sex of animals	Time of termination post final dose (hours)	Samples taken and investigations performed
Mass balance and tissue distribution				
Phase 9	10	4M, 4F	168	Urine, faeces, cage wash; tissues, carcass; RBCs and plasma at termination; metabolites in excreta including cage wash
Phase 10	200	4M, 4F	168	
Toxicokinetics				
Phase 11	10	4M, 4F	168	RBCs, plasma (samples collected at 5, 15, 30 minutes, and 1, 2, 4, 8, 12, 24 hours and then at 24-hour intervals until termination); metabolites in plasma
Phase 12	200	4M, 4F	168	
Biliary elimination				
Phase 13	10	4M, 4F	48	Bile, urine, faeces, cage wash, GIT with contents, carcass; metabolites in bile
Phase 14	200	4M, 4F	48	
Tissue distribution				
Phase 15	10	4M, 4F	1	Tissues, RBC, plasma, carcass
	10	4M, 4F	6	
Phase 16	200	4M, 4F	8	
	200	4M, 4F	24	
Repeated administration				
Phase 17	15 × 10	4M, 4F	168	Urine, faeces, cage wash; tissues, carcass; RBCs and plasma at termination; metabolites in excreta including cage wash

M: Male; F: Female; RBC: Red blood cell Source: Punler & Green, 2017

Male and female rats receiving phenyl-labelled fluazaindolizine at the high dose of 200 mg/kg bw for mass balance examinations displayed some transient signs of toxicity such as piloerection, hunched posture, salivation, or were cold to the touch. There is no explanation as to why these signs were common in this group but not observed in others which were administered the same dose, even repeatedly.

One male rat from the high-dose group in the toxicokinetic experiment had to be replaced just after dosing because it was regarded as unfit to continue in the study and one bile duct-cannulated female from the low–dose group had to killed for humane reasons after 24 hours; in both these cases it was after administration of the IP-labelled test article. It is not clear whether these two losses were due to the toxicity of fluazaindolizine or to an experimental incident. One other male from the bile duct-cannulated groups receiving the low dose IP-labelled test article did not produce bile and had also to be replaced.

Following a single oral administration of [^{14}C]fluazaindolizine to male and female rats, the excretion pattern was similar for both radiolabelled forms. The data for the phenyl-labelled fluazaindolizine is shown in Table 3, but results with the other radiolabel position were not much different.

Near complete elimination was demonstrated, with the major part having already been excreted within the first 24 hours. Total residues at termination, after 168 hours, were very low, accounting for only 0.2%–0.4% of AD. At the low dose, the radioactivity was excreted by male rats in more or less equal amounts in urine (including cage wash) and faeces, whereas females given the low dose tended to eliminate a slightly greater proportion via urine. This slight difference became more apparent at the high dose level since the amount excreted in the faeces by male rats rose slightly and urinary excretion further decreased. In contrast, the female excretion pattern remained nearly identical to that observed with females at the low dose.

On balance, these findings might suggest a limited oral absorption with perhaps further saturation at higher doses in males. However, no firm conclusions can be drawn from these excretion and mass balance experiments alone since the possible impact of enterohepatic circulation could not be elucidated. For biliary excretion, see below.

The excretion pattern following multiple oral administrations was similar to that following a single oral administration indicating that there were no meaningful differences with regard to toxicokinetics even though a reported total recovery of 117.5% in males and 119.2% in females made direct quantitative comparison to the single-dose experiments difficult.

Table 3. Excretion and mass balance at termination (group mean percentage of AD) in the experiments with phenyl-labelled fluazaindolizine

Dose frequency	**1 dose**		**1 dose**		**(14 doses unlabelled) followed by 1 labelled dose**	
Dose	**10 mg/kg bw**		**200 mg/kg bw**		**10 mg/kg bw**	
Sex	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Urine 0–24 h	36.5	46.0	31.6	45.8	42.6	43.5
Subtotal urine, 0–168 hours	45.3	53.7	37.1	51.2	52.5	52.6
Faeces 0–24 h	41.4	34.1	50.4	33.1	46.6	48.7
Subtotal faeces, 0–168 hours	52.0	41.1	59.3	41.3	55.2	57.0
Cagewash 0–24 h	4.1	6.0	2.2	5.9	7.0	**7.7**
Subtotal cagewash	5.8	7.3	2.9	7.1	9.8	9.6
Carcass, residues, GIT	0.2	0.2	0.4	0.4	0.2	0.2

Source: Punler & Green, 2017

Because of the very low tissue residues at 168 hours post dose, no reliable conclusions can be drawn with regard to the distribution of radioactivity to various organs and tissues following administration of the low dose. In the groups receiving the IP-labelled fluazaindolizine at the high dose (200 mg/kg bw), the highest concentrations of radioactivity were detected in the liver, followed by skin, bone, and plasma. Quantifiable concentrations of radioactivity in the kidneys of females but not in males might reflect the observed difference in excretion behaviour with the urinary route being more important in female rats. By far the highest absolute tissue concentration in females was observed in RBCs but only in a single animal, so no robust conclusion could be based on this observation.

When the phenyl-labelled fluazaindolizine was applied at the high-dose level, total radioactivity in males was highest in skin, followed by liver, thyroid and plasma. In females, some radioactivity was detected in the pituitary, thyroid and liver, with lesser amounts in the uterus and plasma. More reliable information can be retrieved from the tissue distribution experiments with much shorter observation periods after dosing as reported in Table 8 below.

The plasma toxicokinetics of fluazaindolizine were similar across sexes and both radiolabels. Absorption appeared to be proportional to the radioactivity administered as the area under the concentration–time curves (AUCs) increased between about 16-fold and 23-fold relative to the 20-fold increase in dose. While the time to reach mean peak concentration (T_{max}) was prolonged in high-dose rats (c 3–6 hours) compared to the low-dose rats (c 0.25–0.625 hours), plasma half-lives were similar in all groups, ranging from 8–11.4 hours in low dose rats and 8.4–13.4 hours in the high-dose groups (see Tables 4 and 5).

Table 4. Selected toxicokinetic parameters measured in rat plasma after single oral administration of imidazo-labelled fluazaindolizine (mean values, except median shown for T_{max})

	Oral dose [mg/kg bw]	C_{max} [µg equiv./mL]	T_{max} [hours]	Half-life [hours]	$AUC_{0\rightarrow\infty}$ [µg eqiv. × h/mL]
Males	10	72.0	0.625	11.4	623
	200	459	6.0	8.41	10 100
Females	10	71.8	0.375	9.67	550
	200	523	3.0	13.4	12 500

$AUC_{0\rightarrow\infty}$: Total area under the concentration–time curve; Source: Punler & Green, 2017

C_{max}: Maximum concentration achieved; T_{max}: Time at which C_{max} is achieved

In red blood cells, C_{max} and AUC, at the low dose level, were a seventh to a tenth of values found in plasma; by contrast T_{max} and half-lives of RBCs and plasma were in the same order of magnitude. Following administration of the high dose, observations were similar, with the exception that there was a remarkable sex difference in T_{max}, with a median of 10 hours in males but just one hour in females.

Table 5. Selected toxicokinetic parameters measured in rat plasma after single oral administration of [phenyl-^{14}C(U)]-fluazaindolizine (mean values except median for T_{max})

	Oral dose [mg/kg bw]	C_{max} [µg equiv./mL]	T_{max} [hours]	Half-life [hours]	$AUC_{0\rightarrow\infty}$ [µg eqiv × h/mL]
Males	10	66.7	0.25	7.96	537
	200	444	4.0	8.68	8 540
Females	10	77.6	0.25	9.02	630
	200	515	6.0	10.4	10 600

$AUC_{0\rightarrow\infty}$: Total area under the concentration–time curve; Source: Punler & Green, 2017

C_{max}: Maximum concentration achieved; T_{max}: Time at which C_{max} is achieved

With phenyl-labelled fluazaindolizine, the C_{max} and AUC in RBCs were, once again, markedly lower than in plasma, but half-lives and T_{max} were in the same order of magnitude. On balance, the kinetic parameters suggested rapid absorption and moderately fast distribution and elimination. There was no evidence of a particular affinity to the blood cells.

Bile cannulation experiments indicated that total oral absorption, for both radiolabels, was in the range 52% to 60% of AD in the low-dose groups and 45% to 50% of AD in the high-dose groups. This estimate is based on mean total radioactivity in bile, urine, carcass, plasma, RBCs, cage wash (assuming that it was mostly of urinary origin) and the GI tract (see Tables 6 and 7). Since in general the oral absorption at the low dose is more relevant in risk assessment, the lowest low-dose figure of 52% should be used for that purpose.

Some saturation of absorption at the high dose was confirmed, particularly apparent in males. In contrast, a slight predominance of the urinary route for elimination in females, as suggested by the mass balance experiments, was not found reproducible in bile duct-cannulated rats. When a low dose of the phenyl-labelled material was applied, residual radioactivity in plasma and carcass was remarkably high.

Table 6. Recovery of radioactivity (mean percentage of AD, normalized to 100% recovery) from the various compartments in the period up to after 48 hours after administration of [imidazo(1,2-a)pyridine-2-^{14}C]fluazaindolizine to bile duct-cannulated rats

Matrix/group	Low dose		High dose	
	Males	Females	Males	Females
Urine	42.5	35.8	26.5	29.0
Bile	8.1	4.6	10.9	7.0
Faeces	42.9	47.3	55.5	52.0
Cage wash	4.7	7.3	5.7	4.9
Plasma	0.6	0.8	0.4	0.8
Red blood cells	0.1	0.1	0.1	0.1
GI tract with contents	0.4	0.5	0.5	1.9
Carcass	1.7	2.5	1.3	2.1
Total absorption estimate	58	52	45	46

GI: Gastrointestinal; Source: Punler & Green, 2017

Table 7. Recovery of radioactivity (mean % of administered dose, normalized to 100% recovery) from the various compartments within or after 48 hours following application of [phenyl-^{14}C(U)]-fluazaindolizine to bile duct-cannulated rats

Matrix/group	Low dose		High dose	
	Males	Females	Males	Females
Urine	42.5	26.6	24.8	31.5
Bile	6.8	4.7	18.2	10.0
Faeces	42.0	51.0	48.7	47.6
Cage wash	3.3	3.0	3.6	3.9
Plasma	1.7	4.9	0.7	1.1
Red blood cells	0.3	0.6	0.1	0.2
GI tract including contents	0.7	4.1	0.6	1.1
Carcass	4.7	10.5	2.1	2.5
Total absorption estimate	60	54	50	50

GI: Gastrointestinal; Source: Punler & Green, 2017

The tissue distribution experiments with both radiolabel positions revealed that the highest concentrations of radioactivity, in all groups and at all time points (1 or 6 hours after low dose administration as well as at 8 or 24 hours after high dose treatment) were measured in plasma, exceeding those in all other tissues. Appreciable levels of radioactive residue were detected in the liver and, to a lesser extent in kidneys and lungs. With imidazo-labelled fluazaindolizine, the pituitary of high-dose females at 24 hours post dosing (but only in this group) contained similar levels of radioactivity to the plasma. As an example, concentrations obtained with the this label are presented in Table 8.

A decline in the radioactive concentrations at the second time point measurement was apparent in most tissues and, along with the outstandingly high levels of radioactivity in the bladder of males at six hours (low dose) and eight hours (high dose), this might reflect rapid elimination. In general, radioactive residues rose with dose, but in most tissues the increase was sublinear, that is less than the 20-fold increment from low to high dose.

Table 8. Mean tissue concentration of radioactivity (in µg equiv./g tissue or mL) after single oral administration of [imidazo(1,2-a)pyridine-2-^{14}C]fluazaindolizine at low or high dose levels (outstandingly high levels shown bold; levels at high dose have been rounded)

	Sex and dose							
	Males				Females			
	10 mg/kg bw		200 mg/kg bw		10 mg/kg bw		200 mg/kg bw	
Organ/tissue	**1**	**6**	**8**	**24**	**1**	**6**	**8**	**24**
Adrenals	5.9	3.0	81	28	8.9	4.3	90	20
Bladder	4.8	**21.0**	**638**	65	7.7	7.5	138	33
Bone marrow	6.6	2.8	45	16	7.0	2.9	60	16
Bone (mineral)	2.8	1.4	26	9	3.7	1.2	23	9
Brain	0.7	0.4	8	2	1.0	0.6	7	2
Fat (renal)	2.2	1.6	26	7	3.4	1.9	30	8
Heart	9.0	4.8	87	26	12.5	6.5	102	25
Kidneys	14.2	6.1	**160**	42	**30.3**	10.4	**179**	55
Liver	**32.4**	**24.1**	**174**	60	**54.8**	**26.4**	**185**	78
Lungs	13.1	6.2	104	34	17.5	8.9	119	32
Muscle	1.8	1.5	28	8	2.5	1.6	28	7
Ovaries					12.6	7.0	97	33
Pancreas	4.9	2.2	55	13	5.9	3.4	54	14
Pituitary	11.4	6.4	107	41	11.1	5.4	105	**145**
Plasma	**53.7**	**27.1**	**473**	**151**	**80.3**	**35.5**	**494**	**156**
Red blood cells	9.9	4.0	64	23	12.1	3.6	72	33
Skin	3.0	3.8	65	22	4.2	4.1	67	25
Spleen	3.0	1.6	33	10	4.5	2.2	37	10
Testes	2.3	2.8	45	16	-	-	-	-
Thymus	3.5	2.6	39	14	4.8	2.9	50	15
Thyroid	8.4	4.3	125	37	8.8	5.2	97	40
Uterus	-	-	-	-	7.0	9.0	94	32

Source: Punler & Green, 2017

It must be emphasized, however, that 30%–80% of the applied radioactivity, in the different groups, was obtained from the GI tract and its contents, which represents the nonabsorbed part of the administered dose at these early time points. In contrast, the results from the measurement of radioactivity in tissues on the day of dosing as described above may be considered to indicate the tissue distribution of the absorbed portion.

Metabolism of fluazaindolizine was limited. Unchanged parent was the main component in all samples analyzed. In total, unchanged fluazaindolizine accounted for 68.1%–86.8% in the excreta, while it amounted to 93.8%–94.6% of TRR in plasma and in liver and kidney made up 69.3%–79.2% of TRR.

All metabolites detected at levels greater than 1% of AD were identified. Excreted metabolites included IN-REG72, IN-UHD20, IN-UHD21, IN-F4106, IN-QEK31, IN-A5760 and both a sulfate and a glucuronide conjugate of IN-A5760. In addition to the parent (which accounted for 20.7%–44.9% of AD, depending on sex, dose and position of radiolabel), the most abundant metabolite in urine was IN-QEK31, accounting for 5%–8% of AD when the imidazo-labelled compound was administered. With the phenyl-labelled compound the sulfate conjugate of IN-A5760 was a significant urinary metabolite accounting for 3.8%–7% of the total AD. In faeces IN-UHD20 was most abundant after the parent, with amounts varying from 0.5% to 6% of AD. The main metabolite in plasma, liver and kidney was IN-F4106 , representing 2.5%–3.1% TRR in plasma; 17.4%–26.5% TRR in liver and kidney. In addition,

IN-RYC33 was found in minor amounts (up to 0.2% of AD) in female rats' urine and bile, whereas glucuronide or sulfate conjugates of IN-REG72, IN-UHD20 and IN-UHD21 were identified only in bile. The metabolite pattern in excreta was not markedly changed by previous repeated administrations of unlabelled test material at the low dose (Punler & Green, 2017).

(b) Oral route, mouse

In parallel to the pilot study in rats (Punler & Green, 2016a), the kinetics and metabolic fate of fluazaindolizine were also examined in a single experiment in CD-1 mice, but in contrast to the rat, this preliminary study in a small number of animals was not followed by more comprehensive investigations in the mouse. Two preparations of the test compound, labelled in different positions as shown in Fig. 1 to yield [*imidazo(1,2-a)pyridine-2*-^{14}C]fluazaindolizine and [*phenyl(U)*-^{14}C]fluazaindolizine, were employed. These were each administered by oral gavage to two animals per sex as a single dose of 10 mg/kg bw. A radiochemical purity of greater than 99% had been confirmed in the pilot study in rats for both test items. The vehicle was 0.1% Tween 80 in 0.5% methylcellulose solution and the dosing volume was 10 mL/kg bw.

The animals were placed singly in all-glass metabowls to collect urine and faeces over the whole post-observation period of 168 hours, after which the mice were humanely killed. Collection of expired air was confined to the first 48 hours post dosing. Radioactivity was determined in excreta, cage wash and exhaled air, as well as in blood and plasma (obtained from a terminal whole-blood sample), liver, kidneys, renal fat, spleen, GI tract and remaining carcass; this was done to obtain information on elimination and mass balance. In addition, metabolism was studied by examination of urine, faeces and cage wash with a combined approach using several methods such as comparative chromatography with reference standards, precise mass measurement and/or diagnostic liquid chromatography–mass spectrometry (LC/MS) fragmentation.

The animals showed no adverse reactions to treatment. With either radiolabel elimination of the administered radioactivity was rapid and quantitative with faeces being the predominant route of excretion. Following administration of [*imidazo(1,2-a)pyridine-2*-^{14}C]fluazaindolizine, elimination in male mice was above 92% within 24 hours, whereas female mice excreted nearly 95% within 48 hours. With the phenyl radiolabel, about 93% of total radioactivity in both sexes had been excreted within the first 48 hours post dose. An overview of elimination parameters and mass balance is provided in Table 9. As in rats, the excreted material in cage wash was most likely of urinary origin. Nonetheless, faeces was more important for elimination in mice than in rats. Even though biliary excretion was not measured and, thus the possible impact of enterohepatic circulation is unknown, the available data suggest a lower oral absorption of fluazaindolizine in the mouse than in the rat. Exhalation, as in the rat, was negligible.

Table 9. Elimination of radioactivity (rounded mean values in %) in the pilot ADME study with fluazaindolizine in the mouse following administration of 10 mg/kg bw to two animals per sex and label

	Radiolabel position, sex			
	Imidazopyridine		Phenyl	
Matrix	Males	Females	Males	Females
Urine (0–168 hours)	11	18	8	16
Faeces (0–168 hours)	86	67	80	67
Cage wash	3	11	8	13
Total recovery	99	96	96	96

Source: Punler & Green, 2016b

In blood, plasma or the few organs and tissues that were examined, the remaining radioactive residue levels at termination were very low, in general, below the concentrations measured in the pilot rat study. and mostly below the limit of quantification (LOQ). The highest concentrations were determined in the livers of females after dosing with the imidazo-labelled compound, but even this concentration only amounted to 0.009 µg equiv./g of liver tissue. This concentration appears much lower than in rats

(see Table 8), but the comparison is not meaningful because radioactivity in the rat study was measured at one or six hours post dosing rather than the168 hour sampling used in the mouse study. With the phenyl radiolabel, the highest tissue radioactivity was 0.01 µg equiv./g, also found in the liver of female mice: Liver residues in males and plasma levels in both sexes were slightly lower but in the same order of magnitude.

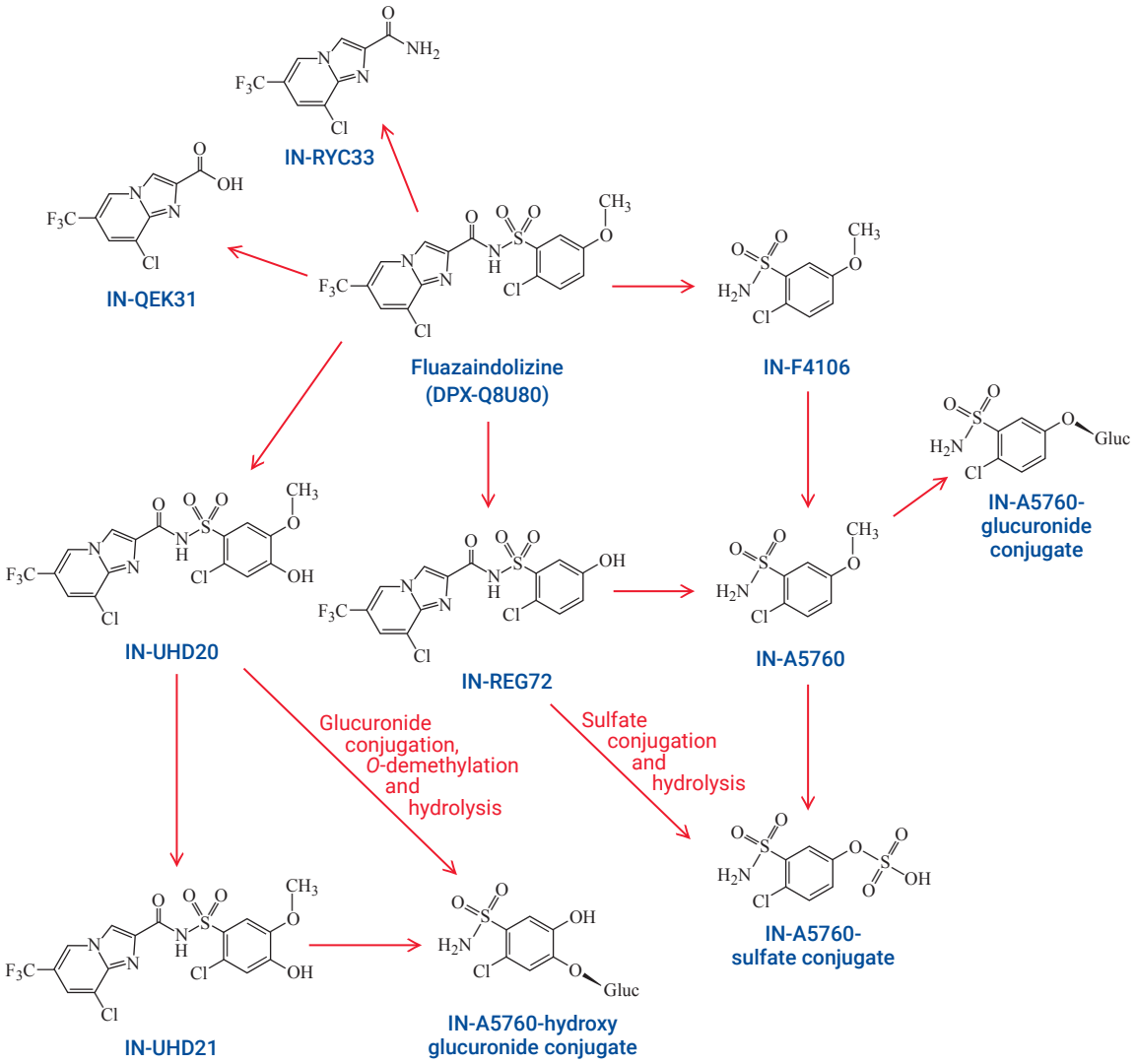
The most abundant component in urine, faces or cage wash was always the unchanged parent, accounting for 65% to 75% of the detected material in the different matrices with slightly differing figures for sex and radiolabel. Apart from that, seven metabolites were identified. These were the same as in rats: IN-QEK31, IN-REG72, IN-A5760 (and its sulfate conjugate), IN-UHD20 and IN-UHD21, whereas the hydrolysis product IN-F4106 was detected only in traces in cage wash. Metabolites occurring at highest concentrations in excreta were IN-QEK31 (up to 3.9 % of AD in males), IN-REG72 (up to 5.4% of AD in females), IN-UHD20 and IN-UHD21 (both up to 7.1% of AD in females and at slightly lower amounts in males). Metabolite A-5760 and its sulfate conjugate were less abundant than in the rat. The main metabolic pathways in the mouse were apparently similar to those in the rat (Punler & Green, 2016b).

1.2 Biotransformation

In vivo metabolism of fluazaindolizine is discussed above in the individual rat and mouse studies. It may be concluded that, in both species, metabolism is limited since the major part of the administered dose was excreted chemically unchanged. So far as the test substance was subject to biotransformation, the main metabolic pathways comprised *O*-demethylation, hydroxylation of the phenyl ring, and hydrolysis of the amide bond, followed by conjugation reactions in some cases.

An overview of metabolism in the rat is shown below in Fig. 2.

Figure 2. Proposed metabolic pathway of fluazaindolizine in the rat



(Redrawn from Punler & Green, 2016a, 2017)

Direct hydroxylation of the phenyl ring of fluazaindolizine in the rat resulted in formation of the metabolite IN-UHD20. Fluazaindolizine and the metabolite IN-UHD20 underwent *O*-demethylation to form metabolites IN-REG72 and IN-UHD21, respectively. Direct hydrolysis of fluazaindolizine, or of IN-REG72 produced either metabolite IN-F4106 or IN-A5760, which both contained only the phenyl ring, or IN-QEK31 which retains the imidazopyridine rings. Metabolite IN-QEK31 was readily excreted and did not appear to undergo further metabolism. Metabolite IN-A5760 was further conjugated, as were IN-UHD20 and IN-UHD21 (Punler & Green, 2016a, 2017).

A comparative study to investigate metabolism of fluazaindolizine in vitro was conducted in hepatocytes derived from various species. The radiolabelled compounds used were the same as in the previously described in vivo studies: [*imidazo(1,2-a)pyridine-2*-^{14}C]fluazaindolizine and [*phenyl(U)*-^{14}C]-fluazaindolizine, both with a radiochemical purity greater than 99%. The in vitro test system were cryopreserved, mixed-gender primary hepatocytes obtained from CD-1 mice, Sprague Dawley rats, New Zealand White rabbits, beagle dogs, and humans, supplied by Bioreclamation IVT (Baltimore, MD, USA).

After thawing, hepatocytes with a viability of 70% or greater (as determined in a trypan blue exclusion assay) were incubated with fluazaindolizine at a concentration of 20 µM for 20, 60 or 120 minutes. All trials were performed in triplicate with each radiolabel. Incubations were terminated by removing aliquots into ice-cold acetonitrile to stop the metabolic reactions. Following freezing, thawing with adaptation to room temperature and subsequent centrifugation, the individual fractions in the supernatants were separated by HPLC. Recovery of radioactivity was determined by liquid scintillation

counting (LSC). Metabolites were identified by qualitative mass spectrometry and compared to a number of reference compounds. Selected samples were also analysed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) with concurrent radiodetection to obtain spectra for comparison with certain significant compounds. Metabolic competence of the hepatocytes was demonstrated by parallel incubation with 10 µM [^{14}C]7-ethoxycumarin and subsequent analysis for metabolites by HPLC.

As with the in vivo studies in rats and mice, metabolism appeared quite limited in this in vitro study, irrespective of the species origin of the hepatocytes. Biotransformation was most rapid and extensive in human hepatocytes, whereas the metabolic rate was the lowest in dog liver cells. For example with the phenyl-labelled substrate, after 120 minutes of incubation 28% of the radioactivity recovered from human hepatocytes could be allocated to metabolites of fluazaindolizine. However, after the same full incubation time with dog cells only 3.6% of the dose taken up by the hepatocytes had been metabolized. In contrast, metabolic rates ranging from 70% (rat hepatocytes) to more than 90% (human and mouse hepatocytes) and even approaching 100% (rabbit and dog liver cells) were obtained for the control substance [^{14}C]7-ethoxycumarin, confirming the metabolic competence of the test system.

Table 10. Metabolic rate and abundance of identified fluazaindolizine metabolites (percentage) with hepatocytes of different origin following 120 minute incubation; main metabolites (>5%) in bold

Metabolite	Origin of hepatocytes; label position									
	Rat		Mouse		Rabbit		Dog		Human	
	IP	Ph	IP	Ph	IP	Ph	IP	Ph	IP	Ph
Metabolites in total	4.4	8.6	9.3	14.3	3.8	7.2	1.7	3.6	24	28
IN-REG72	1.7	2.3	2.4	2.0	ND	(0.5) [a]	0.5	(0.4) [b]	**18.5**	**16.6**
IN-UHD20	1.6	1.2	**6.9**	**9.2**	ND	ND	ND	ND	2.7	2.3
IN-F4106	ND	3.1	ND	ND	ND	2.9	ND	(0.5) [b]	ND	2.4
IN-QEK31	ND	ND	ND	ND	3.8	ND	0.5	ND	ND	ND
IN-REG72 sulfate conjugate	ND	ND	ND	ND	ND	ND	ND	ND	3.2	4.0
Unknown metabolites	1.0	1.4	ND	3.2	ND	3.2	0.7	3.6	ND	2.2

[a] detected after 20 minutes, not confirmed to occur after full-time incubation; Source: Novo, 2018

[b] detected after 60 minutes, not confirmed to occur after full-time incubation;

IP: [*Imidazo(1,2-a)pyridine-2-*^{14}C]fluazaindolizine; Ph: [*Phenyl(U)-*^{14}C]fluazaindolizine

The metabolite pattern was not much different between the different species even though not all metabolites were detected with all hepatocytes (see Table 10) but, as is usually the case, there were quantitative differences. By far the most abundant compound was always the parent but the next most abundant metabolite in terms of percentage was species-specific. No unique human metabolite was found, but extent of conversion appeared highest in human hepatocytes with IN-REG72 being the major metabolite. Indeed, the sulfate conjugate of IN-REG72 was observed only in human hepatocytes in this in vitro study, but this downstream metabolite had previously been found in vivo in rats and mice. It is worth noting that the in vivo metabolite IN-A5760 was not identified in this in vitro study. A few peaks corresponding to unknown compounds were observed which were assumed to represent impurities or degradation products as they were consistently detected also in control incubations with media, but without the hepatocytes. It may be concluded that a similar metabolism for fluazaindolizine may be expected with all the species employed in the toxicological studies as well as in humans (Novo, 2018).

1.3 Plasma levels and metabolites in apical toxicological studies

Plasma concentrations of fluazaindolizine were determined in a small number of animals as part of routine short-term and chronic toxicity studies in rats, mice and dogs. In addition, metabolites were identified and partly quantified in plasma, using reference standards for INREG72, IN-F4106, IN-QEK31, IN-A5760, IN-UHD20 (Q8U80-OH) and INUHD21 (IN-REG72-OH) even though not all of them were available for all studies in which plasma was analyzed. Quantification was performed by HPLC coupled with LC/MS/MS.

The analytical method for determination of the parent compound and these six metabolites in rat, mouse, and dog plasma had been validated in a separate study (Shen, 2020). Also, in that same study, long-time stability of these analytes in frozen plasma from the three species was demonstrated, with IN-UHD21 in dog plasma being the only combination not tested.

A summary of plasma levels of the parent compound in 90-day feeding studies in the different species is shown in Table 11. The calculation of what are described as "dose normalized" plasma concentrations allows a rough estimate to examine whether the (expected) increase in plasma concentrations compared with dose is linear or perhaps sublinear. The latter case might suggest saturation of absorption, but a more efficient metabolism at higher doses could be an alternative explanation. For this "normalization", the ratio between the plasma concentration and the mean daily intake was calculated, assuming as starting point, that the ratio between a plasma concentration of 25 000 ng/mL and a mean daily intake of 25 mg/kg bw per day (as was established in the 90-day study in rats for low-dose males) was a ratio of one to one.

In the mouse, markedly higher plasma levels were measured in females than in males at the lower dose levels of 300 and 1000 ppm. This sex difference might suggest higher oral absorption or slower distribution of fluazaindolizine in particular in female mice. At 1000 ppm some saturation of absorption was already evident, particularly in females, since the plasma concentrations did not exhibit a linear increase with dose. At the two upper dose levels, the ratio between plasma concentration and mean daily intake was clearly sublinear.

A higher mean plasma concentration of fluazaindolizine than in males was also observed in low-dose female rats, whereas no sex differences were apparent at higher dose levels. Saturation of absorption might have occurred in females between 500 and 1500 ppm but this was not reflected in males at any dose level. Even for female rats, this saturation (if indeed it was a real effect) was less pronounced than in mice.

In the dog, by contrast, there were no sex differences in plasma concentrations and no evidence of saturation of absorption was apparent.

Table 11. Summary of fluazaindolizine plasma concentrations and their relation to dose in 90-day dietary studies in different species

Species/ study	Dietary dose (ppm)	Mean daily intake (mg/kg bw/day)		Sampling day	Mean plasma concentrations (ng/mL)		Plasma concentration normalised for dose	
		Male	Females		Males	Females	Males	Females
Mouse, 90 days (Han, 2018)	300	44	50	38	29 780	86 060	0.68	1.72
	1000	146	157		73 180	147 000	0.50	0.94
	3000	444	511		164 000	206 200	0.37	0.40
	7000	1101	1177		237 000	348 000	0.22	0.30
Rat, 90 days (MacKenzie, 2013)	500	25	29	57	24 720	43 700	0.97	1.53
	1500	77	88		89 980	113 000	1.16	1.28
	3000	153	176		177 200	208 200	1.16	1.18
	6000	326	354		343 000	350 400	1.05	0.99
Dog, 90 days (Han, 2014)	125	5	5	61	6 160	6 960	1.23	1.39
	500	18	18		38 250	48 325	2.13	2.68
	1500	58	59		100 425	116 700	1.73	1.98
	4000/ 3000/ 2500 [a]	62	80		129 500	138 333	2.09	1.73

[a] stepwise reduction of dose over the course of the study;

In principle, this pattern was confirmed when plasma concentrations were determined after nearly one year of dietary substance administration in studies of longer duration. In the carcinogenicity

study in mice (Han, 2017), plasma concentrations in females at the lower dose levels of 100 or 300 ppm were two- or three-fold higher than in males, and still markedly higher at 1000 ppm, but were no longer different at the maximum dietary concentration of 2000/3000 ppm. Accordingly, some saturation of absorption became apparent in female mice at 300 ppm (equal to 47 mg/kg bw per day) and above but this was not observed in males.

In a long-term study in rats (Moon, 2018), plasma concentrations in females were markedly higher than in males at all dose levels. As in mice, some evidence of saturation of absorption was also seen in females from 500 ppm (c 20 mg/kg bw per day) and above, but not in male rats.

In a one-year study in dogs (Han, 2016), in contrast, plasma concentrations were linear with dose and any sex differences were not obvious.

Parent fluazaindolizine was by far the most prominent analyte found in plasma of all species tested. A number of metabolites such as IN-REG72, IN-F4106, IN-QEK31 and IN-UHD20 were also detected; these were the same as identified in the absorption, distribution, metabolism and excretion (ADME) studies in rats and mice. The most abundant was IN-UHD20 in mouse plasma, following administration of the higher doses (Table 12). In contrast, UN-UHD21 (hydroxylated IN-REG72) and IN-A5760 were minor metabolites in plasma. It is worth noting that the dog, in general, showed the lowest rate and range of metabolic activity. This finding is in line with the outcome of the comparative in vitro study (Novo, 2018, see section 1.2 above).

All metabolites were found in plasma at amounts that were orders of magnitude lower than the parent's plasma concentration. As an example, plasma concentrations of the parent and various metabolites in male mice and rats in the 90-day studies are shown in Tables 12 and 13.

Table 12. Plasma concentrations of fluazaindolizine and metabolites in male mice as obtained after 38 days of continuous dietary administration (plasma concentrations in ng/mL)

Dietary dose (ppm)	Mean daily intake (mg/kg bw/day)		Metabolite					
		Parent	IN-REG72	IN-F4106	IN-A5760	IN-QEK31	IN-UHD20	IN-UHD21
300	44	29 780	32	ND	ND	276	136	ND
1000	146	73 180	85	37	< LOQ	88	443	8
3000	444	164 000	346	215	9	531	3 530	< LOQ
7000	1101	237 000	1 527	781	6	1476	17 998	14

<LOQ: Less than the level of quantification; ND: Not detected; Source: Han, 2018

Table 13. Plasma concentrations of fluazaindolizine and metabolites in male rats as obtained after 57 days of continuous dietary administration

Dietary dose (ppm)	Mean daily intake (mg/kg bw/day)		Metabolite					
		Parent	IN-REG72	IN-F4106	IN-A5760	IN-QEK31	IN-UHD20	IN-UHD21
500	25	24 720	252	<LOQ	<LOQ	20.2	ND	ND
1500	77	89 980	1 291	76.9	18.2	75.9	ND	ND
3000	153	177 200	2 292	226	ND	261	ND	ND
6000	326	343 000	2 410	1 000	13.4	1292	ND	ND

<LOQ: Less than the level of quantification; ND: Not detected; Source: MacKenzie, 2013

In addition to these dietary studies of longer duration, the plasma pharmacokinetics of fluazaindolizine was evaluated in female rats (three animals per dose group) during a 14-day period of test compound administration via oral gavage. In this non-GLP study (also reported elsewhere in this monograph) dose levels were either 25 or 300 mg/kg bw per day. Serial blood samples were taken on study days 0, 7, and 13 two hours post dosing, on day 14 prior to dosing, and then on several occasions ranging from 15 minutes to five days following the final dose. Plasma was analyzed for parent fluazaindolizine. In addition, fat samples from these rats were analyzed for fluazaindolizine to investigate if there was any evidence of a potential for bioaccumulation.

Steady state plasma concentrations were achieved after the first dose, implying that the compound was entirely cleared within 24 hours. After administration of the final dose on day 14, the half-life of fluazaindolizine was 18 and 19 hours respectively in the groups that had received 25 and 300 mg/kg bw per day. This was a little longer than in the ADME study of Punler & Green (2017) at 10 and 200 mg/kg bw, but those figures were obtained after administration of a single dose. In female rats the time point of maximum concentration in plasma (T_{max}) was 30 minutes and five hours, at 25 and 300 mg/kg bw per day, respectively, and thus similar to the value previously identified in ADME studies (see Tables 4 and 5). The C_{max} was 6.4 times higher in the group receiving 300 mg/kg bw than for the low-dose group (25 mg/kg bw). Accordingly, comparing values the increase in C_{max} was sublinear relative to dose.

Fluazaindolizine did not appear to preferentially partition into fat, as fat to plasma ratios were generally below one (Nabb, 2018; revised by Fallers, 2020).

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

An overview of all acute toxicity studies in rats in which fluazaindolizine was administered via the oral, dermal and inhalation routes is provided in Table 14.

Table 14. Summary of acute toxicity studies with fluazaindolizine in the rat

End-point/ study type	Batch/lot tested, purity	LD_{50}/LC_{50}	Further remarks	Reference
Acute oral	SG0312632, 98.5%	1187 mg/kg bw	100% mortality at 1500 mg/kg bw	Fallers, 2017a
	D201398-2, 98.4%	940 mg/kg bw	Only one dose tested in female rats; 1/3 found dead, no clinical signs or necropsy findings in survivors	Fallers, 2018
	DPX-Q8U80-181, 94.8%	940 mg/kg bw	Different batches tested at different dose levels;	Fallers, 2019
	DPX-Q8U80-200, 96.9%	1248 mg/kg bw	Mortality observed at 940 mg/kg bw and above, approaching 100% at 1500 mg/kg bw and above; clinical signs observed in decedents but not in survivors	
	DPX-Q8U80-093, 98.5%			
	DPX-Q8U80-229, 98.0%			
	DPX-Q8U80-230, 98.0%	All at 1187 mg/kg bw		
	DPX-Q8U80-231, 98.0%			
	DPX-Q8U80-236, 98.3%			

End-point/ study type	Batch/lot tested, purity	LD_{50}/LC_{50}	Further remarks	Reference
Acute dermal	DPX-Q8U80-068, 99.6%	> 5000 mg/kg bw	No evidence of systemic effects or topical findings	Lowe, 2013a
	81354FB107, 98.7%	> 2000 mg/kg bw	No evidence of systemic effects or topical findings	Slonina, 2018a
Acute inhalation (4-hour, nose only exposure)	DPX-Q8U80-068, 99.6%	> 5.8 mg/L	No mortality; clinical signs observed only during treatment	Ng, 2013
	ENBK-169184-057, 97.8%	> 5.3 mg/L	No mortality but clinical signs lasting for up to five days	Kegelman, 2020

Acute oral toxicity

Study 1

In an acute oral toxicity study following the up-and-down procedure (OECD TG 425), fluazaindolizine (purity 98.5%;Batch no. SG0312632) was administered to young adult female Crl:CD(SD) rats. The test article had been dissolved in 0.1% Tween 80 in 0.5% methylcellulose solution and the dosing volume was 20 mL/kg bw. In a first step, a low dose of 940 mg/kg bw was administered by gavage to three animals. In addition, another female received the high dose of 1500 mg/kg bw. In a second (separate) experiment, two further rats were administered, again, 1500 mg/kg bw. The surviving animals were observed for 14 days post dosing for mortality, body weight effects and clinical signs. They were necropsied and subjected to gross examination.

All rats receiving the low dose survived until scheduled study termination without exhibiting any clinical signs or body weight losses. In contrast, all females which had been administered 1500 mg/kg bw died (in one case, had to be killed for humane reasons) on days 1 or 2. Prior to death, prostration, laboured breathing, hypoactivity, decrease in muscle tone, and a lack of righting reflex were observed and the animals became moribund. However, there were no noteworthy gross pathological findings for any rat.

Based on the mortality pattern, an LD_{50} of 1187 mg/kg bw was calculated. Thus, fluazaindolizine was of moderate acute oral toxicity and the dose response appeared rather steep. The absence of any grossly visible pathological changes in the decedents is surprising (Fallers, 2017a).

Study 2

In a subsequent experiment, three female rats of the same (Crl:CD(SD) strain received, by oral gavage, a single dose of 940 mg/kg bw (as in the first study), but of different fluazaindolizine lot (D201398-2; purity 98.4%). In contrast to the study described above, a higher dose was not included and the surviving animals were observed and weighed more often.

One of the rats was found dead on day 2 post dosing but no clinical signs were reported as preceding this death. In the surviving females, no clinical signs and no body weight losses were recorded up to termination on day 15. As in the previous study, no remarkable pathological findings were recorded at necropsy for any animal. Because of this single death, an LD_{50} of 940 mg/kg bw was estimated, confirming the moderate acute oral toxicity of fluazaindolizine (Fallers, 2018).

Study 3

In a third acute oral toxicity study, several lots of fluazaindolizine were administered as single doses to female rats. Dose levels were 940, 1500, 1750 or 2000 mg/kg bw, but not all seven lots were tested against all of them. For instance, the maximum dose was given only with lot DPC-Q8U80-181 to one animal, which died, so such a high dose was not administered in any further experiment. Surviving animals were observed for 14 days as in previous studies before being killed and necropsied.

Overall the mortality pattern was similar to that in the previous studies for acute oral toxicity. At the three high doses, all or nearly all animals died: 1/1 at 2000 mg/kg bw, 4/5 at 1750 mg/kg bw, and 5/5 at 1500 mg/kg bw. Twenty-three female rats in total received a dose of 940 mg/kg bw, of which six were found dead or had to be killed in extremis.

For most lots, an LD_{50} of 1187 mg/kg bw was calculated, but in a few cases the LD_{50} might be a step higher (1248 mg/kg bw) or lower (940 mg/kg bw). An overview is provided in Table 14. For two of the lots an LD_{50} could not be calculated because too few animals had been tested. Where an exact value is needed for risk assessment or classification and labelling purposes, the lowest LD_{50} should be used.

Clinical signs as reported before were confined to the rats which subsequently died; such effects were not observed in the survivors. Gross findings were unspecific and do not provide a clue to explain the fatalities (Fallers, 2019).

Acute dermal toxicity

Study 1

In an acute dermal toxicity study (limit test), 5000 mg/kg bw of fluazaindolizine (purity 99.6%; Lot no. DPX-Q8U80-068) was applied as a stiff paste, moistened with distilled water to give a 60% mixture weight for eight (w/w), to the clipped dorsal skin of five male and five female Sprague Dawley-derived rats. Twenty-four hours later, the animals' bodies were unwrapped, gauze pads with test substance removed and dosing sites gently cleaned.

The animals were observed for 14 days post dosing for mortality and clinical signs. Body weight was determined prior to administration, one week later and at scheduled termination after two weeks before the animals were necropsied and subjected to gross examination.

All rats survived until study termination. There were neither signs of systemic toxicity nor of local irritation, apart from one female in which mechanical damage to the skin due to unwrapping was noted to persist for three days. All animals gained weight. At necropsy there were no remarkable gross pathological findings in any rat.

In this study, fluazaindolizine was of low acute dermal toxicity. In both sexes, the LD_{50} was greater than 5000 mg/kg bw (Lowe, 2013a).

Study 2

A second acute dermal toxicity study was performed in three female Sprague Dawley-derived rats by means of the fixed-dose procedure. In contrast to the first study of this type, the test material was more representative of the technical grade active ingredient resulting from the manufacturing process. A dose of 2000 mg/kg bw fluazaindolizine (purity 98.7% ; Lot no. 81354FB107) was applied for 24 hours to the clipped dorsal area, at first of just one rat. Since no corrosion or toxicity was observed, the same dose was then applied to two additional animals. There followed a period lasting 14 days during which the rats were monitored for mortality, clinical signs and behavioural changes before they were killed and necropsied. Body weights were determined prior to substance application, on day 7 and at termination.

All the rats survived until scheduled sacrifice, appeared healthy and active and gained weight. No clinical signs and no dermal irritation were noted. Necropsy did not reveal pathological changes visible at gross examination. Thus the dermal LD_{50} in this study was greater than 2000 mg/kg bw (Slonina, 2018a).

Acute inhalation toxicity

Study 1

An acute inhalation toxicity study was performed, as a limit test, in five male and five female Crl:CD(SD) rats. The animals were exposed (nose only) for four hours to an aerosol of fluazaindolizine (purity 99.6%; Lot no. DPX-Q8U80-068) that was generated by suspending the test substance in air with a jetmill. The test chamber aerosol concentration was 5.8 ± 1.4 mg/L. Mass median aerodynamic diameter (MMAD) was measured twice and yielding results 3.6 and 3.3 µm.

Following exposure the rats were observed for 14 days for mortality and clinical signs before they were killed and given a gross pathological examination. During exposure their response to alerting stimuli was checked. The animals were weighed prior to treatment, on days 1, 2, 3, and 7 after exposure and at scheduled termination.

No deaths occurred during the study. Accordingly, the LC_{50} was greater than 5.8 mg/L in both

sexes demonstrating a low acute inhalation toxicity for fluazaindolizine. During exposure, laboured breathing was observed in one male and ruffled fur in one female. There were no further clinical signs of toxicity. All animals, except one female, lost weight on the day of exposure. From the next day onwards, however, they all gained weight again until scheduled termination. There were no remarkable gross findings in any rat at necropsy (Ng, 2013).

Study 2

For this end-point a second study was available in which, again, five male and five female Crl:CD(SD) rats were exposed (nose only) for four hours to an aerosol of fluazaindolizine (purity 97.8%; Lot ENBK-169184-057) that is more representative of manufactured technical grade fluazaindolizine and was generated by suspending the test substance in air with a jetmill. The test chamber aerosol concentration was 5.3 ± 0.32 mg/L; the MMAD provided the same figure of 3.9 µm when measured twice. After exposure the rats were observed for 14 days for mortality and clinical signs before they were killed and given a gross pathological examination. During exposure, their response to alerting stimuli was checked. The animals were weighed prior to treatment, on days 1, 2, 3, and 7 following exposure and at scheduled termination.

All animals survived the treatment and, thus, the LC_{50} was greater than 5.3 mg/L in both sexes confirming the previously reported low acute inhalation toxicity of fluazaindolizine. However, in this study more clinical signs were apparent than in the previous one. When removed from restrainers after the exposure period, abnormal gait (in 9/10 animals), lung noise and abnormal breathing, high posture, lethargy and red nasal and ocular discharge were noted. A few of these signs were still apparent in a small number of animals on the following days, but all had resolved by day 5 at the latest. All animals lost weight on the day of exposure but gained weight again afterwards. An additional interim body weight loss in one female was confined to day 4, but because of its isolated occurrence, was most likely incidental. Gross necropsy did not reveal any remarkable lesions (Kegelman, 2020).

(b) Dermal irritation

Study 1

The skin-irritating properties of fluazaindolizine were examined in New Zealand White (NZW) rabbits. The test substance (purity 99.6%; Lot no. DPX-Q8U80-068) was applied, using 0.5 g in total, to the intact skin of three female animals and retained under a semi-occlusive dressing for four hours. The test was performed with one rabbit and results observed before the two remaining animals were treated. Skin reactions were assessed immediately after removal of the patch, and then again 30–60 minutes later, and at 24, 48, and 72 hours thereafter. Body weight was determined prior to treatment and on the last day of scoring, that is after three days.

No signs of skin irritation were observed in this study at any time point. The mean scores for erythema and oedema at 24, 48 and 72 hours were all 0.0 for all rabbits. It may be concluded that fluazaindolizine was not irritating to rabbit skin in this in vivo experiment. In addition, there were no further clinical signs and all animals gained weight (Lowe, 2013b).

Study 2

A very similar study was performed more recently in three female NZW rabbits but with a different lot of the test substance (purity 98.7%; Lot no. 81354FB107). This was applied in the same quantity as in the previous study and the study design was virtually identical.

Slight erythema (score of 1) was observed in all animals after 30 to 60 minutes and in two of them at the 24-hour reading. Oedema was noted in two female rabbits after 30 to 60 minutes and was still apparent in one of them after 24 hours. At 48 and 72 hours skin changes were no longer present. The mean score for the 24-. 48- and 72-hour readings was 0.3 in two rabbits for erythema and also 0.3 in one animal for oedema. All animals appeared active and healthy and gained weight (Slonina, 2018b).

(c) Ocular irritation

Study 1

The potential for eye irritation due to fluazaindolizine was studied in vivo in NZW rabbits. Under local ocular anaesthesia (tetracaine hydrochloride), around 70 mg (corresponding to a volume of 0.1 mL) of the test substance (purity 99.6%; Lot no. DPX-Q8U80-068) was instilled into the conjunctival sac of the right eye of three females. Application of the test substance was performed in a stepwise procedure starting with one animal, followed by two more rabbits. Left eyes remained untreated and served as controls. Rinsing of the eyes was not reported. To avoid pain and discomfort, the rabbits were injected a systemic analgesic (buprenorphine hydrochloride) prior to instillation and on the next morning.

The ocular reactions were assessed approximately 1, 24, 48 and 72 hours after administration of the test substance. An additional eye examination was performed prior to treatment and at 24 hours following substance application and this consisted of instilling fluorescein dye solution into the eyes and inspecting for corneal damage made apparent under an ultraviolet light source. Body weight was determined prior to treatment and on the last day of scoring, that is after three days.

The animals appeared healthy and active throughout the study but two of them lost some weight.

Corneal opacity, conjunctival chemosis or signs of iritis were not observed during the study. A slight conjunctival reddening was observed in two animals at the one-hour scoring. At the same time point discharge was noted from the treated eye of one of these females. However, at 24 hours and thereafter the eyes of all animals appeared normal again. Accordingly, the mean scores as calculated from the 24-, 48-and 72-hour readings were 0.0 for corneal opacity, for iritis and for conjunctival redness and chemosis. On balance for this study, fluazaindolizine can be regarded as not irritating to the eyes of rabbits (Lowe, 2017).

Study 2

In a second study in NZW rabbits, 69 mg of fluazaindolizine from the manufacturing process (purity 98.7%; Lot no. 81354FB107) was applied in a volume of 0.1 mL to the right eye of three healthy females. To an extent, the study design was similar to that in the study described above (Lowe, 2017), including the measures taken to avoid pain and discomfort. However, the study differed significantly in one respect, since the animals were examined for signs of ocular irritation not only at 1, 24, 48 and 72 hours after treatment, but also on post instillation days 4, 7, and 10. A fluorescein dye evaluation was performed as before, only twice, prior to treatment and 24 hours thereafter.

In contrast to the study above, some eye irritation was seen. Weak corneal opacity (score 1) was noted in all three rabbits 24 hours after administration of fluazaindolizine. Whereas this sign had disappeared in two females 24 hours later, even more pronounced opacity (score 2) was observed in the third rabbit at the 48-hour reading. However, this also diminished and was no longer apparent at 72 hours or thereafter.

No iritis was seen in any rabbit, but conjunctival redness varying in severity (scores 1 or 2) was noted to persist in all rabbits until days 3, 4, or even day 7. Chemosis was observed in all females over the first 72 hours and was still present in two rabbits on day 4, with scores ranging from 1 to 3. Conjunctival discharge was also common but its duration varied from 24 hours to as much as four days.

Based on the 24-, 48-, and 72-hour readings the individual scores for corneal opacities ranged from 0.3 to 1.0, for conjunctival redness form 1.0 to 1.7, and for chemosis from 1.3 to 2.3. On balance, these findings suggest fluazaindolizine possessed a slight potential of for eye irritation when test material more representative of the current manufacturing process was evaluated.

The animals appeared healthy and active throughout the study and all gained some weight. However, since terminal body weight was determined after 10 days rather than three days, as in the first study, a possible early-onset impact of treatment on weight development can be neither confirmed nor excluded (Slonina, 2020).

(d) Dermal sensitization

The skin sensitizing potential of fluazaindolizine was investigated in a number of studies. Different methods were applied and the results were partly contradictory, apparently depending on the batches of fluazaindolizine applied.

Study 1

In a first maximization test following the protocol of Magnusson and Kligman, 20 male Hartley albino Guinea pigs were allocated to the test group and 10 additional animals served as negative (vehicle) controls. The test animals received fluazaindolizine (purity 99.6%; Lot no. DPX-Q8U80-068) for intradermal and topical inductions, as well as for the subsequent challenge; sham controls were also challenged with the test substance but after administration of just the vehicle and Freund's complete adjuvant during the induction steps. A positive control was not included, but sensitivity of the test system was being regularly checked in the performing laboratory.

Prior to the main experiment described above, 12 male Guinea pigs were employed for preliminary irritation testing. Fluazaindolizine concentrations of 1%, 3%, and 5% in polyethylene glycol (PEG) or in Freund's complete adjuvant (mixed with distilled water at 50% by volume) were injected intradermally. The 1% solution was found to produce faint to moderate irritation. When applied to the skin surface, a 65% (w/w) mixture in PEG caused no, or only very slight, irritation. The three lower concentrations between 16% and 49% produced no effect.

Based on these results, intradermal induction was performed with a 1% test substance preparation in PEG or Freund's complete adjuvant. The subsequent topical induction one week later was conducted with 0.5 g of a 65% test substance preparation, moistened with PEG, to which the animals were exposed for 48 hours. For epicutaneous challenge 22 days after the intradermal induction, Guinea pigs were administered fluazaindolizine at concentrations of 22% and 65% in parallel for 24 hours. Anonymized readings were made 24 and 48 hours later.

After intradermal induction all animals in the control and test groups showed faint to moderate erythema around the different injection sites. Blanching or dark discolouration at the injection sites were also common. Following topical induction, very faint to faint erythema was seen one hour after patch removal in all test and control animals. In five test group and one control animals, some desquamation was noted.

Following the later challenge with 65% fluazaindolizine, very faint erythema was observed at the 24-hour reading, but only one test animal (1/20) and this had resolved 24 hours later. No other skin reactions were seen in any other test or control animal. Accordingly, it can be concluded that fluazaindolizine did not cause skin sensitization effects in this Guinea pig maximization test.

All animals appeared healthy and gained weight over the course of the study (Lowe, 2013c).

Study 2

Another maximization test following the protocol of Magnusson and Kligman and therefore with a very similar study design to *Study 1* above, was run using male Hartley albino Guinea pigs, 20 allocated to the test groups with 10 additional animals serving as negative (vehicle) controls. A positive control was not included but sensitivity of the test system was regularly being checked in the performing laboratory.

Twelve more male Guinea pigs had been employed for a preliminary irritation testing revealing that 33% fluazaindolizine was the highest not-irritating concentration when applied to skin. A topical concentration of a 65% (w/w) mixture in PEG caused slight irritation. Following intradermal injection, a 5% solution was found to produce faint to moderate irritation.

Based on these results, the intradermal induction was performed with 5% test substance (purity 94.8%; Lot No. DPX-Q8U80-181) in PEG or in Freund's complete adjuvant (50% mixture by volume in distilled water). The subsequent topical induction one week later was conducted with 0.5 g of a 65% test substance preparation in PEG, to which the animals were exposed for 48 hours. For epicutaneous challenge 22 days after the intradermal induction, the Guinea pigs were exposed to fluazaindolizine in concentrations of 11% and 33% in parallel for 24 hours. Readings were made 24 and 48 hours later. For

control animals, only the vehicle (with or without the adjuvant) was applied for inductions and the test substance alone used for the challenge.

After intradermal induction, all animals of the control and test groups showed graduated slight to moderate erythema (grades 1 to 2) around the injection sites. Following topical induction, very faint to moderate erythema was seen one hour after patch removal in nearly all test animals, and also in one animal in the vehicle control group suggesting slight irritation.

Following challenge with 33% fluazaindolizine, faint to moderate erythema was observed at the 24-hour reading in 8/20 test animals. Twenty-four hours later erythema still persisted in seven of these. Following challenge exposure to 11% fluazaindolizine, the severity of the effects was weaker, nevertheless very faint to faint erythema was seen in 10/20 test animals after 24 and 48 hours. Only minimal skin reactions were observed in the control group. On balance, fluazaindolizine exhibited skin sensitizing effects in this Guinea pig maximization test even though no firm conclusion on its potency can be drawn. It must be emphasized that a different batch of test substance was used than in the previous study (Lowe, 2013c) and the purity of this test substance was markedly lower; 94.8% compared with 99.6% in the previous study.

All Guinea pigs gained weight during the study and no clinical signs were reported (Merrill, 2017).

Study 3

Four different lots of fluazaindolizine were tested in parallel for skin sensitizing potential using a local lymph node assay (LLNA) in mice. For this purpose, five groups of five or six female CBA/JHsd mice were dosed for three consecutive days on both ears with 0% (vehicle control) or 90% solutions of the four batches in PEG. The following lots were applied: DPX-Q8U80-068 (purity 99.6%), DPX-Q8U80-093 (98.5%), DPX-Q8U80-181 (94.8%), and DPX-Q8U80-200 (96.9%). For a concurrent positive control, groups of six female mice were dosed for three consecutive days with 25% hexylcinnamaldehyde (HCA) in PEG.

On test day 6 of the assay, mice received [^{3}H]thymidine by tail vein injection and were killed approximately five hours later. Cell proliferation in the draining auricular lymph nodes of the ears of each mouse from the test substance groups was then evaluated and compared to the vehicle control group.

No clinical signs of toxicity and no test substance-related differences in mean body weight compared to the vehicle control group were observed at any test concentration.

A statistically significant increase in cell proliferation compared to the vehicle group was observed at the 90% test concentration of fluazaindolizine of Lot Q8U80181. At 3.56 the stimulation index (SI) was greater than 3.0, but the EC3 value (the estimated concentration required to induce a three-fold increase in cell proliferation in an LLNA), considered to be the threshold positive response, could not be calculated in this study, since only a single concentration of this Lot had been tested. There were no statistically significant increases in cell proliferation in the 90% test concentrations observed with the other three Lots on test, for which the SI values ranged from 1.62 to 1.82.

The positive control, HCA, produced the expected dermal sensitization response in mice, with an SI of 12.08.

Based on these data, fluazaindolizine Lot Q8U80181 must be considered a dermal sensitizer, confirming the positive outcome of Merrill (2017) reported above. The remaining three lots of fluazaindolizine were not sensitizing under the conditions of this assay. For one of them (DPX-Q8U80-068), this result agreed with that reported by Lowe, (2013c) from the previous negative maximization test in Guinea pigs (Hoban, 2017a).

Study 4

In a further LLNA test, another lot of fluazaindolizine (Lot no. D201398-2, also identified as TSN315809; purity 98.1%) was tested, but at different concentrations of 0% (vehicle control), 5%, 45% or 90% in PEG. Apart from that, the animal system, study design, positive control substance and all other procedure were the same as in *study 3*, above, by Hoban (2017).

Based on the rate of radioactive decay, measured in disintegrations per minute (dpm), compared to the vehicle control group, SI values of 0.69, 1.49, and 1.39 were calculated for the three concentrations, respectively, with the positive control giving an SI of 19.18, (a clear positive result). Because the SIs for the test substances were below the threshold, an EC3 value, for which there is in any case no need, could not be calculated.

Under the conditions of this study fluazaindolizine Lot no. D201398-2 proved negative for skin sensitization. This outcome provides additional evidence that the positive responses observed in both the LLNA and the maximization test should not be attributed to this compound in general, but to certain batches and, most likely, particular impurities of fluazaindolizine (Hoban, 2018a).

An overview of the available irritation and skin sensitization studies with various batches of fluazaindolizine is shown in Table 15.

Table 15. Summary of irritation and skin sensitization studies with fluazaindolizine

End-point	Batch/lot tested, purity	Test method and test system	Results	Reference
Skin irritation	DPX-Q8U80-068, 99.6%	NZW rabbit, in vivo	No evidence of skin irritation	Lowe, 2013b
	81354FB107, 98.7%	NZW rabbit, in vivo	Slight and transient erythema and oedema	Slonina, 2018b
Eye irritation	DPX-Q8U80-068, 99.6%	NZW rabbit, in vivo	Not irritant	Lowe, 2017
	81354FB107, 98.7%	NZW rabbit, in vivo	Slightly irritant (conjunctival redness, chemosis, discharge)	Slonina, 2020
Skin sensitization	DPX-Q8U80-068, 99.6%	Magnusson & Kligman test in Guinea pigs	Negative (not sensitizing)	Lowe, 2013c
	DPX-Q8U80-181, 94.8%	Magnusson & Kligmann test in Guinea pigs	Positive (sensitizing)	Merrill, 2017
	DPX-Q8U80-068, 99.6%	Local lymph node assay (LLNA), mouse	Negative	Hoban, 2017a
	DPX-Q8U80-093, 98.5%	Local lymph node assay (LLNA), mouse	Negative	Hoban, 2017a
	DPX-Q8U80-181, 94.8%	Local lymph node assay (LLNA), mouse	Positive	Hoban, 2017a
	DPX-Q8U80-200, 96.9%	Local lymph node assay (LLNA), mouse	Negative	Hoban, 2017a
	D201398-2, 98.1%	Local lymph node assay (LLNA), mouse	Negative	Hoban, 2018a

At the request of JMPR the sponsor provided an explanation that the manufacturing process resulting in fluazaindolizine of lower purity (such as Lot No. DPX-Q8U80-181) observed to have sensitizing properties, is no longer in use.

(e) Phototoxicity

No data were submitted, however, it was mentioned by the sponsor that a new, guideline-compliant in vitro study was ongoing or at least in preparation.

2.2 Short-term studies of toxicity

(a) Oral administration

Mouse

Study 1

In a 28-day feeding study, fluazaindolizine (purity 99.2%; Lot no. FE114893-110) was administered to main (G1 to G5) and satellite (G1S to G5S) groups of Crl:CD1. Main groups consisted of five mice/sex per dose, satellite groups three mice/sex per dose. Nominal dietary concentrations were 0, 300, 1000, 3000, or 6000 ppm. For the main dose groups G2–G5, these dietary concentrations were equivalent to the following exposures: 52, 169, 514 and 1105 mg/kg bw per day for males, 58, 196, 634 and 1286 mg/kg bw per day for females. Intakes in the three-mouse satellite groups were similar.

The animals were monitored twice daily for mortality and clinical signs. Body weight, body weight gain, food consumption and efficiency were evaluated regularly. Blood was taken for haematological and clinical chemistry analyses at termination before the mice were killed and necropsied. Organ weights were determined and many organs and tissues preserved for histopathology. However, full-scale histopathological examination was performed only on top-dose and control animals, with the exception of the livers of male mice and kidneys of females which were microscopically assessed in all dose groups. The satellite groups were used mainly as a source of plasma to allow subsequent analysis for the concentrations of parent fluazaindolizine and its metabolites. However, it seems that this analysis was either eventually not performed or the results were not submitted. However, sufficient data of this sort is available, has been compiled and is presented in section 1.3 above, so this analysis is not regarded as necessary. In addition, body weight, food intake and organ weights were determined in the satellite groups and the animals were subjected to gross examination at necropsy.

All animals survived until scheduled termination and no clinical signs were observed that could be attributed to treatment. However, high-dose males lost weight during the first week of treatment. These losses resulted in a markedly lower mean body weight gain over the whole study period in this group compared to the controls (0.5 g against 1.7 g in the main group animals, 0.2 g against 1.1 g in satellite group animals). In contrast, the mean body weight gain in other male groups was similar to the controls. Because of the low number of animals in each group, the absence of statistical significance (surprising when the large difference is taken into account) is not a convincing argument that this finding was not treatment-related. Standard deviation was smaller in the high-dose males than in the controls. In high-dose females, lower body weight gain was also noted, but only among the satellite animals (0.2 g against 2.1 g in the control group) and therefore in females it was considered incidental. Food intake was similar in all groups, irrespective of sex. However, as to be expected because of lower body weight gain, food efficiency was clearly reduced in high-dose males and in high-dose satellite females. On balance, at least in male mice, the effect on the nutritional parameters is considered substance-related and adverse even though it was confined to what was a very high dose of more than 1000 mg/kg bw per day.

Minimal, but statistically significant, decreases in red cell mass parameters and increases in reticulocytes were observed in high-dose males and females which might suggest the red blood cells (RBCs) as a potential target. In addition, decreased total protein, characterized by decreased albumin (both sexes), and decreased globulin (females only) were present in the 514 mg/kg bw per day females and in high-dose males and females. Based on their minimal nature and in the absence of corresponding clinical signs, liver weight changes or pathological findings, these alterations in clinical chemistry parameters were not considered adverse, in contrast to the haematological findings.

Organ weights were apparently unaffected by test substance administration and there were no gross lesions which could be attributed to treatment. Histopathology revealed hepatocellular hypertrophy in high-dose males with four out of five animals affected, but not in females or in any other male group. In the absence of an organ weight increase, further microscopic lesions or clear-cut clinical chemistry findings, this observation might point to a treatment-related effect but cannot be regarded as adverse. In high-dose females, minor kidney findings were noted, for instance basophilic tubules, tubular necrosis in one mouse and unilateral (in two animals) or bilateral (in one mouse) dilatation of the renal pelvis; all of these might be early indications of nephrotoxicity.

On balance, the NOAEL in this 28-day oral feeding study with fluazaindolizine in the mouse was 3000 ppm (equivalent to 514 mg/kg bw per day). At the next highest dose of 6000 ppm, body weight gain and food efficiency was compromised in males, there were indications of effects on RBCs in both sexes and some potentially adverse microscopic kidney lesions were observed in females (Sudha, 2021).

Study 2

In a 90-day feeding study, fluazaindolizine (purity 99.6%; Lot no. DPX-Q8U80-068) was administered to male and female Crlj:CD1 (ICR) mice (10 mice/sex per dose) at concentrations of 0, 300, 1000, 3000 or 7000 ppm (equal to 0, 44, 146, 444 and 1101 mg/kg bw for males, 0, 50, 157, 511 and 1177 mg/kg bw for females).

Parameters evaluated included body weight, body weight gain, food consumption and efficiency, occurrence of clinical signs, ophthalmology (performed close to termination), organ weights, and gross and microscopic pathology. Blood samples for haematology and clinical chemistry were taken immediately prior to termination. The kidneys and livers (with gallbladder) from all animals were assessed under the microscope, whereas histopathology of all other collected organs and tissues was confined to the high-dose and control groups.

In addition to the main treatment groups, satellite groups of five male and five female mice were fed the test substance at identical dietary dose levels for approximately two months. From these animals, blood was collected on day 58 for analysis of plasma concentrations of fluazaindolizine and its metabolites. These samples were sent to the DuPont Haskell laboratory and the results are reported under section 1.3 above, and results shown in Tables 11 and 12; they are not further considered here.

All mice survived until scheduled termination and there were no clinical signs or ophthalmological findings that could be attributed to treatment. Occasional findings were minor in nature, transient and did not show a dose–response relationship. Food consumption was not affected in any group, however the overall mean body weight gain in high-dose males was by nearly 50% lower than in the control group, confirming a similar observation seen in the preceding 28-day study in the mouse (*study 1* above). The significantly lower body weight gain in top-dose males resulted in a reduction in mean total body weight in this group. Difference from the control group tended to increase with ongoing duration of treatment but statistical significance was achieved only once, after 84 days of feeding. In the same group, food efficiency was markedly compromised and the difference from the controls was statistically significant. Fluazaindolizine administration had no effect on body weight, body weight gain or food utilisation in female mice, or in the males at lower dose levels up to 3000 ppm. Thus the affected body weight development in males due to reduced food efficiency was probably treatment-related but can be regarded as a sex-specific, high-dose phenomenon.

Haematological changes were confined to a lower haematocrit (Ht) and a decrease in haemoglobin (Hb) in high-dose males, the latter of which that even achieved statistical significance. As an isolated finding with no counterparts in any other group, these minor alterations were considered of equivocal toxicological relevance even though similar observations had been reported in the 28-day study in the same strain of mouse. Since the extent of these decreases was smaller in this study, and the observations were confined to the male sex despite a longer exposure period, some adaptation or counter-regulation by the animals might have taken place. Clinical chemistry changes were also very minor but, on balance, suggest a potential weak impact on liver function. Slight but statistically significant decreases in albumin and in albumin/globulin (A : G) ratio were noted in males and females in the 3000 and/or 7000 ppm groups. Albumin reduction resembled what had previously been reported in the 28-day study. More specific for hepatotoxicity might have been an increase in high dose males in the activity of γ-glutamyltranspeptidase (GGTP), which is a highly liver-specific, but slowly reacting, enzyme. Activity of GGTP was below the LOQ in all other groups but could be quantified in the males receiving 7000 ppm. Dose-related decreases in total bilirubin at the two upper dose levels in males and in all treated female groups could be treatment-related, but in contrast to an increase, such negative changes in this parameter are not usually considered adverse.

Organ weight changes, presumed to be treatment-related, were confined to an increase in mean relative liver weight in males fed 7000 ppm. A marginal but statistically significant reduction in mean absolute heart weight and in the weight of accessory sex organs (prostate, seminal vesicles and coagulating glands combined) was also observed in this group, but was most likely due to the lower

terminal body weight seen at this dose level since the relative organ weights were not significantly different from the control group means. However the case for a reduction in these organ weights was supported when weights were expressed relative to brain weight even though the toxicological relevance of this calculation is to some extent equivocal.

The only gross observations that might be related to treatment were the abnormal shape of the left kidney in one of 10 females, and pelvic dilatation in a second female fed 7000 ppm. Both were associated with microscopic lesions. Dose-related microscopic changes were observed in liver (centrilobular hepatocellular hypertrophy and cytoplasmic basophilia), gallbladder (inflammatory cell infiltration, epithelial hypertrophy/hyperplasia, and hyalinosis in both sexes, as well as fibrosis and eosinophilic crystals occurring only in males), and kidney (fibrosis and tubular hypertrophy). Male and female mice fed 3000 and/or 7000 ppm of fluazaindolizine were affected. A summary histopathological findings presumed to be substance-related is shown in Table 16.

Table 16. Selected histopathological findings in the 90-day study with fluazaindolizine in mice (Number affected per group, n = 10/sex per dose)

	Dietary concentration (ppm)									
Organ: finding	**0 (control)**		**300**		**1000**		**3000**		**7000**	
	M	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Liver:										
Hypertrophy	0	0	0	0	0	0	0	0	4	2
Basophilic cytoplasm	0	0	0	0	0	0	1	0	3	2
Gallbladder:										
Inflammatory cell infiltration	2	0	1	1	0	2	3	4	6	4
Epithelial hypertrophy or hyperplasia	0	0	0	0	0	0	2	0	7	4
Fibrosis	0	0	0	0	0	0	0	0	4	0
Hyalinosis	0	0	0	0	0	0	3	0	9	5
Eosinophilic crystals	0	0	0	0	0	0	1	0	2	0
Kidney:										
Tubular hypertrophy (medulla)	0	0	0	0	0	0	2	4	9	7
Fibrosis	0	0	0	0	0	0	0	0	1	3
Infarction	0	0	0	0	1	0	1	1	2	5

M: Males; F: Females Source: Han 2018

The NOAEL in this study was 1000 ppm (equal to 146 mg/kg bw per day), based on histopathological changes in the gallbladder and kidney of male and female mice at the two upper dose levels of 3000 and 6000 ppm. Liver (with gallbladder) and kidney were identified as target organs. Unfortunately, no urinalysis was performed as part of this study and a potential impact on renal function remains unknown (Han, 2018).

Rat

Study 1

A wide range of end-points was investigated in a 14-day study in Crl:CD(SD) rats that was different from all the other short-term studies in that the test substance was administered by oral gavage. In the main experiment, fluazaindolizine (purity greater than 99%; Lot No. Q8U80-016) was suspended in 0.1% Tween 80 in 0.5% aqueous methylcellulose solution and applied once a day for up to two weeks to groups of five male and five female rats per dose at a volume of 10 mL/kg bw. The dose levels were 0 (vehicle control), 25, 300 or 1000 mg/kg bw per day. Because of excessive weight losses, the maximum dose had to be reduced after one week to 500 mg/kg bw per day.

An additional three females per dose (but not in the vehicle control group) were employed for serial blood and terminal fat sampling for toxicokinetic investigations; these are reported in section 1.3

above. Due to severe toxicity, meaningful evaluation of toxicokinetic parameters was not feasible in the high-dose group even though some measurements were performed until day 7. Accordingly, toxicokinetics is reported and discussed in the respective part of the monograph only for the low- and mid-dose levels.

Genotoxicity was investigated by means of a micronucleus assay in peripheral blood of animals from the male and female control and high-dose groups. Examination of low- and mid-dose animals for this end-point is not made clear in the study report. In addition, satellite groups of five male and five female rats were given a single dose of 2000 mg/kg bw. A positive control group receiving cyclophosphamide was also included. The genotoxicity part of the study is discussed in section 2.4 Genotoxicity, later in this monograph.

During the in-life phase, rats were monitored twice daily for mortality, appearance, clinical signs and abnormal behaviour. The animals in the main study groups were weighed daily and subjected to detailed clinical examination on days 2, 7 and 13, one to three hours post dosing. Haematological and clinical chemistry examinations (including urinalysis) were performed at termination. At terminal kill, all animals were necropsied and underwent gross evaluation. Bone marrow smears were prepared but apparently not assessed. A number of organs were taken and weighed and more were preserved for histopathological examination that was eventually confined, to the control and high-dose groups. From all main study animals, liver samples were taken to determine the total cytochrome P450 contents in microsomal suspensions. Hepatic peroxisome suspensions were prepared in which β-oxidation activity was measured.

All animals from the top dose group receiving 1000 mg/kg bw per day (including those females scheduled for kinetic investigations) were found dead or had to be killed in extremis. Three high-dose males were found dead, one each on days 9, 10 and 12, whereas the two remaining rats were prematurely killed on day 12. Three female rats were found dead , one each on days 5, 10 and 11, with the remaining five animals being killed in a moribund state on day 12. Clinical signs were confined to this high-dose group and comprised lethargy, polyuria, stained fur and swollen nose. Severe body weight losses were noted in both sexes.

In the groups receiving the low and mid dose levels of 25 or 300 mg/kg bw, in contrast, all rats survived, clinical signs were not observed and body weight and its gain were not affected, suggesting a rather steep dose response.

A number of haematological (suggesting severe anaemia and marked leukocytosis) and clinical chemistry findings were noted at the high dose but were considered to reflect the moribund state of the animals rather than a clear-cut, specific effect due to fluazaindolizine. At 300 mg/kg bw per day, some slight decreases in RBC parameters with the beginning of a regenerative response (a minimal increase in reticulocytes), and mild leukocytosis were noted in both sexes. Alterations in clinical chemistry parameters in the mid-dose group were confined to an increase in triglycerides in male rats and a decrease in glucose in females.

Due to the moribund state of the animals and the severe body weight losses, reliable evaluation of organ weight changes at the maximum dose level was not possible. There was a variety of gross and histopathological changes in high-dose males and females including gastric ulceration, secondary nasal inflammation and ulceration due to regurgitation, or degenerative findings in the haematopoietic and lymphatic systems, along with anorexia. Whilst it is certain they were related to treatment, substance-specific pathology cannot be distinguished from findings due to the animals' generally poor condition.

At the mid-dose level liver and kidney weights were increased in males and tended to be higher also in females. Differences from the control group were statistically significant only in some cases, so the small number of animals must be taken into account and statistical analysis not given too much weight. No gross lesions were noted and the only histological finding attributed to treatment was minimal erosion in the glandular stomach of two males.

Taking into account the moribund state of high-dose animals, investigations into liver enzyme induction in this group were inconclusive. Among animals receiving 300 or 25 mg/kg bw per day, total cytochrome P450 was not affected, but there was an increase in β-oxidation activity by about 25% compared to controls, in mid-dose males and females.

On balance the low dose of 25 mg/kg bw per day was the NOAEL in this study. Adverse findings at the next highest dose level of 300 mg/kg bw per day comprised indications of anaemia, organ weight increases and limited evidence of liver toxicity as suggested by alterations in clinical chemistry parameters. The top dose of 1000 mg/kg bw per day, even after reduction to 500 mg/kg bw per day, was clearly excessive and not tolerated by the animals. This is not surprising since the initial maximum dose was not much below of the LD_{50} as obtained in the acute oral studies reported above. Because of its unique design, the unusual dose spacing and small number of animals examined for a diversity of end-points, the regulatory value of this non-GLP study from 2010 is limited (Nabb, 2018; revised by Fallers, 2020).

Study 2

A combined 28-day study and one-generation study was performed in Crl:CD(SD) rats to which fluazaindolizine (purity 99.2%; Lot No. FE114893-110) was administered via the diet. In this part of the monograph only the 28-day feeding study is reported, the reproductive results are presented in section 2.5 Reproductive and developmental toxicity.

Five male and five female rats per group received the test substance for 28 consecutive days at dietary levels of 0, 500, 2500 or 5000 ppm before being. The total group size was 15/sex per dose. The remaining rats from the same groups continued on the test diets, mated and were then parental animals in this one-generation study. For the 28-day subset, the following mean daily intakes were calculated: 0, 37, 179, and 361 mg/kg bw for males and 0, 38, 195, and 369 mg/kg bw for females.

The animals assigned to the 28-day subset were monitored daily for mortality and signs of toxicity and were subjected to a detailed clinical inspection once a week. The rats were weighed weekly and food intake was also determined at weekly intervals, followed by calculation of food efficiency and mean daily compound intake. Prior to commencement of treatment and again during week 4 of fluazaindolizine administration, the rats from this subset were examined by means of an abbreviated functional observational battery (FOB) for possible effects on sensory function (reflexes, responses to approach, touch or auditory stimuli), grip strength and motor activity.

On the final day of the treatment period, rats were placed in metabolism cages and, following fasting for at least 15 hours, urine was collected. At termination, blood samples were taken for haematology and clinical chemistry. The animals were killed then and gross examinations carried out. A number of organs were removed and weighed. Histopathology was performed on these and many more organs and tissues but, with the exception of the urinary bladder that was evaluated microscopically in all groups, microscopic examination was confined to the control and top-dose groups.

There were no premature deaths during the four-week treatment period and no clinical signs could be attributed to substance administration at any dietary dose level. Body weight gain and, as a result total body weight, were reduced in high-dose males. For the whole 28-day period, body weight gain was suppressed by 16% compared to controls, but by nearly 28% in week 1. An initial but transient effect on body weight gain was also observed in males receiving 2500 ppm, but this had resolved completely after 28 days and thus was not regarded as adverse. A similarly transient reduction in body weight gain at the beginning of treatment was noted in high-dose females, but less pronounced than in males and not observable at 500 or 2500 ppm. Food consumption and food efficiency were both reduced in high-dose males and females over the entire treatment period. These reductions were by around 7%–15% compared to the control groups. No such effect was seen in either sex at the lower dose levels.

Neurological assessment, haematology, clinical chemistry and urinalysis did not reveal any differences between the groups that could be attributed to treatment. Likewise, no gross lesions were found at necropsy. Increases in some relative organ weights (brain, heart, testes) in high-dose males were explained by the lower terminal body weight in this group. No organ weight changes were noted in female rats. However, microscopic findings in the urinary bladder presumed to be treatment-related were detected in high-dose males and females. These changes were characterized by transitional cell hyperplasia resulting in a thicker bladder mucosa and were reported to occur in two out of five males and three out of five females.

On balance the NOAEL in the 28-day feeding subset of this complex study was 2500 ppm (equivalent to 179 mg/kg bw per day), based on affected nutritional and body weight parameters and microscopic findings in the bladder (Munley, 2017a).

Study 3

In a combined general short-term toxicity and subchronic neurotoxicity study in Crl:CD (SD) rats, fluazaindolizine (purity 99.6%; lot No. DPX-Q8U80-068) was administered for 95–99 days at nominal dietary concentrations of 0, 500, 1500, 3000 or 6000 ppm to groups of 16 male and 16 females rats. Mean daily intakes of 0, 28, 84, 166, and 348 mg/kg bw were calculated for male rats, and 0, 31, 97, 189, and 376 mg/kg bw for females. From five animals per sex and dose, 0.5 mL of blood was sampled on day 57 and sent to the sponsor to be analyzed for the parent compound and metabolites in plasma. The results are reported above in Table 13 of section 1.3.

Animals were monitored daily for mortality and clinical signs and weighed prior to the start of treatment and then,weekly. At weighings, rats were also subject to a detailed clinical examination. Ophthalmoscopy took place for all animals on day 0 and prior to termination. When neurobehavioural parameters were examined in selected animals, these rats were weighed once more. Food consumption was measured on a weekly basis and food efficiency and mean daily compound intake calculated.

The animals on study were assigned to two different subsets: general subchronic toxicity (10 rats/sex per dose) and neuropathology evaluation (six rats/sex per dose). With regard to the in-life evaluation of neurobehavioural parameters there was some overlap between animals from these two groups. Examinations by FOB (for example, grip strength, hind-limb foot splay and rearing) and motor activity assessments were performed during the adaptation period before commencement of treatment and then during weeks 4, 8 and 13. Examined were not just the neuropathology-subset rats but also four animals/sex per dose (always the same individuals) from the general toxicity subset.

Haematological, clinical chemistry and urinalysis parameters were determined in all animals of the general toxicity subset prior to scheduled termination. At the end of the study period animals were killed and necropsied. Organ weight were measured for adrenals, brain, heart, kidneys, liver, ovaries, prostate, seminal vesicles (with coagulating glands), spleen, testes (with epididymides) and uterus. All weighed and a number of additional organs were preserved and prepared for histopathology, but in the event most organs were examined microscopically only in the control and high-dose groups. However, histopathology of kidneys and urinary bladder were performed on all animals from the general toxicity subset. Gross lesions were also assessed under the microscope, irrespective of the dose group.

The rats selected for neuropathological examinations, in contrast, were deeply anaesthetized on days 98 or 99 and underwent whole-body in situ perfusion. Following evisceration and recording of any gross lesions, the remaining carcasses were sent to another laboratory with special expertise in neuropathology. There, different parts of the brain (forebrain, cerebrum with hippocampus, midbrain, cerebellum, pons, and medulla) and of spinal cord were taken, sectioned and prepared for neurohistopathology. Likewise, the eyes (with optic nerve), gastrocnemius muscle, a number of root fibres, ganglia and peripheral (sciatic, tibial and sural) nerves were sectioned and examined under the microscope. The neurohistopathological assessment of all these samples was confined, again, to control and high-dose groups since no differences were noted that would have made the additional evaluation of the low- and mid-dose groups valuable.

All animals survived until scheduled termination and no clinical signs could be attributed to treatment. Ophthalmoscopy and neurological examinations by means of FOB or motor activity measurements did not reveal any evidence of substance-related effects in any group.

In high-dose males and females, mean terminal body weight was below that of controls by 14% and 9%, respectively. This was due to body weight gain being lower than in controls by 20% (males) and 22% (females) in this dose group. At the same high dose, overall food consumption and also food efficiency were reduced in both sexes by 10%–12%. It seems likely that the adverse impact on body weight development was partly due to lower food intake, but also to a separate direct effect on metabolism of fluazaindolizine at high doses. Nutritional parameters were not affected at lower dose levels up to 3000 ppm.

Haematological findings were confined to the high dose level of 6000 ppm and comprised marginal but significant reductions (less than 10% below control values) in RBC count in males and of haematocrit (Ht) and haemoglobin (Hb) in both sexes, which resemble similar observations in other studies with fluazaindolizine in rodents (for example Han, 2018; Nabb, 2018). Because of their small size, the health relevance of these presumably treatment-related effects was considered borderline.

In addition, total leucocyte count was increased in top-dose males, but difference from the control group was not statistically significant and a similar tendency was apparent in the low-dose group suggesting that there was no dose–response relationship.

The only change in clinical chemistry parameters that could be attributed to treatment was a dose-related reduction in cholesterol in females receiving the test item at 3000 ppm (by 26%) and 6000 ppm (by 32%). In the absence of corroborative findings in clinical chemistry or histopathology, such a decrease was not regarded as adverse. The most outstanding urinalysis finding was a marked and statistically significant increase in urine volume in males at 3000 and 6000 ppm (86% and 190% above controls, respectively). Secondary to this effect, total protein concentration in urine was reduced in these two groups, as well as urine specific gravity. The observation of a higher volume of more diluted urine is in line with an apparent impact of fluazaindolizine on kidneys and/or the urinary tract, as already known before and also demonstrated in this study by histopathological evidence. In addition, urine pH was increased in high-dose males.

There were no gross lesions at necropsy which could be attributed to the test substance. Some changes in relative organ weights were noted at the high dose, mainly in males, but these were considered secondary to the reduced body weight.

Those histopathological findings that were presumed to be treatment-related were all found in the kidneys and urinary tract of male and female rats from the upper dose level groups, and these are summarized below in Table 17. Apparently, male rats were more often affected than females.

Table 17. Selected histopathological findings in the urinary tract in a three-month feeding study in rats (n = 10 rats per sex and dose)

	Dietary concentration (ppm)									
Finding	**0 (control**		**500**		**1500**		**3000**		**6000**	
	M	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Kidney:										
Pelvic dilatation [a]	1	0	1	1	0	1	1	0	4	2
Transitional cell hyperplasia [a]	0	0	0	0	0	0	1	1	6	3
Pyelitis	0	0	0	0	0	0	0	0	3	0
Pyelonephritis [a]	0	0	0	0	0	0	1	0	4	3
Urinary bladder:										
Transitional cell hyperplasia	0	0	0	0	0	0	0	0	5	0

[a] Unilateral and bilateral occurrence combined; M: Male; F: Female; Source: MacKenzie, 2013

The NOAEL in this study was 1500 ppm (equal to 84 mg/kg bw per day), based on effects on the kidneys in both sexes and the urinary tract in general in male rats at the upper two dose levels, supported by urinalysis findings. In addition, at the maximum dose level, nutritional parameters were compromised in male and female rats and there was some evidence of weak anaemia. Fluazaindolizine was apparently devoid of a neurotoxic potential when fed at dietary doses of up to 6000 ppm for about three months (see also section 2.6a below) (MacKenzie, 2013).

Dog

Study 1

A preliminary range-finding and palatability study was initiated in a small number of male and female beagle dogs (two/sex per dose) which received fluazaindolizine (purity: 99.6%; Lot No. SG0311305) at intended dietary concentrations of 0, 1000, 10 000 and 40 000 ppm. However, because of the immediate occurrence of severe toxicity, substance administration was suspended on day 2. In the high-dose groups, one of the two male dogs was found dead on the first day of treatment. This animal however produced no remarkable gross findings and only very few organs underwent microscopic examination. Minimal papillary mineralization was observed in the kidneys. In addition, congestion and moderate lymphoid depletion was seen in the jejunum. Perhaps because this very early death was not clearly attributable to treatment, this animal was replaced by a new male. The replacement dog, however, was also found

dead on day 3. At that time, treatment had already been terminated since clinical signs such as vomiting, subdued behaviour, asthenic gait and lateral position were noted in this animal as well as in the other male dog which had received the maximum dose from the beginning, and was still alive. In the second decedent, papillary mineralization of the kidneys occurred, too, and some dark-red abdominal fluid was noticed. When the remaining male dog from this high-dose group was killed in a moribund state on day 9, (one week after cessation of treatment) emaciation was apparent. This was most likely due to markedly reduced food consumption. In line with that, no stool was excreted and atrophy of a few organs such as the heart or the thymus was noted at necropsy. Microscopic kidney findings comprised tubular dilatation and degeneration and, again, papillary mineralization. A few haematological and clinical chemistry parameters were altered, partly reflecting the moribund state. It is worth noting that the two female dogs apparently tolerated this very high dose of 40 000 ppm better and survived, but vomiting was also observed. These same females were employed again when the study was continued.

In the surviving animals, the study was restarted on day 15, but at strongly reduced dietary dose levels of 0, 250, and 1000 ppm in male dogs and 0, 250, 1000, and 4000 ppm in females. Administration continued on 28 subsequent days. The revised dietary concentrations resulted in mean daily intakes of 0, 11 and 38 mg/kg bw per day in males and 0, 9.5, 37 and 139 mg/kg bw per day in females.

After this restart, no premature deaths occurred and no clinical signs of toxicity were observed. Ophthalmological examination did not reveal remarkable findings. Whereas there were no dose-related effects on body weight or food consumption in male dogs, food intake tended to be lower in females and one dog at the top dose of 4000 ppm consumed much less food than the other female on this dose, and even lost some weight. Body weight of this female dog decreased between study days 21 and 43 by nearly 14%, whereas a minimal increase was seen in the other female receiving 4000 ppm, similar to what was seen in the control, low- and mid-dose groups.

Haematology did not reveal findings that could be attributed to treatment. Alterations in clinical chemistry parameters comprised increases in akaline phosphatase (ALP) in both females receiving 4000 ppm and in one animal of each sex at 1000 ppm. Also an increase in alanine transaminase (ALT) in one high-dose female and a mild decrease in cholesterol in female dogs at the two upper dose levels suggested an impact on the liver, even though reductions seen in cholesterol were not considered adverse. Urinalysis did not elicit any remarkable findings.

There were no gross lesions at necropsy but the one high-dose female exhibiting some weight loss, appeared thin and its thymus was very small. Organ weights varied very much among the animals and did not allow firm conclusions to be drawn, especially taking into account the small number of animals on study. Histopathological findings were confined to single cell necrosis and pigmentation of Kupffer cells in the liver of one high-dose female, but microscopic examinations were rather limited in this preliminary study.

Even though the study authors proposed a NOAEL of 1000 ppm (equal to 37 mg/kg bw per day), this figure does not appear robust enough because only two animals per sex and dose were studied, and the uncertainties over organ weights even in the presence of clinical chemistry findings. However, it is not necessary to identify an NOAEL for such a range-finding study. The mortality pattern among the animals receiving the initial high dose of 40 000 ppm suggests that this dietary dose was acutely toxic and that the animals suffered acute poisoning and died as a result. Under the assumption that the maximum dietary dose might roughly correspond to a mean intake of around 1000 mg/kg bw, this data provides some evidence that the acute oral toxicity of fluazaindolizine in dogs may be at similar levels as were seen in rats (Lee, 2013).

Study 2

In a three-month feeding study, fluazaindolizine (purity: 99.6%; Lot No. SG0311305; purity: 99.6%) was administered to groups of four male and four female beagle dogs at dose levels of 0, 125, 500, 1500 or 4000 ppm. Due to strongly reduced food consumption in the highest-dose group, the dietary concentration fed to this group was decreased first to 3000 ppm on day 24. Subsequently, after one animal had died, treatment was suspended in this group on day 29 for 10 days and then continued at a dietary level of 2500 ppm. Because of these intercurrent changes, the results obtained in the high-dose group must be regarded with some reservations. Calculated mean daily intakes of fluazaindolizine were

approximately 0, 5.5, 20, 59 and 68 mg/kg bw in male dogs, and 0, 5.1, 21, 61, and 93 mg/kg bw in females, a surprising difference in intake between high-dose males and females.

The animals were monitored daily for mortality and signs of toxicity, including a detailed clinical examination at weekly intervals. Ophthalmoscopy was performed prior to treatment and near the end of the treatment period. The dogs were weighed once a week and food consumption measured daily throughout the study. Haematological and clinical chemistry analyses including urinalysis were performed before the commencement of treatment, and at weeks 4, 8, and 13 of substance administration. In addition, bone marrow smears were prepared at terminal necropsy but not evaluated. During week 9 blood samples were taken and plasma sent to the sponsor for determination of fluazaindolizine and some of its metabolites. The results of these analyses are shown in Table 11 in section 1.3.

At termination, on days 91–93, the dogs were killed and necropsied. Adrenals, brain, epididymides, heart, kidneys, liver (with gallbladder), ovaries, pituitary, prostate, testes, thymus, thyroid (with parathyroids), spleen, and uterus with cervix were weighed. All these and a number of additional organs and tissues were taken from all animals and subjected to subsequent full histopathological examination. At necropsy, liver samples of approximately 7 g were taken, deep-frozen and sent to the sponsor for subsequent biochemical evaluation.

There was one unscheduled death in this study and this was considered possibly treatment-related. One high-dose female was found dead on day 28 (four days after the first reduction in dose) having displayed lateral position, tonic convulsion, stiff appearance and dyspnea prior to death. Pathological examination suggested some liver involvement but similar lesions were seen in surviving dogs at scheduled termination. Preceding death, the animal had lost weight but the same effect was observed in the other animals of this group before dose reduction. Haematological and clinical chemistry examinations did not reveal any apparent cause of death.

There were no clinical findings in the other animals, except the effects on nutritional parameters described in the following paragraphs. The few ophthalmological findings were not dose-related and thus not attributed to treatment.

There were clear effects on body weight and body weight gain in both sexes at the high dose, which persisted after dose reduction, but also effects in males receiving the next highest dose of 1500 ppm. The reduction in body weight was partly reversible when dosing was suspended. The results are summarized below in Table 18. The absence of statistical significance for body weight gains in males does not contradict the assumption that the effects at the two upper dose levels were treatment-related, given the few animals in the various groups. In particular in females, a remarkable variability in body weight gain among the groups was noted. An outstanding increase in body weight gain was noted at the mid-low dose of 500 ppm.

To an extent, the adverse effects on body weight were certainly due to reduced food consumption. In male dogs, food intake was clearly lower in the high-dose group from the beginning of treatment and throughout the study period, with the possible exception of the interval in which the administration of fluazaindolizine had been interrupted. At the next lower dose level of 1500 ppm, food consumption tended to be lower until week 5, but was then in the same range as the control group. It might be worth noting that food intake was always highest in the male group that received the low dose of 125 ppm.

In females, lowered food intake became apparent in the high-dose group at latest in the third week of treatment and remained lower than in the controls for the rest of the study, apart from the partly substance-free interval in weeks 6 and 7 when it was higher. No effect was seen at 1500 ppm and food intake was always the highest in the group receiving 500 ppm, in which body weight gain was also particularly high.

In addition to reduced food consumption, food efficiency was strongly compromised in high-dose males and females and will have contributed to the body weight losses.

Table 18. Mean body weights at selected time points (kg, rounded) and overall body weight gain (g) in the 90-day feeding study with fluazaindolizine in beagle dogs

	Dietary concentration (ppm)									
Parameter	**Control**		**125**		**500**		**1500**		**4000/3000/2500**	
	M	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Body weight: day 0	6.17	5.99	5.97	5.67	6.29	5.97	6.06	5.99	6.25	5.84
day 28	6.68	6.27	6.76	6.07	6.90	6.59	6.32	6.29	5.36 [a]	5.03 [a]
day 42	6.94	6.42	7.05	6.15	7.13	6.85	6.40	6.36	5.90 [b]	5.28 [b]
day 90	7.40	6.45	7.57	6.55	7.68	7.36	6.83	6.49	5.28*	4.94
Overall body weight gain	1229	459	1601	889	1388	1390*	768	497	−975	−627*

M: Male; F: Female; Source: Han, 2014

[a] Dosing suspended due a single death occurring 4 days after first dose reduction because of reduced food consumption;
[b] Treatment continued at further reduced dose level; * $p < 0.05$, by Dunnett's test

Haematological parameters were not obviously affected by treatment at doses up to 1500 ppm, neither in males nor females. At the maximum dose level a decrease in RBC parameters was noted in a single male with some regenerative response also apparent. In line with that, in the same animal, spleen weight was increased and splenic congestion and extramedullary haematopoiesis were noted. It cannot be excluded that this anaemia was treatment-related even though the group means of red cell parameters were not much affected.

Blood clinical chemistry revealed a decrease in total protein and its albumin fraction in high-dose males and females, as well as markedly lower cholesterol and calcium levels. As the study progressed these effects also became apparent in the groups receiving 1500 ppm, although statistical significance was only occasionally achieved. Activities of ALT, aspartate transaminase (AST), and GGTP were increased in top-dose males, and so was SDH activity in high-dose females. Alkaline phosphatase activity was significantly higher in males from 1500 ppm upwards and tended to increase in female dogs receiving the maximum dose. On balance, these findings indicate an impact on liver function. Urinalysis did not provide consistent findings.

Gross pathological findings were confined to the high-dose groups. Spleen enlargement was observed in the single male with anaemia and in two high-dose females. Likewise, the only organ weight change presumed treatment-related was an increase in mean spleen weight at the maximum dose. In one male from this group the thymus was small; this and an oedematous pancreas and ascites in another one might suggest liver toxicity. Atrophy of the heart was noted (microscopically) in two high-dose males and two high-dose females but was considered secondary to the suppression of body weight.

Histopathological findings that could be attributed to treatment were observed in both sexes at the two upper dose levels, and these are summarized below in Table 19.

Table 19. Selected histopathological findings in the three-month feeding study in beagle dogs (n = four per sex and dose)

	Dietary concentration (ppm)									
Finding	**0 (control)**		**125**		**500**		**1500**		**4000/3000/2500**	
	M	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Liver:										
Single cell necrosis	0	0	0	0	0	0	1	1	4	4 [a]
Increased pigmentation	0	0	0	0	0	0	1	0	4	4 [a]
Periportal vacuolation	0	0	0	0	0	0			3	1
Glycogen depletion	0	0	0	0	0	0	1	0	4	1
Gallbladder:										

Finding	Dietary concentration (ppm)									
	0 (control)		125		500		1500		4000/3000/2500	
	M	F	M	F	M	F	M	F	M	F
Pigment in lumen	0	0	0	0	0	0	0	0	2	1
Kidney:										
Hyaline/pigment deposition in tubular epithelium	0	0	0	0	0	0	0	0	4	2
Spleen:										
Congestion	0	0	0	0	0	0	0	0	4	3
Extramedullary haematopoiesis	0	0	0	0	0	0	0	1	2	1

[a] Including the animal found dead on day 28; M: Male; F: Female; Source: Han, 2014

The biochemical investigations of liver samples revealed an increase in total cytochrome P450 enzymes at the highest dose level. The activities of CYP2E and CYP3A were increased in a dose-related manner. In contrast, a significant decrease in hepatic β-oxidation activity was observed in samples from the highest-dose group. According to the study author, this last finding was surprising since usually an increase would have been expected because of higher enzyme activity, at least when a substance was a peroxisome proliferator. All these enzyme-related findings were probably treatment-related, but they cannot be regarded as necessarily adverse.

In this study, a NOAEL of 500 ppm was established (equal to 20 mg/kg bw per day). It was based on mild histopathological and clinical chemistry findings suggestive of liver toxicity, and a lower body weight gain in males at 1500 ppm. Biochemical investigations also suggested an effect of fluazaindolizine on liver function in dogs.

The maximum dietary level of 4000 ppm, even after reduction to 2500 ppm, was clearly excessive and caused severe body weight loss due to lower food intake and reduced food efficiency, but also a number of additional effects, on, for example blood, spleen, heart, and kidney. Overt liver toxicity was evident. One female dog even died receiving the reduced top-dose level (Han, 2014).

Study 3

In a one-year study, fluazaindolizine (purity 98.5%; Lot no. SG0312632) was administered to groups of four male and female beagle dogs at daily dietary dose levels of 0, 125, 500, 1000 or 2000 ppm. The calculated mean daily intakes of the test substance were 0, 4.4, 20, 36 and 66 mg/kg bw for male dogs, and 0, 4.6, 17, 37 and 70 mg/kg bw for females.

The animals were monitored twice daily for mortality and signs of toxicity and subjected to a detailed clinical examination at weekly intervals. Ophthalmoscopy was performed prior to treatment and at the end of the treatment period. The dogs were weighed once a week. Food consumption was measured daily but food efficiency and intake of test article calculated on a weekly basis. Haematological and clinical chemistry analyses including urinalysis were performed before the beginning of treatment and at weeks 13, 26 and 52. Also, blood from the moribund animal (see below) was sampled on day 101. Additional blood samples (c 1 mL) were taken from all surviving dogs during week 51 and plasma samples were sent to the sponsor for analysis of fluazaindolizine and some of its metabolites. The results have been briefly reported in section 1.3, above. After terminal kill, dogs were subjected to necropsy and gross examination. Organ weights (same organs in the 90-day study reported above) were determined and a wide range of organs and tissues were examined histopathologically.

A single death was attributed to treatment. A high-dose female was found moribund and had to be killed on day 101, after more than three months of continuous fluazaindolizine consumption. Before premature termination this dog had suffered from severe weight loss that had begun on day 14 of treatment; weight loss was 32% in total and, accordingly, the dog appeared thin. In what would appear to be correspondence with this, food intake was markedly reduced. It is worth noting that another female from this group experienced transient weight loss and also appeared thin between days 14 and 105 of substance administration, but then gradually recovered. Returning to the prematurely terminated

female, deep respiration, paleness and lateral position were also noted. Haematological findings such as reductions in RBC count, Hb and Ht by 10% to 20%, and a nearly 10-fold increase in reticulocyte count were indicative of regenerative haemolytic anaemia, as well as some pathological findings reported below. Clinical chemistry analysis and microscopic examination following necropsy revealed liver toxicity in this animal.

All the other animals survived and no clinical signs (including ophthalmological findings) occurred which could be attributed to treatment. Mean body weights were transiently lower in high-dose males and females. This was mainly due to body weight losses in some animals during the first three months of substance administration. In the following three months however, body weight gain in both sexes was highest in the groups receiving 2000 ppm. Body weight data are summarized below in Table 20.

It should be taken into consideration that the single animal that was found in a moribund state was still included in the calculation and, accordingly, contributed to the extreme weight loss in females in the first three month interval. On balance, initial body weight loss at the highest dose level was regarded as an adverse but reversible effect of fluazaindolizine in dogs, but individual differences in vulnerability to this particular effect are likely.

Table 20. Mean body weights at selected time points (kg, rounded) and overall body weight gain (g) in the one-year feeding study with fluazaindolizine in beagle dogs

		Dietary concentration (ppm)									
		0 (control)		125		500		1000		2000	
Parameter		M	F	M	F	M	F	M	F	M	F
Body weight:	day 0	6.63	5.49	6.41	5.51	6.19	5.65	6.20	5.51	6.55	5.68
	day 91	8.22	6.99	7.72	5.91	7.30	7.24	7.02	6.31	6.40	5.15
	day 365	8.80	7.72	8.57	6.38	8.41	8.06	8.32	6.98	8.11	7.26
Body weight gain:	days 0–91	1593	1502	1304	401	1103	1590	822	802	−156	−522
overall	days 0–364	2170	2231	2155	879	2211	2408	2124	1477	1550	1394

M: Male; F: Female; Source: Han, 2016

In line with reductions in body weight, food intake was markedly lower in high-dose males and females but only during the first three months of substance administration. Thereafter, food consumption normalized and was similar to that of controls and other treatment groups. Food efficiency displayed a similar pattern with decreases only during days 0–91. Furthermore, food efficiency was markedly lower in low-dose females when calculated over the whole study period, and this might explain the outstandingly low overall body weight gain (see Table 20). However, in the absence of a dose–response relationship this finding cannot be attributed to treatment.

Haematological parameters were not affected in male dogs at any dose level. In females, mean values for RBC count, Ht and Hb were significantly decreased after 13 weeks, but also after 26 weeks. Because the finding at 26 weeks cannot be attributed solely to the poor state of the animal that was killed on day 101, the changes observed cannot just be attributed to that animal's moribund state. However, these differences from the control group, presumed to be treatment-related, had completely resolved at termination, suggesting the development of some tolerance to the effects of fluazaindolizine.

A number of significant changes in clinical chemistry parameters were noted in high-dose males and females even though statistical significance was not always achieved. These findings comprised lower serum albumin concentrations as well as reductions in cholesterol and calcium in both sexes. Significant decreases in calcium and albumin were also observed at the mid-high dose of 1000 ppm, but only in male dogs. Occasional increases in the activities of ALT, AST, SDH and GGTP were observed in both sexes at 2000 ppm. A dose-related increase in ALP was very common and, in males only, gained statistical significance from 1000 ppm upwards. On balance these findings suggested an impact on liver function. No significant changes, however, were observed up to 500 ppm. Urinalysis did not reveal any findings that could be attributed to treatment.

Organ weight changes that were presumed to be substance-related were few and all confined to

the maximum dose level. Absolute liver weight in high-dose males was increased even though statistical significance was achieved only for the relative organ weight. This agrees with the observation of the liver as a target organ of fluazaindolizine in dogs. In addition there were significant increases in the absolute weight of the adrenals in high-dose males and of relative adrenal weight in high-dose females. The adrenal findings, however, were not corroborated by pathological changes and therefore, considered toxicologically equivocal.

Gross findings were attributed to administration of fluazaindolizine only in the one premature decedent. In this high-dose female, enlargement of the spleen, jaundice and black contents in the lumen of the digestive tract might agree with anaemia, liver toxicity and bleeding. In all other animals, occasional gross findings were considered incidental, mainly in the absence of any dose–response relationship.

Histopathology revealed a small number of liver findings that confirm the results from the 90-day study reported above. No evidence of treatment-related findings was obtained from other organs. The relevance of sperm granuloma is equivocal because it was also seen at the low dose and in the control group in the preceding 90-day study. Findings that might be presumed substance-related are summarized in Table 21. Apart from that, further liver changes such as Kupffer cell hyperplasia and multifocal vacuolation of stellate cells were observed in the prematurely killed female only, and in the same animal, evidence of extramedullary haematopoiesis was obtained from its liver and spleen.

Table 21. Selected histopathological findings in a one-year feeding study in Beagle dogs (n = four dogs per sex and dose)

	Dietary concentration (ppm)									
	0 (control)		125		500		1000		2000	
Finding	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Liver:										
Single cell necrosis	0	0	0	0	1	0	0	1	0 /	2 [a]
Centrilobular hepatocyte pigmentation	0	0	0	0	0	0	1	0	4	3
Epidymidis:										
Sperm granuloma	0	-	0	-	0	-	0	-	2	-

M: Male; F: Female; Source: Han, 2016

[a] Including the animal found moribund and killed on day 101

The premature death of one female dog, transient body weight losses, lower food consumption and food efficiency in both sexes, and transient haematological findings (suggestive of mild anaemia) also in surviving females were observed at the top dose of 2000 ppm in this study. In addition, liver toxicity was demonstrated at this dose by alterations in clinical chemistry parameters, higher organ weight and histopathological findings; this appeared more pronounced in males than in females. The NOAEL was the next lowest dietary dose of 1000 ppm (equal to a mean compound intake of 36 mg/kg bw per day). Very few of the changes in clinical chemistry at this dose in males may be related to treatment; because of their small divergence from control values and lack of concomitant microscopic findings or statistically significant liver weight increases, they were not considered adverse.

Since this NOAEL (36 mg/kg bw per day) was above the NOAEL in the 90-day study (20 mg/kg bw per day), but below the LOAEL in the 90-day study in beagle dogs (Han, 2014), it can be regarded as the relevant overall NOAEL for short-term toxicity of fluazaindolizine in the dog, although different batches of the test compound were used in the studies. The Meeting felt that this approach was justified since the dogs were supplied by the same breeder and the two studies were run in the same laboratory (Han, 2016).

(b) Dermal application

In a four-week study, fluazaindolizine (purity 98.5%; Lot no. SG0312632) was moistened with distilled water and dermally applied to groups of 10 male and 10 female Crl:CD(SD) IGS rats at nominal dose levels of 0 (vehicle control, distilled water), 125, 250, 500 or 1000 mg/kg bw per day. The animals were

treated for six hours per day on 28 consecutive days.

The rats were monitored for mortality and clinical signs of toxicity. Body weight and body weight gain, food intake and food efficiency were determined. Detailed clinical and ophthalmological observations were performed, including weekly skin irritation scoring. Haematological and clinical chemistry examinations, including urinalysis, were performed close to the end of the treatment period. At termination the animals necropsy and gross examination was performed. A range of organs was taken, weighed and prepared for histopathological examination, however subsequent examination was confined to the control and high-dose groups.

There were no unscheduled deaths and no clinical signs that could be attributed to treatment. No signs of irritation were observed. There were no remarkable changes in any parameter under investigation. The few macroscopic observations noted were incidental. Thus, both the systemic and the local NOAEL in this study was 1000 mg/kg bw per day, the highest dose tested (Bauter, 2014).

(c) Exposure by inhalation

No study was available.

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

A carcinogenicity study was performed in Crlj:CD1 (ICR) mice over a duration of 18 months. In the main study, groups of 60 animals per sex were fed fluazaindolizine (purity 98.5%; Lot No. SG0312632) at concentrations of 0, 100, 300, 1000 or 3000 ppm. As a maximum dietary dose, 2000 ppm had been originally foreseen but this amount was fed for only 28 days and was then increased to 3000 ppm in both sexes, apparently because of an U.S. EPA recommendation. Overall, these dietary doses were equal to (rounded) mean daily intakes over the whole study period of 0, 15, 43, 142, and 427 mg/kg bw for males and 0, 17, 54, 177 and 525 mg/kg bw for females. Satellite groups of five animals per sex received the same dietary doses to provide blood samples for determination of plasma levels of fluazaindolizine and its metabolites after approximately 12 months of feeding, but were then killed without further examination. The plasma samples were sent to the DuPont Haskell laboratory and the outcome of the analytical work is briefly addressed in section 1.3 and not considered here.

The mice were observed daily for mortality and occurrence of clinical signs. A detailed clinical examination was performed once a week, whereas ophthalmological assessment took place only prior to the commencement of treatment and then again close to its cessation. Food consumption and body weight were determined on a weekly basis for the first three months of the study and every four weeks thereafter to allow calculation of body weight gain and food efficiency.

Blood smears were prepared after 12 and 18 months from all surviving animals of the main groups and also from any killed in extremis over the course of the study. Examination for changes in total and differential white blood cell count was confined to those blood smears obtained at scheduled termination from the control and high-dose groups, and if available, from premature decedents. Full haematological analysis was not performed because it is not compulsory in studies of this type. Likewise, clinical chemistry parameters in blood or urine were not determined.

All animals surviving until scheduled termination after 78 weeks and all available mice which were found dead or had to be killed during the treatment period were subject to necropsy and gross examination. Adrenals, brain, heart, kidneys, liver (with gallbladder), ovaries, spleen, testes (with epididymides) and uterus with cervix were weighed. All these and an extensive selection of further organs from the control and high-dose groups were subjected to histopathological examination. In addition, kidneys and eyes (in both sexes), liver with gallbladder (in males only) and mammary glands in females were examined under the microscope in all animals from all groups. The same holds true for all gross lesions and for those organs in which a significant increase in amyloidosis was observed at the top-dose level, in practice this was the jejunum in male mice and the mandibular salivary glands in females.

On balance, the mice tolerated the administration of fluazaindolizine up to the highest dietary dose level of 3000 ppm very well and there were only very few findings that could be attributed to treatment. Survival was sufficient in all groups. Survival rate varied only slightly from 75% to 82% in male groups and between 68% and 73% in females, without any dose–response relationship. No evidence of treatment-related mortality, or of a more frequent occurrence of clinical signs was obtained at any dose level.

Mean body weight and body weight gain, food intake and food efficiency were not affected by treatment. During some study intervals these nutritional parameter values did decrease in female mice receiving the upper intermediate dose of 1000 ppm but since no reduction was seen at the top dose, these findings were considered incidental.

Ophthalmological examination revealed an increase in cataracts in male mice in treated groups when compared to the controls whereas the opposite was noted in females (Table 22). There was no clear dose–response relationship, but on the other hand, some saturation of absorption at 300 ppm and above is likely (see section 1.3). More convincing arguments against a treatment-related adverse effect due to fluazaindolizine are the lack of statistical significance and the absence of dose-dependent microscopic eye lesions. In particular, the number of male mice exhibiting lenticular degeneration was not increased. To conclude, an adverse effect on cataract formation due to substance administration is not likely.

Table 22. Cataract incidences in male and female mice after nearly 18 months of dietary exposure to fluazaindolizine (number of individuals affected/number examined)

	Dietary concentration (ppm)				
Sex	**0 (control)**	**100**	**300**	**1000**	**3000**
Males	0/50	3/50	6/45	6/46	6/47
Females	15/43	10/46	8/42	8/43	8/41

Source: Han, 2017, revised 2021

The limited haematological information gained from blood smears did not indicate any effect that could be attributed to treatment.

There was no significant increase in gross lesions with dose and no statistically significant changes in organ weights were noted.

Gross and histopathology did not reveal a dose-related increase in tumour frequency in either sex. The only neoplastic finding that might warrant more detailed consideration was lymphoma. In male mice lymphoma was observed mainly in the group receiving 300 ppm, occurring at a higher frequency in various organs such as the adrenals, the Peyer's patches in the GI tract, some lymph nodes, rectum, salivary gland, testes, urinary bladder and the lungs, as well as in nasal cavity, but no dose–response relationship was apparent. Lymphoma was usually observed in the same animal at different sites. For instance, in male mouse 3M0011 from the 300 ppm group that was found dead on day 232, lymphoma was detected in the adrenals, the aorta, and coagulation glands, in the duodenum, the epididymis, femur, in the kidneys, liver and lungs, in several lymph nodes and some other organs. These findings reflected systemic lymphoma in very few animals with the number of affected mice differing between dose groups. Between the control and high-dose groups, in which all animals were subject to comprehensive histopathological assessment however, no difference was found and so no treatment-related effect can be assumed.

There was no increase in any single organ-specific, non-neoplastic lesion that could be attributed to administration of fluazaindolizine in either males or females. However, in high-dose males, amyloid deposition was increased in a number of organs. By implication, the local accumulation of insoluble protein fibrils that was noted might eventually affect the function of the organs that are thus affected. Statistical significance was only achieved for the jejunum, with amyloidosis seen in 13 out of 60 males receiving the maximum dietary dose of 3000 ppm, as compared to a prevalence of only 6 out of 60 among control animals. Other affected organs comprised the parathyroid glands and the spleen, with incidences for both organs of 10/60 at 3000 ppm and 4/60 in the control group. The incidence was approximately doubled in the duodenum (14/60 versus 7/60) and in the lungs (8/60 vs. 4/60). Amyloidosis of the adrenals, heart, kidneys, liver and thyroid was also found in more high-dose animals than in control males.

Similar observations were made in top-dose females, but the pattern of affected organs was somewhat different. A more frequent amyloid deposition was noted in the mesenteric lymph nodes (15/60 at 3000 ppm compared with 9/60 in the control group), the stomach (10/60 against 6/60 in controls), the thyroid (9/60 against 5/60 in controls) and in the pancreas (4/60 against 1/60 in the controls). In the mandibular salivary glands incidence of amyloidosis in the high-dose female group (8/60) even achieved statistical significance from that in the control group (2/60).

On balance, exacerbation of amyloidosis (common and usually age-related) in mice of both sexes, even though in different organs, was considered the only potentially adverse effect of fluazaindolizine observed in this study. Based on a clear increase in amyloid deposition in a number of organs at the top dose of 3000 ppm, with a statistically significant increase over the control group for the jejunum in male mice and the mandibular salivary glands in females, the NOAEL was identified at the next lowest dose of 1000 ppm (equal to a mean daily intake of 142 mg/kg bw) . No evidence of oncogenic potential was obtained for fluazaindolizine in this study. It is worth noting that the effects on kidneys and gallbladder observed at a similar dose level (3000 ppm) in the 90-day study in the same mouse strain, at the same laboratory, were apparently not reproducible even though one might have anticipated similar effects to be seen here. However, since such findings were expected to occur in the long-term study also, a dose selection for the long-term study that might otherwise be challenged as too low, appears reasonable (Han, 2017, revised 2021).

Rat

In a combined chronic toxicity and carcinogenicity study, groups of 70 Crl:CD(SD) rats per sex received fluazaindolizine (purity 98.5%; Lot No. SG0312632) at dietary concentrations of 0, 150, 500, 1500 or 4500 ppm for 24 months. Mean daily compound intakes of 0, 6.5, 25, 76 and 241 mg/kg bw were calculated for male rats, the values for females being of 0, 6.8, 27, 78 and 254 mg/kg bw per day. In addition, satellite groups of 10 animals per sex were given the same dietary doses, but were killed after just 12 months.

The animals were monitored twice daily for mortality and occurrence of clinical signs. A detailed clinical examination of all animals took place once a week. An ophthalmological examination was conducted on all animals during the week before treatment commenced, after nearly one year of feeding and prior to scheduled termination. The method was indirect ophthalmoscopy under pharmacologically induced mydriasis.

Body weight was determined prior to and at the first day of treatment (day 0), then once a week during the first 13 weeks of feeding, every other week thereafter and finally immediately before termination. Food consumption was measured for the individual weighing intervals on a cage basis (two animals of the same sex were housed together) and mean daily food intake was determined from this data. Food efficiency and mean daily compound intake were calculated on the basis of body weight and food intake, this weekly during the first three months and then every other week.

Blood sampling for haematology and clinical chemistry and urinalysis were performed in the satellite groups at weeks 13, 26 and at interim termination of these animals after one year. In addition, 0.5 mL of blood was taken from the satellite group animals at week 49 to obtain plasma from which to determine the concentration of parent compound and its metabolites. Plasma samples were sent to the sponsor and analysed. The results are briefly mentioned in section 1.3 above.

In the main study groups, blood smears for differential leucocyte counts were prepared from all surviving rats at 12 and 18 months and at scheduled termination after two years, but also from any animals that had to be killed in extremis. It appears that evaluation was only performed on blood smears taken at 24 months from control and high-dose animals.

At scheduled terminations after 12 or 24 months, also when animals were found dead or had to be killed for humane reasons, rats were necropsied and gross examination conducted. With the main study and the satellite groups, organ weights of adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes (with epididymis, thyroid (with parathyroids) and uterus were measured. A wide range of organs and tissues was taken from all animals on study, preserved and prepared for subsequent histopathology. Full histopathological examination of all organs and tissues was confined to the control and high-dose animals, both from the satellite and main study groups and including any premature decedents. However,

all gross lesions and selected organs were also assessed in the low and intermediate dose groups. The selected organs were: kidneys, urinary bladder, stomach and pituitary gland at interim termination of the satellite animals after 12 months. In the main study groups, the adrenals, kidneys, nasal cavity and pituitary were examined microscopically at all dose levels in both sexes, as well as the heart and testes from all males.

In the satellite groups there were no unscheduled deaths until interim kill at 12 months. In all main study groups, survival at study termination after two years was below 50%. In principle, such a high mortality rate extending to the control group (or, as is the case here, just the controls), might put the quality and reliability of a study into question, but in this case the animals found dead (or needing killing in extremis) were subject to comprehensive examinations so sufficient information on the toxicity and carcinogenicity of fluazaindolizine was obtained. Table 23 provides a summary of death rates, including details where survival fell short of 50% in the various groups. It is worth noting that significant differences in survival became apparent in high-dose males and high-dose females compared to the respective controls. However, opposing patterns were seen in the sexes: a better-than-control survival rate in top-dose males but a higher mortality rate in female rats receiving 4500 ppm than in controls. From this one might assume that the administration of fluazaindolizine has a beneficial effect on longevity in male rats, but an adverse effect in females. There is no explanation for this observation since the cause-of-death analysis did not provide any clues. No firm conclusion can be drawn as to whether the decreased mortality in males or the opposite finding in female rats could be in fact considered treatment-related.

There were no clinical signs of toxicity which could be attributed to treatment, except an increase in the number of animals with pale urine (presumably less concentrated) among the high-dose males, which agrees with the urinalysis findings. To be precise, nine out of 80 top-dose animals were affected, compared to not more than one animal in the control, low and intermediate dose groups.

Ophthalmological examination did not reveal any evidence of dose-related differences between the various groups.

Table 23. Survival in the main study groups (n = 70 per sex and dose) in the two-year feeding study with fluazaindolizine in rats

	Dietary concentration (ppm)				
Parameter	**0 (control)**	**150**	**500**	**1500**	**4500**
			Males		
Survival at termination (day 728)	29%	33%	40%	37%	47%*
Last day when survival was 50% or above	644	637	665	686	707
Found dead during study (*n*)	27	25	19	24	16
Killed moribund (*n*)	23	22	23	20	21
			Females		
Survival at termination (day 728)	41%	41%	31%	27%	29%*
Last day when survival was 50% or above	672	700	630	651	623
Found dead during study (*n*)	8	15	11	11	9
Killed moribund (*n*)	33	26	37	40	41

n: Number of individuals; Source: Moon, 2018

* statistically significant by trend test (males) or pairwise comparison (females); $p < 0.05$

Mean body weight and body weight gain were marginally lower in high-dose males and females than in controls. The differences from the control group were more pronounced in female rats where the final body weight in the group receiving 4500 ppm was 91% of the control mean, with overall body weight gain reduced by 13% . However, none of these differences reached statistical significance. Food consumption was not altered but food efficiency tended to be lower in top-dose females.

Haematology revealed only two remarkable changes which could be related to treatment; these were either not considered adverse or their relevance was unknown. A significant reduction in RBC count and ht was noted in high-dose males of the satellite group after three months, confirming similar

evidence from other studies in rats (MacKenzie, 2013; Nabb, 2018). However, with the longer duration of treatment in the current study this effect disappeared since no significant differences from the control means were apparent after 26 or 52 weeks.

When blood smears were examined in animals from the main group at termination, the percentage of monocytes was significantly reduced in high-dose males compared to the controls (0.3% against 2.3%). Surprisingly, the opposite was seen in females in which the monocyte count was significantly higher in the high-dose group (2.4% against 0.3% in the controls). Unfortunately further microscopic examination of blood smears from other groups, or those taken at earlier time points, had not been performed, so could not assist in interpreting this unusual finding. Limited historical control data (HCD) was based on a total of just three previous studies. Nevertheless comparison with this HCD suggests that the male control group in the current study was well within the range (2.2%–2.6%) whereas the percentage of monocytes in the female control group was unusually low (HCD gave 1.3%–4.9%). If this data is taken into consideration, at least the reduction of monocytes in males seems plausible, but there is no clue as to what this finding might mean from a clinical pathology point of view.

There were occasional and transient alterations in few clinical chemistry parameters but the only consistent and presumably treatment-related finding was a decrease in cholesterol concentrations in male rats at the two upper dose levels. Consistent with the interpretation of a similar reduction (but in females) in the 90-day study in rats (MacKenzie, 2013), this change was not considered adverse because typical findings seen concomitantly with lower cholesterol levels, such as overt liver toxicity or malabsorption, were absent in this case.

Urinalysis revealed treatment-related and consistent changes only high-dose males. A significant increase in urine volume was accompanied by a lower total protein concentration and reduced osmolality.

At necropsy, there were no macroscopic findings in the satellite groups that could be attributed to treatment. In the main study animals, which were fed for a longer time period of up to two years, the only presumably substance-related gross findings were confined to the kidneys of high-dose male rats. In four of these animals the renal pelvis was dilated. A similar change was not visible in the control group, the low- or mid- dose groups. In addition, an irregular kidney surface was noted in 10 of the males in this group compared with incidences of 2/70 among the control animals and four, one, or two cases in the other dose groups. Irregular kidney surface was also observed in seven high-dose females compared to only one in the control group and one or none in the other dose groups. There were no further noteworthy gross findings in female rats.

No differences that were both dose-related and significant were seen in absolute organ weight between treated and control groups. In female rats, relative kidney weights were significantly increased at the top dose; the mean value in this group exceeded the control mean by about 19%. This change may reflect the histopathological findings in this organ, but it is perhaps surprising that no significant effect on kidney weight was seen in males where the impact of the test substance on the kidney and, subsequently on urine parameters, was generally even more pronounced than in the female.

There was no increase in any neoplastic findings at any dose level, neither in male rats nor females. Non-neoplastic histopathological findings that may be presumed treatment-related and adverse were confined to the kidneys of male and female rats receiving the highest dose of 4500 ppm. These microscopic lesions are summarized below in Table 24. Histopathology also revealed an increase in frequency and severity of eosinophilic globules in the olfactory epithelium of the nasal cavity in high-dose males and females. In the absence of microscopically visible injury to olfactory tissue, this rarely-described finding was not regarded as adverse. An increased incidence of squamous cell hyperplasia in the limiting ridge of the stomach was observed in high-dose males and females, achieving statistical significance in the latter. Because its degree was mostly minimal to slight, and in the absence of further stomach findings, this effect was considered perhaps treatment-related but not adverse.

Table 24. Selected microscopic kidney findings in the main study groups (n = 70 per sex and dose) in the two-year feeding study with fluazaindolizine in rats (number of rats affected)

	Dietary concentration (ppm)									
	0 (control)		150		500		1500		4500	
Parameter	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Renal cyst	6	1	9	2	8	2	2	0	17*	8*
Deformed papilla	0	0	0	0	0	0	0	0	14*	15*
Papillar necrosis	0	0	0	0	1	1	1	0	9*	5*
Dilated pelvis	18	5	20	7	23	2	19	5	59*	23*
Haemorrhage in pelvis	0	0	0	0	0	0	0	0	10*	0
Dilated tubules (medulla)	0	5	0	0	0	0	2	2	18*	16*
Interstitial fibrosis	0	0	0	0	0	1	0 / 1	1	6*	2
Urothelial hyperplasia	4	15	6	6	5	11	9	11	22*	27*
Mineralization	4	10	6	23*	2	18	4	16	20*	21*
Chronic progressive nephropathy: all cases	58	13	59	17	60	9	54	14	63	40*
severe cases	12	0	12	0	14	1	8	1	8	8

M: Males; F: Females; Source: Moon, 2018

* statistically significant by Cochran–Armitage test for trend or by Fisher's exact test, $p < 0.05$

In this long-term study in rats, the kidney was identified as the main target organ of fluazaindolizine. The NOAEL was 1500 ppm, (equal to a mean daily intake of 76 mg/kg bw), based on nephrotoxicity at the highest tested dose level of 4500 ppm and demonstrated by a number of clear-cut histopathological findings and a few gross lesions in both sexes, by an increase in relative kidney weight in females and marginal urine findings in males. In addition, body weight gain and food efficiency were marginally affected at this high dose and an impact on survival in females cannot be completely excluded. There was no evidence of carcinogenicity of fluazaindolizine in rats under the conditions of this study (Moon, 2018).

2.4 Genotoxicity

(a) In vitro studies

Fluazaindolizine was extensively tested in a battery of appropriate tests. In total, nine studies in vitro were available and these are summarized in Table 25 and reported in detail below. Additionally in silico predictions with regard to bacterial mutagenicity were provided.

Five reverse mutation assays in bacteria were available in which different batches were tested in the same four strains of *Salmonella typhimurium* and one *Escherichia coli* strain, always by means of the plate-incorporation method in the absence and presence of metabolic activation by rat liver supernatant fraction obtained by centrifuging at 9000 *g*, referred to as S9 mix.

Table 25. Summary of in vitro studies on genotoxicity of fluazaindolizine

End-point (method)	Test system	Concentration range [a], S9 mix activation	Result	Reference
Gene mutation in bacteria (Ames test)	*S. typhimurium* strains TA1535, 1537, 98, 100 and *E. coli* strain WP2 *uvr*A; various batches tested in the individual studies	100–5000 µg/plate, ± S9	Negative	Myhre, 2012
		1.5–5000 µg/plate, ± S9	Negative	Wagner, 2013
		33.3–5000 µg/plate, ± S9	Negative	Myhre, 2017a
		33.3–5000 µg/plate, ± S9	Negative	Faranda, 2018
		33.3–5000 µg/plate, ± S9	Negative	Myhre, 2019

End-point (method)	Test system	Concentration range [a], S9 mix activation	Result	Reference
Gene mutation in mammalian cells (HPRT assay)	Chinese hamster ovary (CHO) cells	31.3–375 µg/mL, −S9, 31.3–300 µg/mL, +S9	Negative	Dutta, 2017a
		15–150 µg/mL, ± SD mix	Negative	Dutta & Van Dyke, 2018
Chromosome aberration test	Human peripheral lymphocytes	100–600 µg/mL [b]	Positive (4-hour exposure, ± S9 mix)	Roy & Jois, 2013
		100–450 µg/mL [b]	Positive (4-hour exposure, ± S9 mix)	Kellum, 2017a

[a] Only concentrations which could be successfully evaluated are mentioned here; full range of tested concentrations might be wider (see detailed description below);

[b] Depending on exposure time (4 or 20/22 hours) and activation conditions

Gene mutation in bacteria

Study 1

In the first Ames test, fluazaindolizine (purity 99.6%; Lot No. DPX-Q8U80-068) was applied to all tester strains at concentrations of 100, 333, 1000, 3333 and 5000 µg/plate. The test item was dissolved in dimethyl sulfoxide (DMSO) which was also used as the negative control substance. In the absence of S9 mix the following positive control substances were employed: 2-nitrofluorene (for TA98), sodium azide (TA100 and TA1535), acridine mutagen ICR-191 (TA 1537), and 4-nitroquiniline-*N*-oxide for the strain of *E. coli*. In the activation experiments, 2-aminoanthracene was used with *Salmonella* strains TA100, TA1535, and TA1537 and for *E. coli* WP2 *uvr*A, whereas benzo[*a*]pyrene was the positive control substance in the experiment with *Salmonella* strain TA98. Mutagenicity testing was performed in triplicate at all concentrations.

Some cytotoxicity was noted at the two highest concentrations but only with strains TA100 and TA1537. No precipitation was seen.

No increase in revertants was observed with any of the tested concentrations of fluazaindolizine and the positive control substances gave the expected increases.

Under the conditions of this study, fluazaindolizine was not mutagenic to bacteria in the Ames test up to the limit concentration of 5000 µg/plate (Myhre, 2012).

Study 2

In a similar study, a different lot (purity 98.5%; Lot No. DPX-Q8U80-093) of fluazaindolizine was tested in another laboratory but in the same four strains of *Salmonella typhimurium* and in *Escherichia coli* strain WP2 *uvr*A. The test item concentrations ranged from 1.5 to 5000 µg/plate. The vehicle was DMSO which was also used as negative control substance. In the absence of S9 mix, the following positive control substances were employed: 2-nitrofluorene (for TA98), sodium azide (for TA100 and TA1535), 9-aminoacridine (for TA 1537), and methyl methanesulfonate for the *E. coli* strain WP2 uvrA. In all activation experiments, 2-aminoanthracene served as the positive control substance. Mutagenicity testing was performed, at all concentrations, in the initial test in duplicate and in the confirmatory test in triplicate. The initial test with TA1537 with metabolic activation was repeated because the positive control substance had not produced the expected increase in revertants.

No cytotoxicity or precipitation was seen with any tester strain at any concentration.

All tester strains proved negative in the initial assay. Likewise, no increase in revertants was seen with any of the tested concentrations of fluazaindolizine in any strain in the confirmatory assays. and the positive control substances gave the expected positive responses.

Under the conditions of this study, fluazaindolizine was not mutagenic to bacteria in the Ames test up to the limit concentration of 5000 µg/plate (Wagner, 2013).

Study 3

A third batch of apparently lower purity test article (purity 94.8%; Lot No. DPX-Q8U80-181) was tested in the same four strains of *Salmonella typhimurium* and the same strain of *Escherichia coli* as above. Concentrations ranging from 33.3 to 5000 µg/plate were employed. The vehicle was DMSO which also served as the negative control. The following positive control substances were used: 2-nitrofluorene (for TA98), sodium azide (for TA100 and TA1535), acridine mutagen ICR-191 (for TA 1537), and 4-nitroquiniline-*N*-oxide for *E. coli* strain WP2 uvrA. In activation experiments, 2-aminoanthracene was used with *Salmonella* strains TA100, TA1535 and TA1537, and for *E. coli* WP2 *uvr*A, whereas benzo[*a*] pyrene was the positive control substance in the experiment with *Salmonella* strain TA98. Subsequent to a toxicity-mutation test on duplicate plates, a definitive mutagenicity test was run in which testing was performed in triplicate at all concentrations.

Weak cytotoxicity became apparent from background lawn reduction with all tester strains at the two upper concentrations of 3333 and 5000 µg/plate, but a reduction of greater than 50% of revertant colonies (which would suggest more pronounced toxicity) was confined to *Salmonella* TA100 when tested in the presence of metabolic activation. Precipitation was consistently observed from 3333 µg/plate upwards.

No increase in revertants was observed in any tester strain at any concentration and the positive control substances produced the expected mutagenic responses.

It may be concluded that this batch of lower purity test article was also negative in the Ames test up to the highest tested concentration of 5000 µg/plate (Myhre, 2017a).

Study 4

In the fourth study of this type, the same tester strains of *Salmonella typhimurium* and *Escherichia coli* were used and the same positive and negative (vehicle DMSO) control substances as in the studies by Myhre (2012 and 2017); the main change was that different batch of fluazaindolizine (purity 98.1%; Lot No. D201398-2) was tested. The method was once again a plate-incorporation assay with and without metabolic activation and the concentrations applied ranged from 33.3 to (nominal) 5000 µg/plate. The actual top concentration was 4925 µg/plate, due to miscalculation when adjusting for purity. Again, a toxicity/mutation test was run on duplicate plates and the definitive mutagenicity test in triplicate.

Cytototoxicity was observed for strain TA1537 at the maximum concentration but a reduction in background bacterial lawn was noted for all tester strains at nominal concentrations of 3333 and 5000 µg/plate. No increase in revertants was seen with any strain at any concentration and the positive control substances gave the expected responses.

Under the conditions of this study, fluazaindolizine was negative in the Ames test (Faranda, 2018).

Study 5

In the most recent Ames test, a fifth batch (purity 97.4%; Lot No. 164712-5) was tested at concentrations ranging from 33.3 to 5000 µg/plate. The vehicle was once again, DMSO which served as the negative control, while the positive control substances were the same as used before (see Myhre, 2012). The test design was identical to the previous studies.

Results with this batch of fluazaindolizine were as before with no increase in revertant colonies being noted. Some cytotoxicity was demonstrated in tester trains TA100, TA1535 and TA1537, confined to concentrations of 3333 µg/plate and above, depending also upon activation conditions.

Under the conditions of this study it was confirmed that fluazaindoline was devoid of potential to cause gene mutations in bacteria (Myhre, 2019).

The absence of a mutagenic potential in bacteria was also suggested by in silico methods. In a quantitative structure–activity relationship (QSAR) study for which there is no guideline so far and GLP is not relevant, fluazaindolizine and five impurities (R2Y16, QJA58, RXZ20, TKE32, and WCW73) were predicted negative for bacterial (Ames test) mutagenicity in two independent computational systems. Analyses were performed by DEREK Nexus (v. 2.2.1 Ames mutagenicity model, v 6.0.1) and by OASIS TIMES (v. 2.29.1 Ames mutagenicity, v. 16) that also takes metabolic activation into account.

Derek NEXUS was developed by Lhasa Ltd, Leeds, UK; OASIS TIMES was developed at the Professor As. Zlatarov University in Bourgas, Bulgaria.

All predictions were negative but it must be acknowledged that, for OASIS TIMES the structures of all six molecules were out of domain, making the DEREK predictions more reliable. However, all negative predictions agreed with the negative outcome of the five in vitro Ames tests reported above (Zhang, 2019).

Gene mutation in mammalian cells

Study 1

The potential of fluazaindolizine to cause gene (point) mutations in mammalian cells was studied with two different batches in the same test system, Chinese hamster ovary cells, by means of the HPRT assay. In the first of these studies, the test item (purity 94.8%; Lot No. DPX-Q8U80-181) was dissolved in DMSO (also used as negative control substance) and applied to CHO cells in a preliminary toxicity test at concentrations ranging from 3.91 to 2000 µg/mL. All testing was performed in duplicate. Based on the observations of precipitation and cell survival, concentrations from 62.5 to 800 µg/mL, in the absence and presence of S9 mix for metabolic activation, were chosen for the first mutagenicity test, with significant cytotoxicity above this level preventing meaningful evaluation of mutagenicity at higher concentrations.

Without S9 mix, cultures treated with concentrations up to 375 µg/mL did not increase mutant frequency whereas the positive control substance (ethyl methanesulfonate) gave the expected positive response.

In the presence of S9 mix less than four concentration levels could be evaluated due to poor survival and the assay had to be repeated. In this second experiment, performed only with activation, the concentrations ranged from 31.3 to 400 µg/mL and only cultures receiving treatment with five concentrations between 31.3 and 300 µg/mL were scored. No significant increase in mutant frequency was observed up to the highest of these concentrations. The positive control substance benzo[*a*]pyrene, in contrast, caused a marked increase in mutant colonies.

On balance, the HPRT assay with fluazaindolizine was negative, even though severe cytotoxicity precluded testing at high concentrations. It is not clear whether this toxicity was due to the active compound or an impurity, bearing in mind the relatively low purity of the batch used (Dutta, 2017a).

Study 2

In a similar study, fluazaindolizine dissolved in anhydrous DMSO (purity 99.6%; Lot No. DPX-Q8U80-068) was applied to CHO cells at concentrations up to 1500 µg/mL in a preliminary toxicity test. Because of marked cytotoxicity and precipitation, only concentrations between 15 and 150 µg/mL were selected for the HPRT assay, to be used both in the absence and presence of S9 mix. Testing was performed in triplicate.

All cultures exposed to the concentrations chosen for the HPRT test could be scored. No increase in mutant frequency was observed at any concentration of fluazaindolizine, either with or without metabolic activation. In contrast, the positive control substances ethyl methanesulfonate (for unactivated conditions) and benzo[*a*]pyrene caused mutagenicity.

Under the conditions of this study, the negative outcome of the HPRT test for mutagenicity to mammalian cells was confirmed. Once again, the severe cytotoxicity of fluazaindolizine was observed at moderate and high concentrations. Since the test material in this study was very pure, cytotoxicity does indeed seem to be an inherent property of the active compound (Dutta & Van Dyke, 2018)

Chromosome aberration test

In an in vitro chromosome aberration test, fluazaindolizine (purity 99.6%; Lot No. DPX-Q8U80-068) was applied, with or without metabolic activation (S9 mix) to peripheral human lymphocytes in two independent experiments. The blood cells had been obtained from two young, healthy and non-smoking women. The test item was dissolved in anhydrous DMSO which also served as negative control. Positive control reagents were mitomycin C for the non-activated experiments and cyclophosphamide in the presence of S9 mix.

Fluazaindolizine exposure times were 4 and 20 hours without activation and just four hours in the presence of S9 mix. The four-hour exposures were followed by a 16-hour recovery period. Accordingly, all cells were harvested at 20 hours after treatment had begun. Two hours prior to sampling, 0.1 µg/mL of colcemid was applied for spindle inhibition.

Based on solubility and preliminary toxicity testing which examined much higher concentrations of up to 2341 µg/mL, the concentrations chosen for the clastogenicity assay ranged from 30 to 700 mg/mL, with exposure over four hours, both with and without S9 mix, and from 7.5 to 300 µg/mL for the long-term exposure. In a repeat test, cell culture were exposed for four hours, under activation, to concentrations ranging from 150 to 550 µg/mL. In a repeat test with 20-hour exposure without metabolic activation, concentrations from 25 to 300 µg/mL were applied. These repeat tests were performed since an insufficient reduction in mitotic index had been observed in the initial chromosome aberration test. Because of the pattern of cytotoxicity observed in all these tests, three concentrations were always selected for various exposure conditions to undergo microscopic analysis (see Table 26).

Duplicate cultures were run for each concentration, exposure time and activation condition. Following harvest, preparation, staining and coding of slides, 500 cells per culture were counted to determine the mitotic index as a measure of cytotoxicity. Whenever possible, 100 well-spread metaphases per culture were evaluated for structural chromosomal aberrations. In the case of positive results, re-analysis was performed. The findings are summarized below in Table 26.

Table 26. Percentages of cells with structural aberrations (mean of two cultures), the results of re-analysis, if performed, is shown in brackets

	Initial test (4 hours, −S9 mix)		**Repeat test (4 hours, +S9 mix)**		**Repeat test (20 hours, −S9 mix)**	
Treatment	**Mitotic index**	**% aberrant cells**	**Mitotic index**	**% aberrant cells**	**Mitotic index**	**% aberrant cells**
Solvent, DMSO	10.7	0.5	12.2	0.0	13.1	0.0
[re-analysis]	[10.7]	[0.5]	[12.2]	[0.0]		
Mitomycin C	4.5	25.0**	–	–	7.4	21.0**
Cyclophosphamide	–	–	5.2	16.0**	–	–
Fluazaindolizine,						
100 µg/mL	–	–	–	–	12.5	0.0
150 µg/mL	9.1	1.0	–	–	–	–
200 µg/mL	–	–	–	–	8.2	0.0
230 µg/mL	–	–	–	–	5.9	1.5
300 µg/mL	6.9	0.5	–	–	–	–
350 µg/mL	–	–	12.6	2.0	–	–
400 µg/mL	–	–	9.8	10.0**	–	–
[re-analysis]			[9.8]	[9.5**]		
425 µg/mL	–	–	5.7	3.5**	–	–
				[8.0**]		
600 µg/mL	4.6	5.0**	–	–	–	–
[re-analysis]	[4.6]	[10.5**]				

** Statistically significant at $p < 0.01$ using Fisher's exact test Source: Roy & Jois , 2013

A significant increase in cells with structural chromosomal aberrations was noted after a four-hour exposure to fluazaindolizine, both with and without metabolic activation. The results were confirmed by re-analysis of the slides. In contrast, no such increase was seen when exposure was longer, but in the absence of S9 mix. The positive control substances produced the expected increases in aberrant cells. No evidence of an increase in polyploid metaphases was observed in any experiment at any concentration.

Under the conditions of this in vitro study, fluazaindolizine induced structural chromosomal

aberrations in peripheral human lymphocytes, both with and without metabolic activation, and must be considered positive in this assay. In contrast, no increase in numerical chromosome aberrations was apparent (Roy & Jois, 2013).

In a second study of this type, a different batch of fluazaindolizine (purity 94.8%; Lot No. DPX-Q8U80-181) was applied to human peripheral lymphocytes in the absence and presence of S9 mix for metabolic activation. The blood cells for culturing were drawn as venous blood from a 33-year-old female volunteer.

The test item was dissolved in DMSO which was also used as a negative control in all experiments. The positive control substances were the same as in the previous study; mitomycin C for the non-activated experiments, cyclophosphamide in experiments with S9 mix.

Based on the solubility, precipitation and toxicity observed in comprehensive pretests, the concentrations chosen for clastogenicity testing ranged from 50 to 500 µg/mL, with four-hour exposure with and without S9 mix, and from 25 to 350 µg/mL with cultures exposed for 22 hours in the absence of metabolic activation. Because cytotoxicity was clearly indicated by a reduction in mitotic index of more than 50% from concentrations of 450 µg/mL upwards with the four-hour exposure, and 200 µg/mL upwards with the 22-hour exposure, cytogenetic evaluation was confined to concentrations of 100, 250, and 450 µg/mL in the four-hour experiments (± S9), and 50, 100, and 200 µg/mL in the experiment with long-term exposure.

Duplicate cultures were run for each concentration, exposure time and activation condition. All cells were harvested at 22 hours after initiation. Three hours before harvest, they were arrested in metaphase stage using colcemid. A total of 500 cells per culture were counted to determine the mitotic index as a measure of cytotoxicity. At the selected concentrations, 150 well-spread metaphases per culture were evaluated for structural chromosomal aberrations and numerical aberrations were also recorded.

A significant increase in lymphocytes with structural aberrations was observed in the cultures exposed for four hours to 450 µg/mL fluazaindolizine, both in the absence and presence of metabolic activation. It was emphasized by the study author that the percentage of aberrant cells in cultures that received high concentration treatment with activation (7.3%) was still within the HCD of the performing laboratory. However, it was by around 10-fold that in the concurrent vehicle controls (0.7%). Without activation a three-fold increase over vehicle controls (4.0% against 1.3%) was noted at 450 µg/mL. It must be taken into consideration that the mitotic index at 450 µg/mL, with and without S9 mix, was reduced to about 50% compared to the vehicle control, suggesting significant toxicity. No increase in aberrant cells was seen after long-term (22 hours) exposure without S9 mix. Likewise, no evidence of numerical chromosome aberrations was obtained. The positive control substance gave the expected positive responses.

Under the conditions of this study, fluazaindolizine proved positive in the in vitro chromosome aberration assay confirming the outcome of a previous study (Kellum, 2017a).

(b) In vivo studies

Because of the positive outcome of two in vitro chromosome aberration studies, further investigations in vivo were needed. Four in vivo studies (three micronucleus assays in the mouse and one UDS test in the rat) were all negative, but another micronucleus assay in rats was inconclusive. With regard to the reliability of results from the mouse micronucleus assays it is worth noting that sufficiently high plasma levels of fluazaindolizine and some of its metabolites had been measured after 38 days and one year, respectively, of continuous feeding in 90-day (Han, 2018) and carcinogenicity (Han, 2017) studies in mice. These findings (see section 1.3) might suggest that the target bone marrow was also exposed in the micronucleus studies in which high doses were applied via oral gavage. Moreover, plasma analysis for fluazaindolizine was included in one of the micronucleus assays itself. An overview on the three guideline-compliant micronucleus assays in the mouse is provided in Table 27, which also gives a number of technical details of the studies.

Table 27. Micronucleus assays with fluazaindolizine in the mouse

Test item, Purity	Species and strain, sex, number evaluated, matrix	Route, number of applications, termination	Dose levels in main study (mg/kg bw)	Results	Reference
Lot No. DPX-Q8U80-093, purity 98.5%	Crl:CD1(ICR) mice, m and f, Five per sex and dose, Bone marrow smears	Single oral dose, Animals killed 24 or 48 hours post dose (one half each)	Males 0, 250, 500 and 800 Females 0, 250, 500, 1000 and 1500	On balance negative. No increase in micronucleus frequency in males and, after 24 hours, in females; limited evidence of a positive effect in females at the overtly toxic dose of 1000 mg/kg bw after 48 hours; mortality and clinical signs in females at the two upper dose levels avoiding evaluation for micronuclei at the top dose level; .	Myhre, 2014
Lot No. DPX-Q8U80-181, purity 94.8%	Crl:CD1(ICR) mouse, m and f Five per sex and dose, Peripheral reticulocytes	Single oral dose, Blood sampling after 48 and 72 hours, Animals killed after 72 hours	Males 0, 175, 350 and 750 Females 0, 175, 350 and 600	Negative. No increase in micronucleated reticulocytes at any dose level; no signs of toxicity; bone marrow exposure demonstrated by measuring of plasma levels.	Myhre, 2017b
Lot No. 81354FB107, purity 98.7%	Crl:CD1(ICR) mouse, m and f Five per sex and dose, Peripheral reticulocytes	Single oral dose, Blood sampling after 48 and 72 hours, Animals killed after 72 hours	Males 0, 175, 350 and 750 Females 0, 175, 350 and 600	Negative. No increase in micronucleated reticulocytes at any dose level; toxicity followed by mortality in one high dose male	Myhre, 2018a

m: Male; f: Female

Study 1

In the first study, fluazaindolizine was suspended in 0.1% Tween 80 in 0.5% aqueous methylcellulose solution and applied by oral gavage at a volume of 10 mL/kg bw. This vehicle was also used as negative control. The positive control group comprising five males and five females received cyclophosphamide (40 mg/kg bw) in deionized water by oral gavage. Termination of these animals took place after 24 hours and the procedure thereafter was the same as described below.

Dose levels for the micronucleus assay had been selected on the basis of preliminary toxicity tests, but surprisingly there was unexpected mortality in females in the definitive genotoxicity test, with nine of 14 females in the group receiving 1500 mg/kg bw found dead. Two females also died at the next lowest dose of 1000 mg/kg bw. Clinical signs at these dose levels comprised abnormal gait, lethargy, tremors, low posture and eyelid ptosis. By contrast, no signs of toxicity were observed in males, but the top dose level in males was only 800 mg/kg bw. Thus the highest dose levels at which micronucleus incidence could be evaluated were 800 mg/kg bw in male mice and 1000 mg/kg bw in females.

After termination of the fluazaindolizine-exposed groups for 24 or 48 hours post dosing, femoral bone marrow smears were prepared and, after staining, at least 2000 polychromatic erythrocytes per animal were evaluated for the presence of micronuclei. Evaluations were conducted on five animals per sex and dose.

There was no significant or dose-related increase in micronucleus formation at any of the dose levels of fluazaindolizine in males, or in the 24-hour exposure experiment in females. In the female group receiving 1000 mg/kg bw and killed 48 hours post dosing, there was a significant increase in

micronucleus frequency. In addition, there was a decrease in polychromatic erythrocytes. It must be borne in mind that these findings were obtained in a group exhibiting marked toxicity and that both figures were still within the HCD range of the performing laboratory.

The positive control substance cyclosphosphamide caused marked increases in micronucleus frequency.

On balance, the test substance was considered to be negative in this in vivo micronucleus assay in mouse bone marrow. The equivocal increase in micronucleus frequency at a dose level near the LD_{50} (based on rat data) was attributed to overt toxicity (Myhre, 2014).

Study 2

In a second study in the same mouse strain, another reagent lot of lower purity (the same batch that tested positive in the in vitro study by Kellum, 2017a) was tested and a different method applied. Micronuclei were not measured in bone marrow smears but in reticulocytes from peripheral blood. This study design would allow different conclusions. An increase in micronucleated reticulocytes, compared to the negative control group would be regarded as indicative of clastogenicity or spindle damage in vivo whereas a significant decrease in the total percentage of reticulocytes among all RBCs might elucidate inhibition of erythropoiesis. Even though the latter is not a genotoxic effect in a strict sense, its implication would point, on one hand, to systemic toxicity and, on the other, might be considered proof that the target bone marrow had been reached by the test substance.

Five to nine animals per sex and concentration were administered fluazaindolizine as a single oral dose. The dose levels of 0, 175, 350 and 600 mg/kg bw (females), and 0, 175, 350 and 750 mg/kg bw (males) were selected on the basis of a range-finding study in which dose levels up to 1000 mg/kg bw had been employed but found to be too high. The test item was dissolved in 0.1% Tween 80 in 0.5% aqueous methylcellulose solution and this vehicle was also used as the negative control. The animals (five per sex) in the positive control group received a single oral dose of 30 mg/kg bw of cyclophosphamide. Four male and four female mice from the low-dose group receiving 175 mg fluazaindolizine/kg bw were killed four hours post dose to measure the plasma concentration of the test item.

At 48 and 72 hours post dosing blood was drawn and samples processed for subsequent flow cytometric analysis of at least 20 000 reticulocytes per sample for the presence of micronuclei. In addition, the ratio of reticulocytes (immature RBCs) to normochromatic erythrocytes (NCEs) was determined as a possible indication of toxicity. Samples from five animals per sex and dose were analyzed after 48 hours. In assessing the 72 hour samples, examination was confined to the vehicle control, high-dose and positive control groups.

Plasma analysis revealed 146 µg/mL of fluazaindolizine in pooled male plasma samples at approximately four hours after treatment with 175 mg/kg bw of the test article, and 211 µg/mL in pooled female samples. From these findings it may be assumed that bone marrow, a well-perfused tissue was adequately exposed to the test article.

No unscheduled deaths and no clinical signs of toxicity were observed in this micronucleus assay. The percentage of reticulocytes among all RBCs was not affected by treatment. No increases in micronucleated reticulocytes was noted at any dose level of fluazaindolizine, whereas cyclophosphamide produced a clear positive effect.

In this mouse study no evidence of micronucleus formation resulting from fluazaindolizine administration was obtained in peripheral blood, and bone marrow exposure may be reasonably assumed from the plasma analysis results (Myhre, 2017b).

Study 3

A very similar study to that described above (*study 2*) with flow cytometric analysis for the presence of micronuclei in peripheral reticulocytes was performed, but with a different batch of fluazaindolizine (see Table 27 for comparison). Dose levels, vehicle, control substances and all procedures were the same as reported above (Myhre, 2017b) but in this case plasma analysis was apparently not performed. There were five animals per sex and dose with the exception of the male and female top-dose groups in which seven mice were treated, just in case of unexpected mortality; in the event only five mice per sex were evaluated for micronucleus frequency.

In the high dose male group receiving 750 mg/kg bw, one animal displayed prostration 3–5 hours post dosing and was found dead the next day. At mid- and low- doses (350 and 175 mg/kg bw) findings in the perineal area (hair loss and scabbing, or a mass, respectively) were noted in one male at each dose level but were considered incidental for lack of a dose–response relationship. No signs of toxicity were observed in female mice up to the highest dose level of 600 mg/kg bw.

No increase in micronucleated reticulocytes was observed at any dose level, either in males or females. In contrast, cyclophosphamide treatment produced the expected positive responses. The percentage of reticulocytes among all RBCs was unaltered in the treated groups.

Under the conditions of this study, fluazaindolizine was negative in the micronucleus assay in peripheral blood (Myhre, 2018a).

Study 4

A further micronucleus assay in peripheral blood was performed as part of a more comprehensive toxicity study in Crl:CD(SD) rats which is reported in greater detail in section 2.2 Short-term studies. Fluazaindolizine of a purity greater than 99% was suspended in 0.1% Tween 80 in 0.5% aqueous methylcellulose solution and administered by gastric intubation at a volume of 10 mL/kg bw to groups of five male and five female rats.

In a single dose experiment the animals were given a high dose of 2000 mg/kg bw of fluazaindolizine. Blood was sampled two days later to analyze for micronucleus formation. A concurrent positive control group of the same size received cyclophosphamide at a dose level of 10 mg/kg bw. Samples were taken from the orbital sinus and 15 000 reticulocytes per animal were examined by flow cytometry, using a commercially available rat micronucleus assay kit.

One death was noted on the day after dosing among females receiving fluazaindolizine. Since the remaining four females and all males survived without any noteworthy clinical signs, it seems that single application of this very high dose was much better tolerated than repeated administration of 1000 or 500 mg/kg bw since all animals receiving these repeated doses were dead by day 12 at the latest (see section 2.2). It is worth noting that the dose used for the micronucleus test was markedly higher than the LD_{50} in the acute oral studies in rats (see section 2.1).

The micronucleus assay was also performed in the main study groups in which the animals had received repeated daily doses of 0, 25, 300 or 500/1000 mg/kg bw for up to 14 days. In these rats, blood sampling for micronucleus frequency analysis took place on the final day of substance administration. It seems that not all groups were evaluated since in males only the results for the negative controls and the high-dose groups were reported. In contrast, female rats from all groups were apparently subjected to micronucleus counting in peripheral blood reticulocytes. The study report does not clarify this situation.

No increase in micronucleus formation over that seen in controls from the main study was seen in male or female rats receiving the single high dose of 2000 mg/kg bw whereas the positive control substance cyclophosphamide gave the expected positive response. However, the comparison to the negative (vehicle) control appears questionable since fluazaindolizine was administered only once, while the negative control group had received the vehicle daily over 14 days. Plasma analysis for fluazaindolizine in the same study (see section 1.3) indicated bone marrow exposure to be very likely.

A doubling in micronucleus frequency over the control group was observed, in contrast, in female rats receiving repeated doses of 1000 (later 500) mg/kg bw per day. However, this finding was attributed to excessive toxicity in this group resulting in spontaneous death or premature termination of these animals following various numbers of doses.

The Meeting agreed on the positive outcome but noted that this non-GLP study was not compliant with the relevant OECD Guideline TG 474 in a number of ways, for instance the inclusion of only one sampling time in the limit dose experiment at 2000 mg/kg bw, scanty reporting of data, the absence of statistical evaluation and of negative and positive historical control data. Overall, this study was regarded as of limited reliability and with regard to its outcome, inconclusive (Nabb, 2018; revised by Fallers, 2020).

Study 5

Fluazaindolizine (purity 98.5%; Lot No. DPX-08U80-093) was tested for potential direct interaction with DNA in an unscheduled DNA synthesis (UDS) assay in primary rat hepatocytes. For this purpose, the test item was dissolved in 0.1% Tween 80 in 0.5% aqueous methylcellulose solution and administered as a single dose by oral gavage to Sprague Dawley rats. The dose levels 0 (vehicle control), 500, 1000 and 2000 mg/kg bw had been selected on the basis of a preliminary range-finding experiment. It is perhaps surprising that a dose much higher than the LD_{50} (see section 2.1) could be applied in this study, but all animals were killed by design less than 24 hours following administration, thus the time for toxic signs to develop might not have been sufficient. The positive control substance dimethylnitrosamine was administered at a dose level of 35 mg/kg bw. The animals were killed either 2–4 hours or 12–16 hours post dosing. For each dose level and each time point of termination, groups were always of four rats per sex.

Hepatocytes were isolated and cultured. At least, six cultures per animal were prepared. Three to six hours after plating on culture dishes cells were washed and exposed for four hours to a medium containing [^{3}H]thymidine. After this cultures were incubated for further 17–20 hours before slides were prepared. Three slides per animal were scored under the microscope (interfaced with an automated colony counter) for silver grains in the nucleus and surrounding cytoplasm in 50 cells per slide. The net nuclear grain count for each nucleus was obtained by subtracting the mean cytoplasmic area count from the nuclear area count. A marked, dose-related and statistically significant increase in this net grain count is considered indicative of unscheduled DNA synthesis. In addition, nuclei with an increased grain count may indicate cells described as in repair.

All treated rats survived until scheduled termination and piloerection was the only clinical sign that was attributed to treatment. There was no increase in net nuclear grain count at any dose level or time point amongst all the treated animals. Also there was no increase in the percentage of cells in repair. The positive control substance produced a clear positive response.

Fluazaindolizine proved negative in the UDS assay in primary rat hepatocytes following in vivo exposure up to the limit dose of 2000 mg/kg bw. There was no evidence therefore of direct interaction of the test compound with rat liver DNA. It must be taken into consideration, however, that this assay is rather insensitive and is no longer part of modern testing strategies (for reservations see for example Kirkland & Speit, 2008, or EFSA, 2017) (Bruce, 2019).

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Study 1

A combined 28-day toxicity and one-generation reproduction study was performed in Crl:CD(SD) rats to which fluazaindolizine (purity 99.2%; Lot No. FE114893-110) was administered via the diet. Fifteen male and 15 female rats per group received the test substance at dietary levels of 0, 500, 2500 or 5000 ppm, but five animals per sex and dose were terminated after 28 days (the general toxicity part of this study is reported in sub-section 2.2. The remaining rats from the same groups were continued on the test diets and mated. The cohabitation period commenced after 28 days of feeding (when the designated parental animals were 84 days old) and ended either when evidence of successful copulation was observed or at latest after two weeks. Male rats from the parental generation were killed after siring litters; the females were allowed to deliver and wean their pups until postnatal day (PND) 21 before being killed. Culling of litters to four males and four female pups each, where numbers allowed, was performed on PND 4. Three pups per sex and litter were killed on PND 21 at weaning, while 10 young rats per sex and dose (that is, one per sex and litter) were continued on the test diet until PND 60.

During lactation and until PND 42 (F1 generation), dietary concentrations were adjusted to ensure that the target doses were approximately achieved. In fact, dietary dose levels were reduced, for this period to 0, 300, 1500 and 3000 ppm. Overall, for the reproduction part of this study, the mean daily compound intakes amounted to 0, 37, 179 and 361 mg/kg bw in parental generation males. In parental females, mean daily intakes varied in the ranges 0, 36–43, 182–204 and 348–416 mg/kg bw in the different reproductive phases (pre-mating, gestation and lactation). In the F1 generation mean daily

intakes in males were calculated to be 0, 37, 188 or 371 mg/kg bw in the period before PND 42; and 0, 43, 209 or 438 mg/kg bw in the period thereafter until termination on PND 60. In F1 females intakes were calculated as 0, 38, 191 or 365 mg/kg bw per day before PND 42, and 0, 44, 216 or 411 mg/kg bw per day thereafter.

The animals assigned to the reproduction subset were monitored daily for mortality and signs of toxicity and were subjected to a detailed clinical inspection once a week. All rats were weighed weekly and food intake was also determined at weekly intervals. Reproductive parameters were recorded. Live and dead pups were counted and sexed. Live pups were weighed and examined for abnormal behaviour and appearance. The F1 animals kept alive after PND 21 (F1 adults) were observed for vaginal patency and preputial separation and weighed when these landmarks of sexual maturation were achieved.

At termination, parental animals and F1 adults were subjected to gross examination. A number of organs were taken and weighed, however histopathology was confined to the kidneys in both sexes and, in female rats, the urinary bladder and vagina.

There were no premature deaths or clinical signs of toxicity in the parental animals that could be attributed to treatment. A slightly lower body weight gain was noted in high-dose males whereas no clear effect on body weight was observed in females. Food intake was not affected.

Fertility and reproductive performance were not altered. Likewise, no impact on pup weight or body weight gain or on sexual maturation was observed.

Organ weights in adult rats were not affected and no gross lesions were attributed to treatment. However, histopathological findings in kidneys and urinary bladder (the latter not examined in all females) were observed in mid- and high-dose males and females from the parental generation (see Table 28) and at the top dose in F1 adults.

In the reproduction part of this study the NOAEL for systemic (parental) toxicity was 500/300 ppm (corresponding to about 37 mg/kg bw per day), based on histopathological kidney lesions in male rats. Similar findings in F1 adults and in parental females were confined to the top dose level of 5000/3000 ppm.

Table 28. Kidney and bladder findings in parental animals in the one-generation study in rats

	Dietary dose (ppm)							
	Males (10 per group)				Females (10 per group)			
Finding	**0**	**500**	**2500**	**5000**	**0**	**500**	**2500**	**5000**
Kidney:								
transitional cell hyperplasia, unilateral	0	0	2	4	0	0	0	5
pyelitis, unilateral	0	0	0	2	0	0	0	2
ulcer (papilla or pelvis, unilateral)	0	0	1	1	0	0	0	2
Urinary bladder:								
transitional cell hyperplasia	ND	ND	ND	ND	0/9 [a]	0/7 [a]	0/6 [a]	7/9 [a]

ND: Not determined; [a] Denominator shows number of rats examined; Source: Munley, 2017a

Since no effects on fertility or reproductive success or on the pups were observed, the reproductive NOAEL was 5000/3000 ppm, the highest dose tested (corresponding to a mean daily intake of about 348 mg/kg bw per day).

There were no effects on pups' growth and development but it is not unequivocally clear whether the microscopic kidney findings in F1 adults can be attributed to dietary intake alone of the test substance after weaning, or might not also due to pre-, peri- or postnatal exposure during lactation. Therefore, the offspring NOAEL was 2500/1500 ppm (corresponding to a mean daily intake of about 179 mg/kg bw) (Munley, 2017a).

Study 2

In a comprehensive two-generation study according to most recent standards, fluazaindolizine (purity 98.5%; Lot No. DPX-Q8U80-093) was continuously administered to two consecutive generations of Crl:CD(SD) rats at dietary concentrations of 0, 150, 500, 1500 or 4500 ppm. These dose levels had been established on the basis of a previous 90-day feeding study (MacKenzie, 2013, see section 2.2) and the one-generation study reported above (Munley, 2017a) in the same rat strain. During certain study intervals, (the lactation periods in F0 and F1 females and up to PND 42 in F1 offspring) the concentrations of active ingredient in the diet were reduced to 0, 90, 300, 900 or 2700 ppm to adjust for increased food consumption and ensure that the nominal target dose levels were met as closely as could be judged. As is common in studies of this type, mean daily intakes during the various experimental stages and with regard to sex varied a lot. For risk assessment purposes it seems appropriate to use the lowest calculated figures for the various dose groups. These figures were consistently calculated for male animals from the F0 generation in the premating phase of this study (days 0–71) and were calculated to be 0, 9, 30, 88, and 265 mg/kg bw per day.

All groups comprised 30 male and 30 female rats. Following ca 10 weeks of test substance administration (the premating period), F0 and F1 males and females were housed together and mated within their respective dose groups. For parental F1 animals, the premating dietary exposure period started at weaning. The males were killed after successfully siring a litter. Dams were allowed to deliver and rear their offspring until weaning on PND 21, but all litters were randomly culled to four pups per sex on PND 4. From each F1 litter, one male and one female were selected randomly to serve as parents for the F2 generation, but subsequent sibling mating was avoided. The remaining F1 and all F2 pups were killed at weaning.

Animals were monitored daily for mortality and apparent clinical signs. A more detailed clinical examination was performed once a week. Body weight and food consumption were also determined on a weekly basis. Litters were examined for live, dead or missing pups at birth, on PND 4 and then weekly until PND 21. On the same occasions all pups were weighed. At termination, gross pathological examination was performed on all adult rats and on weanlings. Uteruses of all dams were examined for presence and number of implantation sites. A selection of reproductive and other organs were taken and these were weighed. From all parent F0 and F1 animals the following organs were prepared for histopathology: cervix, coagulating glands, epididymis, kidneys, ovaries (with oviduct), prostate, seminal vesicles, testes, ureters, urethra, urinary bladder, uterus, and vagina, along with any gross lesions. In principle, histopathology was performed first on 10 males and 10 females from the control and high-dose groups in both the F0 and F1 generations. In case of potential substance-related microscopic findings, the analysis was scheduled to be extended to all rats in that sex from the same generation. In the event, kidneys and urinary bladders from all adult rats from all groups were examined under the microscope. In F1 animals, but not in the F0 generation, this full histopathological examination also included the ureters, the urethra, as well as the prostate in F1 males.

In addition, some specific investigations were performed. In F0 and F1 (young adult) females, estrous cycle parameters were evaluated daily for three weeks prior to cohabitation and up to presumed mating. Ovarian follicles were counted in ten F1 females from each of the control and high-dose groups at termination (on PND 21). Sperm parameters (motility, morphology and concentration of sperm cells or spermatids in the testis and epididymis) were determined for F0 and F1 adult males from all groups at terminal kill. In addition, urinalysis was performed in ten F1 males from the control and high-dose groups. Landmarks of sexual development in F1 pups (vaginal opening or preputial separation) were examined to determine the day when first observed and the body weight measured on the day of achievement.

Study 2: parental effects

There were no unscheduled deaths or clinical signs that could be attributed to the administration of fluazaindolizine. Adverse effects on body weight or body weight gain were confined to the top dose of 4500 ppm. Examples include, during the first week of substance administration in the premating period, body weight gain in high-dose, F0 generation males was by 26% lower than in the control group, but not very different from the control group in succeeding weeks. In F0 generation females a similar first week effect was even more pronounced with body weight gain in the high-dose group lower by 41% than the

control mean. For the whole premating period mean body weight in females was lower by 19% than for controls. Slight reductions in body weight and body weight gain were also noted in this group during gestation and lactation. Similar observations were made in F1 adults receiving the top dose, with the exception that body weight gain during lactation increased after it had been lower during the premating and gestation periods. Reductions in food consumption at the maximum dose level of 4500 ppm in both sexes and generations more or less paralleled the changes in body weight and body weight gain.

Gross necropsy of parental animals in both generations revealed treatment-related findings in the urogenital tract at the top dose. Common findings were dilatation of the renal pelvis and, more rarely, of the ureters. The largest number of affected animals was 16 of 30 high-dose F1 males in which dilated renal pelvis was observed. In addition, an irregular or rough surface and discolouration of the kidneys was sometimes reported.

Two organ weight changes were attributed to treatment. In F0 generation males, absolute and relative kidney weights were increased in the high-dose group by 4.4% and 8.5%, respectively. Statistical significance was only achieved for the latter difference. This finding agreed with histopathological lesions (mucosal hyperplasia) reported below. In addition, significantly higher absolute (by 16.9%) and relative (by 17.6%) spleen weights were observed in F1 females receiving the highest dose. In the absence of microscopic examination of this organ, the background to this change is unknown. The study author suspected a relationship with bacterial pyelonephritis which was considered secondary to the renal toxicity of fluazaindolizine. Since pyelonephritis was increased in F1 females but not in the F0 generation, this hypothesis might explain the increase in spleen weight which was also seen only in the second generation.

Histopathological lesions presumed to be treatment-related were confined to the urogenital tract and were observed in both sexes and generations at the two upper dose levels. Findings comprised mucosal hyperplasia of the urothelium of the renal pelvis, ureters, urinary bladder and urethra, as well as focal erosions and ulcers of the renal pelvic mucosa. Perhaps secondary to these primary effects, was ascending inflammation (pyelitis, pyelonephritis, and sometimes cystitis or inflammation of the prostate) because bacterial infection was common. Renal degenerative changes such as hydronephrosis or a slight increase in frequency and severity of chronic progressive nephropathy were assumed to result from inhibition of normal urinary flow because of the mucosal thickening in the urinary tract. It was likely that rats which had suffered mucosal damage from fluazaindolizine became more prone to bacterial infection, and that any age-related degenerative changes might also be exacerbated. A selection of prominent urogenital findings for adult rats (unilateral and bilateral occurrence combined) is shown in Table 29.

Table 29. Selected microscopic findings in the urogenital tract of F0 and F1 generation adults (30 rats per sex and dose group, if not mentioned otherwise)

	Dose (ppm)									
	0	**150**	**500**	**1500**	**4500**	**0**	**150**	**500**	**1500**	**4500**
Organ: finding	**F0 generation, males/females**					**F1 generation, males/females**				
Kidney:										
Mucosal hyperplasia	0/1	0/0	1/0	0/0	**25/8**	0/1	0/2	1/2	3/1	**23/16**
Mucosal erosion/ulcer	0/0	1/0	1/0	0/0	**13**/2	0/0	0/0	0/0	0/0	**13**/2
Interstitial inflammation	0/0	0/0	0/0	0/0	**7**/1	0/0	0/0	0/0	0/0	**4**/0
Pyelonephritis	1/0	0/1	1/1	0/1	0/0	0/0	0/1	0/1	1/1	**7/11**
Hydronephrosis	0/0	0/0	2/0	0/0	9/3	5/4	7/1	9/1	6/3	**16/15**
Nephropathy	16/2	13/1	13/6	10/2	**22**/5	12/1	13/4	11/2	13/4	**23/13**
Urinary bladder:										
Mucosal hyperplasia	1/1	0/1	1/0	1/1	**14/20**	0/0	0/2	0/1	1/0	**7/16**
Cystitis	Finding not reported for this generation, zero incidence assumed					0/0	0/0	0/0	1/0	**7/8**

	Dose (ppm)									
	0	150	500	1500	4500	0	150	500	1500	4500
Organ: finding	**F0 generation, males/females**					**F1 generation, males/females**				
Ureters:										
Mucosal hyperplasia	Few animals examined (0–10 per group);					0/0	0/0	0/0	0/0	**8/4**
Inflammation	Mucosal hyperplasia in one mid-dose male, inflammation not reported					0/0	0/0	0/0	0/0	**6/10**
Urethra:										
Inflammation	Finding not reported for this generation but examination only in up to 10 rats from control and high-dose groups					0/0	0/0	0/0	0/0	**6/10**
Prostate:										
Inflammation	Finding not reported for this generation, but only 2–12 males per group examined					0/-	0/-	0/-	1/-	**6**/-

Clear increases marked in bold; Source: Munley, 2020

Fully in line with microscopic findings, urinalysis revealed a marked increase in red blood cells and in leucocytes in high dose F1 males as compared to the control group.

Study 2; reproductive toxicity

Sperm parameters in F0 and F1 males were not affected by treatment and there was no impact of the test substance on estrous cyclicity in F0 or F1 dams. Ovarian follicle counts in F1 females from the control and high-dose groups were not significantly different even though slightly lower at the high dose level (mean of 96 compared to 108 in controls). The precoital interval before successful mating, mating itself, fertility and gestation indices were not affected by treatment.

In both generations a number of mated pairs failed to produce a litter. Sometimes, but not always, the causes for reproductive failure could be elucidated. However, based on the distribution of these cases among the groups, no pattern was obvious and so its being an effect of treatment is unlikely.

Abortions were not reported. The number of implantation sites and post-implantation losses were comparable between all groups.

Examinations revealed dystocia to be the cause of death in one high-dose F1 female. Even though this could theoretically be an indication of an endocrine-mediated adverse effect on reproduction due to the test substance, this finding was considered incidental since it was apparently a unique case and mean gestation length was the same or very similar in all groups.

Study 2; offspring effects

There were no effects on litter size, viability, sex ratio or pup survival during lactation. It was the case that the number of decedents among F2 pups (all found dead on PNDs 0–4) was highest in the maximum dose group (see Table 30) but similar differences between groups had earlier been observed in the F1 generation, and there the number of pups that died during lactation was lowest at the top-dose level. Approximately one half of the pups found dead had no milk in their stomachs, suggesting that they had not suckled.

From birth onwards throughout lactation, mean pup weights in the F1 generation were by 5%–7% lower in the high-dose group than in the control group. A similar finding was noted with F2 pups at the top dose towards the time of weaning, but it was less pronounced and the weight difference was not statistically significant.

Table 30. Selected offspring parameters in the two-generation study in rats

	Dose (ppm)									
	0	150	500	1500	4500	0	150	500	1500	4500
Parameter	**F1 generation**					**F2 generation**				
Live pups at birth (per litter)	13.3	13.6	13.9	14.3	13.7	13.9	15.0	14.6	15.1	13.7
Viability index (%)	99.3	97.8	98.4	99.4	99.2	98.7	98.7	98.5	99.8	100
Lactation index (%)	99.2	98.5	99.5	99.6	100	100	95.8	99.6	100	95.6
Mean pup weight (g), day 0	6.6	6.5	6.6	6.6	6.3*	6.4	6.4	6.2	6.4	6.2
Mean pup weight (g), day 21	58.1	58.9	58.2	57.4	53.8*	55.9	56.9	55.3	56.2	51.9
Mean kidney weight in male weanlings (mg)	743	715	712	692	646*	693	708	658	682	616*

* Statistically significant using Dunnett's test at $p < 0.05$; Source: Munley, 2020

At necropsy of weanlings, dilatation of renal pelvis and of the ureters was observed at the top dose in F1 female pups and in the F2 generation pups in both sexes. In contrast to what was seen in parental males, mean kidney weight was significantly lower than for controls in high-dose male weanlings of both generations (see Table 30). Whereas no differences in organ weights among the groups were observed in F1 female weanlings, the relative spleen weight was significantly increased (by more than 18% compared to controls) at the top dose in F2 female weanlings, similar what was observed in adult F1 dams.

Histopathological examination of pups revealed the same microscopic lesions in the kidneys, ureters, urinary bladder and urethra as in the adults. In the F1 generation only the highest dose group was affected, but among F2 weanlings these findings, at least in the kidneys and urinary bladder, were more frequently noted in the group receiving 1500 ppm as well (Table 31). There was also a dose-related increase in severity of these lesions.

Table 31. Selected microscopic findings in the urogenital tract of F2 weanlings

	Dietary dose (ppm)									
	0	150	500	1500	4500	0	150	500	1500	4500
Organ: finding	**Males**					**Females**				
Number examined [a]	26	29	28	24	28	26	29	28	24	28
Kidney:										
Mucosal hyperplasia	0	0	0	**3**	**13**	0	0	1	**5**	**20**
Hydronephrosis	3	5	4	1	3	2	3	3	2	**10**
Pyelonephritis	0	0	0	0	**8**	0	0	1	1	**15**
Urinary bladder:										
Mucosal hyperplasia	0	0	0	**4**	**17**	0	1	1	**5**	**23**
Cystitis	0	0	0	1	**13**	0	1	0	**3**	**19**
Ureters:										
Mucosal hyperplasia	0	0	0	2	**5**	0	0	1	2	**18**
Inflammation	0	0	0	0	**6**	0	0	1	1	**14**
Urethra:										
Mucosal hyperplasia	0	0	0	0	**3**	0	0	1	2	**18**
Inflammation	0	0	0	1	**3**	0	0	1	1	**12**

[a] Related to kidney histopathology, numbers might be slightly lower for other organs; Source: Munley, 2020

Clear increases marked in bold.

The mean time interval until preputial separation or vaginal opening in F1 pups was not affected by treatment suggesting that sexual maturation was not impaired by treatment.

Parental toxicity in this study was observed, in both generations, at the maximum dose level of 4500/2700 ppm. This was evident from lower body weight gain and reduced food consumption, increases in kidney and spleen weight in certain cohorts, but in particular by gross and histopathological findings in kidneys and the urogenital tract, supported by urinalysis. The upper-mid dose level of 1500/900 ppm (equal to a mean compound intake of 88 mg/kg bw per day) was therefore the parental NOAEL.

There was no evidence of reproductive toxicity in this well-performed, two-generation study. Fertility and reproductive performance and success were not compromised at any dose level. Accordingly, the highest tested dietary dose level of 4500/2700 ppm (equal to a mean daily intake of 265 mg/kg bw) was considered the reproductive NOAEL.

Offspring toxicity reflected the effects observed in parental animals. It was characterized by lower pup weight at birth and weight gain throughout lactation, by gross and histopathological lesions of the urogenital tract and organ weight changes, specifically a decrease in kidney weight in male weanlings and an increase in spleen weight in female F2 weanlings. Most of these effects were confined to the maximum dose level. However, there was evidence already of microscopic lesions in the kidneys and urinary bladder at 1500 ppm (see Table 31). Accordingly the NOAEL for offspring toxicity was 500 ppm (equal to a mean daily intake of 30 mg/kg bw) (Munley, 2020).

(b) Developmental studies

Rat

Fluazaindolizine (purity 98.5%; Lot No. DPX-Q8U80-093 / SG0312632) was administered daily by oral gavage to five groups of 22 presumed pregnant Crl:CD(SD) rats, from GD 6 until GD 20. The dose levels were 0, 35, 100, 200 and 400 mg/kg bw per day, selected on the basis of a pilot range-finding study. The control group received just the vehicle, which was 0.5% aqueous methylcellulose containing 0.1% Tween 80. The dosing volume was 10 mL/kg bw.

The dams were observed twice a day for mortality, behavioural changes and signs of overt toxicity. The animals were weighed daily during the treatment period and food consumption was recorded every second day throughout the study. Following terminal kill the dams were necropsied and assessed for gross pathological changes. All the postmortem maternal and fetal examinations were performed without knowledge of the group designation since all dams were assigned a blind identifier number prior to scheduled termination.

In the ovaries the number of corpora lutea was determined. The uteruses were removed and weighed. The number of implantation sites and their distribution were recorded. Live and dead fetuses were counted as well as resorption sites. An attempt was made to distinguish between early and late resorptions based on the detection of morphological structures. Conception rate and pre- and post-implantation losses were calculated. It appears placental weights were not determined in this study.

All fetuses were weighed, sexed by external appearance and evaluated for external anomalies, then killed. One half of each dam's litter was prepared for head and visceral examination and the other half for skeletal examination.

There were no unscheduled deaths in this study and no clinical signs were observed in female rats that could be attributed to treatment. The same holds true for necropsy findings.

Food consumption in high-dose females, over the whole treatment period, was by 12% lower than in the control group. An initial decrease in food intake (by 10% below controls) was also seen in the next group of dams which received 200 mg/kg bw per day, but this effect was confined to GDs 6–8. Since it was not accompanied by body weight losses or a significantly lower body weight gain in this group, only the reduced food consumption in the high-dose group was considered an adverse effect.

An initial body weight loss of 1.1 g on average was noted for high-dose dams from GDs 6–8. Even though the pregnant females gained weight again as the study progressed, their mean body weight gain was lower by 14% than for the control group. The resulting mean body weights during the treatment period were also consistently lower, even though the differences remained relatively small. Body weight and its gain were not affected at lower doses.

Reproductive parameters (conception and implantation rates, pre- and post-implantation losses) as well as the number of resorptions and of viable fetuses did not differ among the groups. One low-dose female and one from the group receiving 200 mg/kg bw per day were not pregnant. Fetal sex ratio was not skewed. However, mean fetal weight was by 8.7% lower at the high dose than in the control group. There was no increase in malformations whereas a small but significant increase in short cervical ribs was observed in high-dose fetuses (see Table 32). The fetal incidence of this finding was still within the historical control range, although at nine fetuses it reached the upper edge of what had been seen in only one study from the HCD database. With regard to the more significant parameter, the litter incidence, the historical control range was exceeded. A zero incidence as observed in the concurrent control group is a common finding and must not be used to disregard the high-dose observations. It is true that this finding is sometimes observed in the presence of maternal toxicity. However, in three of the dams with litters in which the variation was found, terminal body weight was very close to the group mean of 365.6 g and in a fourth case was even much higher (420.1 g). Accordingly, there is no obvious relationship between occurrence of short cervical rib and maternal toxicity when individual data is taken into account.

Table 32. Mean fetal body weight (with SD) and fetal and litter incidences of short cervical ribs in a developmental toxicity study with fluazaindolizine in the rat including historical control data (HCD) for this finding&

	Dose (mg/kg bw per day)				
Parameter	**0**	**35**	**100**	**200**	**400**
Total number of fetuses [litters]	266 [22]	248 [21]	275 [22]	268 [21]	265 [22]
Fetal weight (g), sexes combined, mean standard deviation	5.90 ± 0.44	5.94 ± 0.38	5.87 ± 0.39	5.62 ± 0.34	5.39 ± 0.44**
Short cervical rib, fetal [litter] incidence	0 [0]	2 [2]	2 [1]	1 [1]	6 # [4] #
HCD [a], fetuses	Range: 0–9 ; mean 1.1; zero incidence in 11/17 studies				
HCD [a], litters	Range: 0–3; mean 0.6				

[a] Historical control database consisted of 17 studies conducted between 2010 and 2016; Source: Munley, 2017b

** Statistically significant using Dunnett's test at $p < 0.01$;

Statistically significant using Mann–Whitney exact test with Bonferroni–Holm adjustment at $p < 0.05$

To conclude, the maternal NOAEL in this oral developmental study in Sprague Dawley-derived rats was t 200 mg/kg bw per day, based on reduced food consumption over the whole study period and initial body weight loss in high-dose dams, followed by lower body weight gain throughout the rest of the study. Fluazaindolizine was not teratogenic to rats. Effects on fetuses were confined to the highest dose level of 400 mg/kg bw per day and were mainly characterized by a decrease in mean fetal weight. Accordingly, the developmental NOAEL in this study was 200 mg/kg bw per day. In addition, an increase in short cervical ribs was observed at the same dose level but this minor skeletal variation is usually completely reversible and not regarded as adverse (for review see, for example, DeSesso & Scialli, 2018) (Munley, 2017b).

Rabbit

A range-finding study was performed in New Zealand White rabbits. Four groups of eight time-mated females received fluazaindolizine (purity 95.6%, Lot No. DPX-Q8U80-068) by oral gavage once daily from GD 7 to GD 28 in a dosing volume of 10 mL/kg bw. The dose levels were 0, 30, 100 and 200 mg/kg bw per day. The control group received only the vehicle, 0.5% aqueous methylcellulose containing 0.1% Tween 80. The does were closely monitored for adverse signs, body weight and food consumption were recorded. On day 29 the females were killed and uteruses, placentas and ovaries examined. Fetuses were weighed, sexed and examined for malformations and variations.

Three does receiving the maximum dose aborted on GDs 26 or 28 and one delivered prematurely on GD 29. To some extent, clinical signs had been observed in the animals that aborted later. In the high-dose group, food consumption and body weight gain were reduced and occasional body weight losses observed. An increase in post-implantation loss was noted and fetal weight was lower than in controls.

No evidence of teratogenicity was obtained but it must be acknowledged that only four litters could be inspected in the high-dose group.

No clear signs of toxicity were observed at the low and intermediate dose levels but there was one abortion at mid-dose. It may be concluded that the top dose was too high for a subsequent, definitive study (Charlap, 2012).

In the main study, fluazaindolizine (purity 95.6%; Lot No. DPX-Q8U80-068) was administered daily by oral gavage to four groups of 22 time-mated New Zealand White rabbits from day 7 of presumed gestation to day 28. The control group received only the vehicle, 0.5% aqueous methylcellulose containing 0.1% Tween 80. The dosing volume was 10 mL/kg bw.

Animals were observed twice daily for mortality, behavioural changes and clinical signs of toxicity. During the administration period, they were weighed every day and food consumption was also recorded daily on an individual basis.

Following terminal kill the does were necropsied and assessed for gross pathological changes. The uteruses and ovaries were removed. In the ovaries, the number of corpora lutea was determined. Uteruses were weighed, opened, and the number of implantation sites recorded. Live and dead fetuses were counted as well as resorption sites. An attempt was made to distinguish between early and late resorptions. Placentas were also examined.

Brain, kidneys, and liver were taken from the does, weighed and preserved for subsequent microscopic examination. In addition all gross lesions and stomach and urinary bladder were collected from all animals for histopathology. Following fixation all protocol-specified tissues were further processed in a contract laboratory from where stained slides were returned for histopathological examination in the laboratory where the study had been run. The animals which had to be killed before termination were also subject to gross and histopathological evaluation.

All fetuses were weighed and evaluated for viability and external anomalies. Fetuses were then killed and abdomen and thorax opened for visceral examination, including detailed inspection of the heart and major vessels. Kidneys were sectioned and graded with regard to development of renal papillae. The sex of the fetuses was determined internally by examination of the gonads. Heads were examined by midcoronal slice. The fetuses were then prepared for skeletal examination. All fetal procedures were performed using coded identification so as to conceal their treatment group.

In the high-dose group one female had to be killed in extremis on GD 25 following markedly reduced food consumption from GD 13 onwards that resulted in a body weight reduction by more than 18%. Three further does from the same group aborted on GDs 25 or 26 and were subsequently euthanized. Two of them had suffered from reduced food intake and body weight losses of 12% and more than 18% respectively over the previous two weeks. The study author attributed the abortions to the preceding body weight loss. These findings were considered treatment-related and adverse but, clearly were not acute effects.

Also in the mid-dose and control groups, one doe each aborted, both on GD 28. Similar to the observation in the high-dose group, these females had previously experienced body weight losses of 11.9% and 18.1% due to a reduced food consumption. These decreases were of a similar magnitude to those of decedents from the high-dose group, but the number of affected animals was lower and there was no apparent dose–response relationship. In addition, no adverse effects on food intake or body weight became apparent in the other rabbits from the groups receiving either 30 mg/kg bw per day or just the vehicle. Therefore these isolated cases of body weight loss and subsequent abortions were regarded as incidental and not attributed to treatment.

Body weight gain, as well as food consumption, were transiently decreased in the high-dose group. Between GDs 10 and 13 mean weight gain was slightly lower, followed by even a marginal mean net weight loss, however, increased weight gain then began and continued from day GD 20 until termination. Between GDs 13 and 20, mean food intake by high-dose females was by nearly 18% lower than in the control group, but was not much different before this period and it normalized later. So any adverse effects were in fact confined to the mid part of the treatment period suggesting a delayed effect of substance administration rather than any acute effect (see Table 33). At the lower dose levels nutritional parameters were unaffected.

A common finding in the does which aborted were haemorrhages in the stomach and the urogenital tract. Organ weights were not altered by treatment. Histopathology revealed, in the high-dose group, an increased incidence of minimal or moderate renal tubular degeneration and dilatation (see Table 33). These lesions were most pronounced in those animals which were in a poor condition and/or aborted. In addition, two high-dose females displayed minimal or mild hepatocellular necrosis; one doe was killed in extremis and the other had aborted. The same lesion, to a minimal degree, was observed in one mid-dose animal at scheduled termination.

Table 33. Maternal toxicity in the developmental study in rabbits; body weight gain and kidney histopathology

		Dose (mg/kg bw per day)			
Parameter, finding		**0**	**10**	**30**	**120**
Mean body weight gain (g):	Days 7–10	32	10	14	37
	Days 10–13	41	33	30	17
	Days 13–20	85	86	81	–8
	Days 20–29	79	87	32	125
Kidney findings (number of affected does/number examined)					
	Tubular degeneration	1/22	1/22	1/22	8/22
	Tubular dilatation	1/22	0/22	1/22	6/22

Source: Wirbisky, 2017

Reproductive parameters were not altered by treatment. The number of fetuses available for examinations was lowest in the high-dose group because of the abortions. However, the total numbers of fetuses, 186 (control), 188 (low dose), 177 (mid dose), and 167 (high dose) were sufficient for meaningful conclusions to be reached.

In the high-dose group, mean fetal weight was lower by 6.2% than in the control group and was in fact the lowest among all groups, but the difference did not achieve statistical significance. The fetal sex ratio was unaltered.

Very few external, visceral or skeletal malformations were reported , and with these no pattern or dose–response relationship was apparent. They were therefore considered spontaneously occurring. The same was the case for variations.

To conclude, the mid dose of 30 mg/kg bw per day in this developmental study in rabbits was the NOAEL for maternal toxicity. It is worth considering that the reductions in body weight gain (or even body weight loss) and food consumption resulting in poor condition and abortions became apparent only at a later stage of treatment, that is, they occurred in a delayed manner. It is also unlikely that the histopathological findings in the kidneys and liver were caused by a single exposure. Accordingly, these findings cannot be regarded as acute and are not a suitable basis for setting an acute reference dose (ARfD).

No developmental toxicity was observed up to the highest dose level of 120 mg/kg bw per day which is therefore the developmental NOAEL. Fluazaindolizine was not teratogenic in rabbits (Wirbisky, 2017).

2.6 Special studies

(a) Neurotoxicity

Acute neurotoxicity

In an acute neurotoxicity study, fluazaindolizine (purity 98.5%; Lot No. DPX-Q8U80-093) was administered as a single dose via oral gavage to male and female Crl:CD(SD) rats (12 per sex and dose). The compound was dissolved in 0.5% aqueous methylcellulose containing 0.1% Tween 80 and applied at dose levels of 0, 30, 125, 450 or 1750 mg/kg bw. The control group received just the vehicle. The dose volume was 12 mL/kg bw. Following dosing the animals were observed for at least 14 days.

A neurobehavioural test battery consisting of functional observational battery (FOB) and motor activity (MA) assessments, was conducted on all rats on study prior to dosing, around two hours after dosing (day 1), and on days 8 and 15. On day 17 six rats per sex and dose level were perfused in situ with a fixative. Subsequently, a microscopic neuropathological evaluation of the peripheral and central nervous systems and selected muscle tissues of these animals was conducted in a specialized contract laboratory; these examinations were confined to the control and high-dose groups.

Other parameters under examination included body weight, body weight gain, food consumption, general clinical signs and gross pathology. The animals were weighed prior to dosing and on test days 2, 8 and 15. Due to a technical problem, food intake was determined only for the first two days of treatment and for the interval from day 8 to day 15, but this cannot be considered a major deviation.

There were no test substance-related effects on mortality or clinical signs in either sex at any dose level. One male from the low-dose group was found dead. However without the possibility of a dose–response relationship this single death was considered incidental and could not be attributed to fluazaindolizine administration. It is perhaps a little surprising that all high-dose animals survived since the maximum dose was nearly twice the LD_{50} as established before (see section 2.2).

Male rats administered the two upper doses of 450 and 1750 mg/kg bw slightly lost weight (2.3 g and 6.3 g respectively compared to controls) on the day of dosing and the following day. A lower body weight gain persisted in high-dose males for the whole study period resulting in lower interim (day 8) and terminal body weights. No effects on body weight and its gain were noted at the two lower doses.

In females, mean body weights were lower on day 2 compared to day 1 in all treated groups, but the losses were not dose-related so cannot be clearly attributed to treatment. The highest mean weight loss of 6.9 g was noted in the group receiving 125 mg/kg bw.

In both sexes, food consumption was reduced on days 1 and 2 at the two upper dose levels and, in females, also in the group receiving 125 mg/kg bw.

On the day of dosing motor activity was decreased in the males in the 450 and 1750 mg/kg bw groups since the duration of movement during the two first 10-minute intervals of the observation period was reduced (see Table 34). However, this effect became weaker and resolved within 40 minutes of the 60-minute test session, no longer being present at the subsequent observation on day 8, thus proving the effect was only transient. The data in Table 34 also demonstrate the large variability in this parameter.

Also in males at 450 and 1750 mg/kg bw, body temperature was slightly lower (36.4 or 36.6°C in the mean as compared to 37.2°C in the controls) on the day of dosing but no differences were observed at later measurements. Even though of potential live-threatening nature in particular in small animals as the rat, this effect was too minor and most likely too transient for causing mortality.

Table 34. Duration of movements(s) during 10-minute observation intervals in male rats on day of dosing

		Dose (mg/kg bw)				
Observation periods		**0**	**30**	**125**	**450**	**1750**
Duration of movement (s)	Interval 1	314	301	259	223	171*
	Interval 2	221	232	189	133*	117
	Interval 3	105	132	108	45	65
	Interval 4	39	51	73	38	41

* Statistically significant using 2-tailed ANOVA at $p < 0.05$, Source: Mukerji, 2017

There were no test substance-related effects on any other neurobehavioural (FOB) end-point in either sex at any dose level. Gross necropsy and neurohistopathological examination did not reveal any remarkable findings that could be indicative of a substance-related effect. Accordingly, there was no evidence of fluazaindolizine having any specific neurotoxic potential and the NOAEL for neurotoxicity was 1750 mg/kg bw, the highest dose tested.

The NOAEL for systemic toxicity in this study was 125 mg/kg bw, based on initial body weight losses and reductions in body weight gain and food consumption, as well as transient reductions in body temperature and of motor activity in male rats at dose levels of 450 and 1750 mg/kg bw. Effects on females were much less pronounced, being confined to decreased food intake, which in the absence of other findings, was not considered adverse (Mukerji, 2017).

Subchronic neurotoxicity

A potential for neurotoxicity resulting from repeated exposure was investigated in two studies in Crl:CD (SD) rats; these are reported in detail above in section 2.2 Short-term studies of toxicity.

In the 28-day subset of a combined short-term feeding and one-generation study in the same rat strain, examinations by means of an abbreviated FOB did not reveal any evidence of neurological effects up to the highest tested dietary dose of 5000 ppm (equal to mean daily doses of 361 and 369 mg/kg bw in males and females, respectively) (Munley, 2017a).

In a combined general short-term toxicity and subchronic neurotoxicity study, 10 males and 10 females per dose were regularly subjected to extensive neurobehavioural assessment by means of an FOB and motor activity measurements. At termination of the feeding period of 95–99 days a comprehensive histopathological evaluation of the main parts of the nervous system (brain, spinal cord, root fibres, ganglia and peripheral nerves) was performed in six animals per sex and dose. In all these investigations, no evidence of neurotoxicity was obtained up to the highest tested dietary level of 6000 ppm that was equal to a mean daily intake of 348 mg/kg bw in male rats and 376 mg/kg bw in females (MacKenzie, 2013).

(b) Immunotoxicity

No concern arose from routine studies in which a number of potential immune-related end-points had been investigated, such as spleen and thymus weights, histopathology of the spleen, thymus, lymph nodes, bone marrow, or haematological parameters (white blood cell count). A smaller size of the thymus was seen in dogs, corresponding with weight loss or with a moribund condition.

In addition to the above, one specific study on immunotoxicity in rats was available. Fluazaindolizine (purity 98.5%; Lot No. DPX-Q8U80-093) was fed to groups of 10 male Crl:CD(SD) rats for 28 days at dietary concentrations of 0, 500, 1500 or 5500 ppm (equal to mean daily intakes of 0, 35.5, 106 and 393 mg/kg bw per day). These dose levels were in the same range as in a preceding combined 28-day, one-generation study in rats (Munley, 2017a). A positive control group of the same size received, instead of fluazaindolizine, an oral dose of 25 mg/kg bw per day of the immunosuppressive agent cyclophosphamide monohydrate once a day for the same four week period.

On study day 24 all rats were intravenously injected with sheep red blood cells (sRBCs). At termination on day 29 blood samples were collected and assayed for specific IgM antibodies to these ovine erythrocytes. During the treatment period, the animals were monitored for clinical signs twice a day, with a detailed clinical examination performed once a week. Other parameters examined in this study comprised body weight, food intake, food efficiency, and organ weights of brain, thymus, and spleen. Gross necropsy was performed and some organs retained for subsequent histopathology, but these were apparently not examined microscopically.

There were no unscheduled deaths during the study and no clinical signs could be attributed to treatment. Limited evidence of systemic toxicity was obtained since minimal (but non significant) decreases in body weight and body weight gain were noted at the highest dose level.

No effect due to fluazaindolizine on humoral immune function was observed at any dose level in the serological investigation. Likewise, organ weights were not affected.

Specific IgM antibodies were reduced in the positive control group by 42%, as might be expected for a positive control. In addition, cyclophosphamide monohydrate caused a decrease in mean terminal body weight, and in the absolute and relative organ weights of the thymus and spleen. The latter effects also became apparent at necropsy when small spleen and small thymus were observed in six and nine rats respectively from each group of 10.

On balance, based on the parameters investigated in this study, fluazaindolizine showed no evidence of an immunotoxic potential. The positive control substance cyclophosphamide, by contrast, produced the expected impact on the immune system of male rats (Hoban, 2018b).

(c) Mechanistic studies on endocrine disruption

There were three studies submitted in which the potential endocrine disrupting properties of fluazaindolizine had been addressed.

Study 1

In an in vitro experiment using human adreonocortical carcinoma cell line H295R, cells were incubated for 48 hours with fluazaindolizine (purity 99.6%; Lot No. DPX-Q8U80-068) at concentrations ranging from 0.0001 µM to 100 µM to investigate any possible impact on steroidogenesis by measuring testosterone and 17β-estradiol levels in culture medium by HPLC-MS/MS. At the highest concentration, significant reductions in the release of both hormones were observed, whereas the next lowest dose level of 10 µM did not induce any such effect. The positive control substances forskolin (inducer of steroidogenesis) and prochloraz (inhibitor) gave the expected responses, proving the test system and assay were functioning properly. From this result, inhibition of steroid hormone production by high concentrations of the test substance can be concluded. This positive finding, however, is contradicted under in vivo conditions by the lack of any reproductive effect of fluazaindolizine in the two-generation study in rats (Munley, 2020) up to a mean daily intake of 265 mg/kg bw. It is true that hormones were not measured in any of the routine studies with fluazaindolizine in laboratory animals, but, on the other hand, there were no effects on steroid hormones reported by O'Connor (2018, see *study 2* below) in male rats (Markell, 2013).

Study 2

In an uterotrophic assay in Crl:CD(SD) rats, fluazaindolizine (purity 99.6%; Lot No. DPX-Q8U80-068) was administered at dose levels of 0, 20, 100 or 500 mg/kg bw on three consecutive days by oral gavage to ovariectomized females (six per group). The vehicle (0.1% Tween 80 in 0.5% aqueous methylcellulose) served also as negative control substance, and a further group of same size received the estrogen receptor agonist 17β-ethynyl estradiol as a positive control. For this compound the vehicle was corn oil containing 1% ethanol.

The animals were monitored daily for clinical signs of toxicity. Body weight and food consumption were determined daily. Vaginal cytology was performed daily and uteruses were weighed at termination when the rats were killed 24 hours after the final dose.

There were no unscheduled deaths and no clinical signs were noted. However, food consumption was markedly reduced by around 36% compared with controls in the group receiving the highest dose, and body weight gain in this group was also compromised. However, no cytological changes in the vaginal epithelium were observed that resembled those caused by the positive control substance. At necropsy no gross lesions were seen and uterine weight was unaffected.

In contrast, 17β-ethynyl estradiol induced cytological changes which were typical for pro-estrus or estrus. In all rats receiving this positive control substance, fluid was present in the uterus and mean uterine weight was up to three times higher than in the negative control group.

Based on this mechanistic study, fluazaindolizine was devoid of estrogen-agonistic activity in female rats (O'Connor, 2012).

Study 3

Fluazaindolizine (purity 99.6%; Lot No. DPX-Q8U80-068) was administered once a day by oral gavage at dose levels of 0, 20, 100 or 500 mg/kg bw for 15 days to groups of 15 young (c 9 weeks old at commencement of treatment) male Crl:CD(SD) rats. A concurrent negative control group received the vehicle, 0.1% Tween 80 in 0.5% aqueous methylcellulose; no positive control group was included. The dosing volume was 5 mL/kg bw for all groups. Due to excessive body weight loss the maximum dose level was reduced on day 10 to 350 mg/kg bw per day.

Body weights, food intake and occurrence of clinical signs were recorded daily. Two to three

hours after the final dose the rats were killed and necropsied. The following organs were collected and weighed: liver, thyroid, adrenals, testes, epididymides, prostate, seminal vesicles (with coagulating glands and fluids). Thyroid glands, testes, epididymides and stomach (also taken but not weighed) were preserved for subsequent microscopic examination. Microsomes were obtained from the livers and analyzed for aromatase activity.

At termination, blood was sampled and the following hormones determined in serum: testosterone and dihydrotestosterone, estradiol, luteinizing hormone (LH), thyroid-stimulating hormone (TSH), triiodothyronine (T3) and thyroxine (T4).

The highest dose of fluazaindolizine caused overt toxicity in the male rats. Beginning from day 4 of treatment, body weight in the high-dose group was significantly lower than in the controls. From day 7 onwards the animals exhibited clear body weight losses until day 11. After the maximum dose had been reduced the animals recovered and gained weight again until termination. However, weight gain over the entire study period was still markedly lower. Likewise, mean daily food consumption was reduced in high-dose males by around one third to one quarter compared to the control group. One top-dose animal was found dead on day 12. Treatment-related clinical signs comprised dehydration, high posture and black or brown discharges from the eyes or mouth. No evidence of toxicity was observed in the groups receiving the lower doses, and body weights at these doses were also unaffected (Table 35).

Necropsy revealed no gross lesions in any group. Decreases in organ weights at the highest dose level were considered secondary to the lowered body weight. However, with regard to prostate weight (Table 35), more detailed consideration was warranted.

Table 35. Mean absolute and relative prostate weights in rats following a 15-day gavage application of fluazaindolizine

	Dose (mg/kg bw per day)			
Parameter	**0**	**20**	**100**	**500/350**
Terminal body weight (g)	420.8	427.7	418.3	354.4*
Absolute prostate weight (mg)	685	574*	537*	362*
Relative prostate weight (% of body weight)	0.163	0.134	0.128	0.102*

* Statistically significant using Dunnett's test at $p < 0.05$; Source: O'Connor, 2018

Unfortunately the prostate was not examined histologically in this study. While the clear reductions in prostate weight at the top dose might be due to the general toxicity of fluazaindolizine and the resulting poor condition of the animals at this rather high dose, there is no obvious explanation for the lower organ weights in the low- and mid-dose groups. On the one hand, a mean absolute prostate weight of 685 mg in the controls is higher than found in a historical control database from studies of this type which were conducted in the same laboratory over the same time period. However this database was extremely small, comprising only four studies in which mean absolute prostate weights ranged from 425 to 581 mg, with relative prostate weights between 0.112% and 0.140%. These ranges would cover the figures obtained in the actual study at the low- and mid-dose levels, but because of the very limited number of studies any conclusion based on this evidence is not completely convincing. Other arguments put forward by the study author were the absence of weight changes in other sexual organs in this study and, perhaps more convincingly, the absence of effects (organ weight changes or histopathological findings) in a number of other (dietary) rat studies of longer duration. On balance, the decrease in prostate weight in this assay is at best equivocal.

Microscopic findings in the few organs examined were confined to glandular mucosal erosion and ulcers and subsequent inflammation in the stomach of high-dose animals.

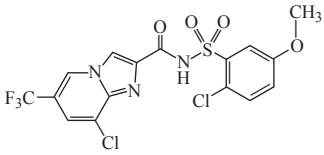
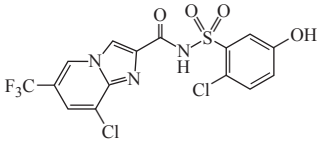
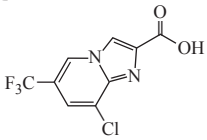
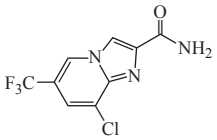
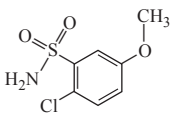
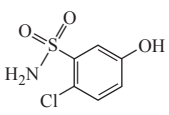
Hormone levels were not apparently affected by treatment, with lower serum T4 levels in high-dose rats (by 20%) being the only exception. Aromatase activity in liver microsomes was increased in the high- and mid-dose groups by 60% and 42% respectively compared with controls, but the study author expressed his doubts on the specificity of the assay and argued that this finding could be also due to the induction of other hepatic enzymes, as for example observed in a 90-day study in dogs (Han, 2014). It must also be observed that a possible increase in the activity of aromatase had no effect on estradiol concentrations, levels of which might be expected to riser in such a situation.

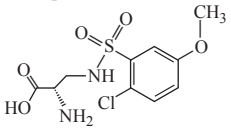
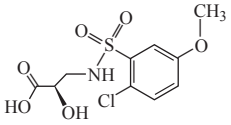
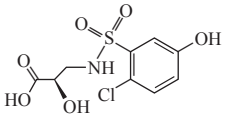
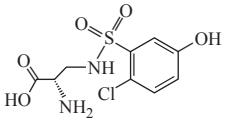
Under the conditions of this study, no clear evidence was obtained for the endocrine-mediated effect of fluazaindolizine on male rats even though a possible decrease in prostate weight at the low- and mid-dose levels and an equivocal increase in aromatase activity were reported. High dose effects on prostate weight and T4 concentrations were attributed to overt systemic toxicity (O'Connor, 2018).

2.7 Studies on metabolites and impurities

A large number of metabolites of fluazaindolizine have been identified not only in the rat (see section 1.2) but also in plants and livestock (DuPont, 2021). A huge toxicological data package consisting of about 75 studies, focussing on nine metabolites (presumably those considered toxicologically relevant by the sponsor) is available. An overview of these metabolites is shown in Table 36, showing their structures, occurrence in rodents (or in vitro), metabolism and the available data. The remainder of this section reports on the individual metabolites, each under its own subheading.

Table 36. Summary on metabolites of fluazaindolizine for which experimental studies have been submitted, parent compound mentioned for comparison purposes

Compound, codes and structure	Rodent metabolite	Genotoxicity studies	General toxicity studies
Fluazaindolizine (parent) 	Not a metabolite but the main compound in excreta accounting for 65%–87% of AD in rats and mice	Full data set: not mutagenic, genotoxic in vitro, negative in micronucleus assays, on balance not genotoxic at least in vivo	Full data set, robust reference values derived
IN-REG72 	Yes: <2% in rat and <6% of AD in mouse excreta) but perhaps major human metabolite (based on in vitro data)	Not genotoxic (negative studies in vitro and in vivo)	No data
IN-QEK31 	Yes: accounting for up to 10% in urine and bile if combined	Not mutagenic, genotoxic in vitro, negative in vivo	Comprehensive data set (acute, short-term, reproductive and developmental toxicity), No more toxic than parent
IN-F4106 	Yes: around or greater than 1% in excreta but major metabolite in rat kidney and liver accounting for 17%–26% of AD	Not mutagenic, genotoxic in vitro, negative in vivo	Comprehensive data set (acute, short-term, reproductive and developmental toxicity), No more toxic than parent
IN-A5760 	Yes: up to 5.4% of AD in mouse excreta, sulfate conjugate accounting for up to 7% in rat urine	Not mutagenic, genotoxic in vitro, negative in vivo	No data
IN-QZY47 	No	Not genotoxic (in vitro data)	Limited database (acute and short-term) suggesting lower toxicity than parent

Compound, codes and structure	Rodent metabolite	Genotoxicity studies	General toxicity studies
IN-TMQ01 	No	Not genotoxic (in vitro data)	Limited database (acute and short-term) suggesting lower toxicity than parent
IN-TQD54 	No	Not genotoxic (in vitro data)	Low acute oral toxicity in the rat, no further data
IN-UJV12 	No	Gene mutations in one strain in the Ames test and evidence of mutagenicity to mammalian cells when a certain batch was tested, not mutagenic in Ames test when material of high purity was used, clastogenic in vitro but not in vivo	Low acute oral toxicity in the rat, no further data
IN-VM862 	No	Not genotoxic (in vitro data)	Limited data set (acute and short-term) suggesting different (more severe) toxicological profile than parent

AD: Administered dose;

(a) Metabolite IN-QEK31; 8-Chloro-6-(trifluoromethyl)imidazo[1,2-a]pyridine-carboxylic acid

This is an important metabolite in particular in crops occurring also in livestock, soil, and water. In rodents, IN-QEK31 is one of the most frequently observed metabolites even though, it was detected in most experiments but only in relatively small amounts in excreta. This low level must be considered alongside the generally limited metabolism of fluazaindolizine (see section 1.2). In male mice, IN-QEK31 accounted for 1.3% of AD in combined urine and cage wash, and for 2.6% of AD in faeces. In females the respective figures were 1.3% and 1.7% of AD (Punler & Green, 2016b). In male rats, the metabolite was found at 4.1% of AD in urine (with cage wash) and 1.5% in faeces. Similar amounts (5% and 1.6%, respectively) were detected for females. In one experiment in bile duct-cannulated rats, combined urinary and biliary levels were higher, (in the order of 8%–10% of AD) in both sexes (Punler & Green, 2017), suggesting that IN-QEK31 could be considered a major rat metabolite.

Based on ADME studies with the parent, it was assumed first that IN-QEK31 would not undergo further biotransformation (see Figure 2 earlier). However, subsequent LC-MS/MS analysis of urine samples obtained from male and female rats which had been given IN-QEK31 at dietary dose levels of 800, 2000 or 10 000 ppm in a two-generation study (Munley, 2019; see below) revealed good evidence of conjugation. Irrespective of sex and dose, a number of glucuronide conjugates were detected in this non-GLP study even though unchanged IN-QEK31 was still the most abundant urinary excretion product. It can be reasonably assumed that these glucuronides will also have been present, if in minor amounts, in other studies with fluazaindolizine (Swain & Ryan, 2018).

MetaboliteIN-QEK31 was produced in small amounts by rabbit and dog hepatocytes in vitro, but was not found after incubation of human hepatocytes or those obtained from rats or mice with the parent compound (Novo, 2018). In routine toxicological studies with all test species this metabolite was detected in plasma, but only in traces, and orders of magnitude below the concentrations of parent fluazaindolizine.

Despite its frequent detection as part of rat metabolism, IN-QEK31 has been subject to extensive toxicological examination for many end-points enabling separate risk assessment. An overview of the available studies is shown in Table 37. The individual studies are briefly reported with findings detailed only in cases where this was considered relevant for risk assessment.

Table 37. Fluazaindolizine metabolite IN-QEK31: overview of the available toxicological and genotoxicity studies

End-point/study type/ test system	Information on study design and further remarks	Results	Reference
Acute oral toxicity, rat	Up-and-down procedure according to OECD 425	Moderately toxic, no signs or abnormalities at 550 mg/kg bw, mortality at 1750 mg/kg bw and above	Fallers, 2017b
Acute dermal toxicity, rat	Limit test	LD_{50} > 5000 mg/kg bw	Merrill, 2014a
Acute inhalation toxicity, rat	Nose-only exposure to a single aerosol concentration for 4 hours	LC_{50} > 5.0 mg/L	Rajsekhar, 2013
Skin irritation, rabbit		Not irritant	Merrill, 2015a
Eye irritation, rabbit		Very mild and transient irritation not resulting in classification	Merrill, 2015b
Skin sensitization, Guinea pig	Magnusson & Kligman test	Not sensitizing	Merrill, 2016a
Mutagenicity in vitro, bacteria	Ames test in *S. typhimurium* and E. *coli*, ± S9 mix	Negative	Wagner, 2014
Mutagenicity in vitro, mammalian cells	HPRT assay in CHO cells, ± S9 mix	Negative	Dutta, 2016a
Genotoxicity (clastogenicity) in vitro	Chromosome aberration in peripheral human lymphocytes, ± S9 mix	Positive with and without activation	Kellum, 2016a
Genotoxicity in vivo (mouse micronucleus assay)	Flow cytometric analysis of peripheral reticulocytes after oral dosing, parallel plasma analysis for IN-QEK31	Negative for micronucleus formation; target tissue (bone marrow), exposure assumed; mortality at 1300 mg/kg bw and above	Myhre, 2018b; Chan, 2016
Short-term toxicity, rat	90-day feeding study including neurobehavioural evaluation, dose levels up to 12 000 ppm	NOAEL 3000 ppm (equal to 183(M)/204(F) mg bw/day); adverse histopathological findings in kidneys and bladder and, in males, reduced bw, and bw gain and food efficiency at the top dose	Hoban, 2016a
Combined short-term and reproductive toxicity, rat	One-generation study with comprehensive evaluation of parental animals but limited evaluation of pups	Parental and offspring NOAEL 3000 ppm (equal to 212 mg/kg bw/day), reproductive NOAEL 12 000 ppm (equal to 812 mg/kg bw/day)	Lewis, 2017
Reproductive toxicity, rat	Comprehensive two-generation study	No NOAEL for parental and offspring toxicity, LOAEL 800 ppm (equal to 48 mg/kg bw/day) based on microscopic kidney findings; no reproductive toxicity up to highest dose of 10 000 ppm (c 596 mg/kg bw/day)	Munley, 2019
Developmental toxicity rat		Maternal and developmental NOAEL 330 mg/kg bw/day	Munley, 2018a

M: Male; F: Female; bw: Body weight

Acute toxicity

In an acute oral toxicity study, fasted female Crl:CD(SD) rats (between one and three per group) received IN-QEK31 at doses of 0, 550, 1750 or 5000 mg/kg bw. The only high-dose animal and two of the three rats administered the mid dose died on test days 1, 2, or 3, respectively; both animals receiving the low dose survived without any clinical signs, body weight losses or gross findings at necropsy. At the two upper dose levels a number of clinical signs such as abnormal gait, laboured breathing, redness of ears and paws, loss of righting reflex, piloerection, dehydration and hypoactivity preceded death. Gross findings that were presumed to be dose-related comprised multiple ulcers and erosions in the glandular stomach. The surviving female in the mid-dose group exhibited hypoactivity and piloerection on day 1 following dosing, but completely recovered during the 14-day postobservation period. The statistical estimate for the LD_{50} was 1750 mg/kg bw but this figure could in practice be lower since two of three female rats receiving this dose died. It seems more appropriate to assume a LD_{50} between 550 and 1750 mg/kg bw, which would suggest a similar acute oral toxicity to that observed for the parent compound (Fallers, 2017b).

An acute dermal toxicity study in five male and female Sprague Dawley-derived rats did not reveal any evidence of toxicity at the limit dose of 5000 mg/kg bw. All rats survived, gained weight throughout the study and IN-QEK31 did not cause any systemic clinical signs, local effects or gross lesions (Merrill, 2014a).

A low acute toxicity of inhalation for IN-QEK31 was demonstrated in male and female Wistar rats (five per sex) which were exposed for four hours (nose only) in a limit test to a mean actual aerosol concentration of 5 mg/L (nominal concentration nearly 15 mg/L, MMAD 2.81 µm). No mortality and no clinical signs of toxicity were observed. Most animals did not gain much weight, or even transiently lost weight, on the first three days following exposure, but all rats had gained weight on days 7 and 14 of the post-observation period. Necropsy revealed no gross lesions (Rajsekhar, 2013).

Metabolite IN-QEK31 proved negative in a skin irritation study in three female New Zealand White rabbits. All animals appeared active and healthy and gained weight over the course of the study. No dermal irritation was observed at any treated site on the body (Merrill, 2015a).

Metabolite IN-QEK31 was tested for eye irritation in three female New Zealand White rabbits. The test was performed in a stepwise fashion and under ocular anaesthesia, by instillation of 0.1 mL (c 67 mg) of the test substance into the conjunctival sac of one eye. All animals appeared active and healthy and gained weight over the course of the study. Signs of eye irritation were mild. Conjunctival redness and chemosis (both of the lowest degree, score 1) and some discharge (score 2) were observed in the treated eyes of all animals. In addition, one rabbit exhibited slight corneal opacity (score 1). All irritation signs had cleared by 48 hours after instillation (Merrill, 2015b).

Metabolite IN-QEK31 was tested for its skin sensitizing potential in 20 male Hartley strain Guinea pigs according to the method of Magnusson & Kligman, (intradermal and topical induction followed by topical challenge). For challenge, the animals were treated with both a 60% (w/w) and a 20% (w/w) mixtures of the test substance in propylene glycol. In a previous test experiment primary irritation testing had been performed on 12 animals to select appropriate concentrations for the different steps. Ten additional animals were used as the vehicle control group. A concurrent positive control group was not included but appropriate historical control data was provided with the report. Very faint erythema was noted in eight (60% mixture) and two (20% mixture) of the exposed sites 24 hours after the challenge. This minimal irritation cleared from the affected sites by 48 hours. Accordingly, it may be concluded that IN-QEK is devoid of skin sensitizing potential (Merrill, 2016a).

Genotoxicity

The mutagenicity of IN-QEK31 in bacteria was investigated in an Ames test (plate incorporation method) in *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and in *E. coli* tester strain WP2 *uvr*A, with and without metabolic activation. In all strains the test substance (dissolved in DMSO) was applied at various concentrations up to 5000 µg/plate. There was no increase in mutant frequency in any experiment. Also, no precipitation was observed and there was no evidence of cytotoxicity. An appropriate positive control substance gave the expected responses. Metabolite IN-QEK31 proved negative for bacterial mutagenicity under the conditions of this assay (Wagner, 2014).

Metabolite IN-QEK31 was tested in CHO cells for its ability to cause forward gene mutation at the hypoxanthine–guanine phosphoribosyl transferase locus (HPRT assay). The test substance was dissolved in DMSO. Experiments were performed at concentrations of up to 1504 µg/mL, both in the presence and absence of metabolically activating S9 mix. No increase in mutant frequency was observed with the metabolite, whereas the positive control substances induced just such a response. No precipitation was observed and survival was sufficient even at the highest tested concentration suggesting that the test compound was not strongly cytotoxic. Metabolite IN-QEK31 was found to be negative in this study (Dutta, 2016a).

In an in vitro chromosome aberration study, cultured human peripheral lymphocytes (obtained from two healthy male donors 30–40 years of age) were exposed to IN-QEK21 for four hours and for 22 hours in the absence of metabolic activation, or for four hours in the presence of S9 mix. Based on preliminary testing for precipitation and cytotoxicity, concentrations of up to 1500 µg/mL were applied in the final experiments and concentrations of 0, 500, 1000, and 1500 µg/ml were used for cytogenetic evaluation. Because of the clear results following the four-hour exposure, the cultures receiving 22-hour treatment were not analysed. Slides, coded for anonymity, were scored for both structural and numerical chromosomal anomalies. At least 300 metaphases per concentration (150 from each duplicate culture, that is, each donor) were analyzed. Appropriate positive control substances were applied.

A statistically significant increase in structural, but not numerical, chromosome aberrations was noted after a four-hour treatment period both with and without metabolic activation at 1500 µg/mL. Also, there was a positive trend with dose (see Table 38). Accordingly, IN-QEK31 must be considered positive in this assay (Kellum, 2016a)

Table 38. Summary of structural chromosome aberration in peripheral human lymphocytes after a four-hour treatment (harvest after 22 hours) with IN-QEK31 or positive control substances

Treatment	Activation	Mitotic index (%)	Cells scored	Percentage with aberrations
Vehicle (DMSO)	−S9	9.4	300	1.0
500 µg/mL	−S9	5.6	300	2.0
1000 µg/mL	−S9	6.2	300	2.3
1500 µg/mL	−S9	4.2	300	7.7*
Mitomycin C, 0.4 µg/mL	−S9	6.5	100	12.0
Vehicle (DMSO)	+S9	6.8	300	0.7
500 µg/mL	+S9	7.3	300	1.7
1000 µg/mL	+S9	4.0g	300	2.0
1500 µg/mL	+S9	6.5	300	4.7*
Cyclophosphamide, 10 µg/mL	+S9	5.6	100	10.5

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*; Source: Kellum, 2016a

* Statistically significant difference using Fisher's test and Cochran–Armitage test, at $p < 0.05\%$

Groups of five to seven Crl:CD1(ICR) mice per sex were administered in two subsequent experiments single oral doses of 0 (vehicle control), 500, 1000, 1300, 1600 or 2000 mg/kg bw of IN-QEK31 to investigate its effect on micronucleus frequency in peripheral reticulocytes by flow cytometric analysis. Blood sampling was performed approximately 48 and 72 hours post dosing and 20 000 reticulocytes per blood sample were analyzed.

Mortality was observed at 1300 mg/kg bw and above. Ultimately analysis for micronuclei was performed only on animals receiving the negative (Tween 80 in aqueous methylcellulose) or positive (cyclophosphamide) control substances, and on those treated with 500, 1000, and 1300 mg/kg bw of the test substance. The administration of the metabolite caused a decrease in the percentage of reticulocytes among red blood cells in male mice compared to the vehicle control group, suggesting that the bone marrow had been reached and some depression of erythropoiesis occurred. This is not a genotoxic but a systemic effect. In females, however, this decrease was not noted. No increase in micronucleated

reticulocytes in peripheral blood of mice was seen after administration of IN-QEK31 up to the highest evaluated dose of 1300 mg/kg bw, neither in males nor in females. In contrast, cyclophosphamide administration (30 mg/kg bw) resulted in a strong decrease in reticulocytes in general, but in an increase in micronucleated reticulocytes. On balance IN-QEK31 proved negative in this in vivo micronucleus assay (Myhre, 2018b).

At the same time as the micronucleus assay of Chan (2016) was performed, an additional proof of exposure study was carried out in which four mice per sex were administered a single oral dose of IN-QEK31 at 500 mg/kg bw. Four hours post dosing the metabolite was detected by LC-MS/MS in quantifiable amounts of 46 100 and 38 200 ng/mL respectively in pooled plasma samples from male and female mice. This result was considered an additional indication of target tissue exposure in the micronucleus assay (Chan, 2016).

Short-term studies of toxicity

Groups of 10 male and 10 female Crl:CD(SD) rats were administered doses of 0, 150, 750, 3000 or 12 000 ppm of IN-QEK31 via their diet for 90 days and examined for a comprehensive range of parameters. The respective mean daily dietary intakes were calculated as 0, 9, 46, 183 and 784 mg/kg bw for males, 0, 10, 51, 204 and 820 mg/kg bw for females. All animals survived until scheduled termination and no clinical signs were observed. Adverse effects were confined, in both sexes, to the maximum dose level and comprised histopathological lesions of the urinary tract. Microscopic kidney changes included dilatation of renal tubules and renal pelvis, tubule degeneration and, in one female only, hyperplasia of the transitional epithelium. In general male rats tended to be more severely affected. In the urinary bladder, hyperplastic transitional epithelium was the predominant finding, It was observed in all males and a few females, along with inflammation and concrements in some male rats. In addition, terminal body weight and overall body weight gain were reduced in top-dose males by 10% to 15%, most likely related to a decrease in food efficiency of a similar magnitude, since food intake was not compromised. The upper intermediate dose of 3000 ppm (equal to 183 mg/kg bw per day) was the NOAEL in this study. The effects on the urinary tract were similar to those observed in a 90-day study in rats with the parent compound (MacKenzie, 2013) but the NOAEL in that study was higher, suggesting a lower potency for the metabolite (Hoban, 2016a).

Reproductive and developmental toxicity

In a combined systemic and reproductive toxicity screening assay, groups of 12 Crl:CD(SD) rats per sex were administered IN-QEK31 at dietary dose levels of 0, 150, 750, 3000 or 12 000 ppm. These doses were selected because of the results in the 90-day study reported above. It must be emphasized that this was in essence a one-generation study; the word “developmental” in its title could be misleading since examination of offspring was confined to viability and body weight until PND 4 , lacking investigations into, for example, skeletal or visceral anomalies or landmarks of sexual maturation.

The rats assigned to the parental generation were approximately 71 days old when dietary treatment was initiated. Following a premating period of two weeks under treatment, males and females were co-housed in pairs for up to two weeks within their respective dose groups to produce the F1 generation. Adult males were killed after siring a litter. Females were further observed during gestation, were allowed to deliver and rear their offspring until PND 4 when the dams and the pups were killed as well.

The parental animals were monitored for mortality, moribundity and occurrence of clinical signs. Body weight and food consumption were recorded weekly. At terminal sacrifice rats were subjected to gross pathological examination. Selected organs were weighed and, along with all gross lesions, retained for histopathology. Ovarian corpora lutea and uterine implantation sites were counted in all successfully mated females. Kidneys and urinary bladders were examined under the microscope in all animals from all groups because these were the major target organs. In males, coagulating glands, seminal vesicles and prostate from all groups were also included. Apart from that, full histopathological examination was confined to five animals per sex, from the control and top-dose groups only. Litters were examined at birth and on lactation day (LD) 4 for viability, clinical signs and individual pup body weights recorded. Following termination, pups underwent gross examination to the extent possible. Stomachs were, at least, assessed for milk spots apparently.

During the in-life phase of the study there were no deaths and no clinical signs that could be attributed to treatment. Body weight gain and food intake were reduced at the maximum dose level in females throughout the whole study, but only the differences in mean body weight and food consumption during the (brief) lactation period were statistically significant and these were accompanied by reduced food efficiency. In high-dose males a transient reduction in body weight gain (by 20%) was noted during the first week of treatment but normalized thereafter. Also, food efficiency was lower in that same week.

Pathology revealed a number of findings that were, however, all confined to the top dose level as well. Absolute and relative organ weights of adrenal glands (without morphological correlate) and of kidneys, were increased in male rats. In females lower heart, liver and spleen weights, most likely reflect lower terminal body weights whereas the lower thymus weight might indicate a stress response. Necropsy revealed kidney dilatation in three males and additionally, in one male only, kidney and bladder calculi, dilatation of the ureters, prostate mass and bladder haemorrhage, all pointing to the urinary tract as the major target of toxicity. Histopathology confirmed this evidence. A high incidence of renal tubular degeneration (partly also regeneration) was noted in high-dose males and the severity of this finding was greater when compared to that seen in other groups. These changes were also present, to a much less extent, in high-dose females. Pelvic and tubular dilatation seen in some high-dose males might also be attributed to treatment. The predominant microscopic kidney findings in females were hyperplasia of the transitional or tubular epithelium in the high-dose group (only occasionally noted in males) and leukocytosis of renal papilla. In the urinary bladder, common findings in males comprised hyperplastic transitional epithelium, mast cell infiltrates and chronic inflammation, but also concrements. Most of these findings were also present in females but at lower incidence. In some high dose females, but not in any other group, lymphoid depletion of the thymus was noted.

Based on organ weight increases in males, gross and histopathological findings in the kidneys and urinary tract in both sexes and changes in body weight and nutritional parameters, the parental NOAEL was 3000 ppm (corresponding to a mean daily intakes of 228 mg/kg bw for males and 212–316 mg/kg bw for females depending on the reproductive stages in this study). For risk assessment purposes, the lowest figure of 212 mg/kg bw should be used as the NOAEL.

All mated females became pregnant and delivered viable offspring. Reproductive performance and reproductive indices were not affected. Sex ratio in pups was not skewed. The only significant reproductive finding was an increase in post-implantation loss in the high-dose group (9.2%) compared to the controls (1.7%) and the other treated groups (5.2%, 3.3% and 4.5%, respectively). This finding might have resulted in the lower mean litter size seen in the high-dose group (13.3 compared to 14.3 in the control group) even though the difference was not significant. On the other hand, pre-implantation loss was much higher in the control group (8.6%) than in the high-dose group (1.8%) demonstrating the high variability of this parameter. Moreover, and in line with the assumptions on variability, the relatively high incidence of post-implantation losses at the top dose was still within the HCD range of the performing laboratory (1.2%–19.9%) and not very much above its mean (7.8%). On balance, the reproductive NOAEL in this study was the highest dose level of 12 000 ppm (corresponding to a mean daily intake of 812 mg/kg bw as calculated for the dams during gestation).

Pup viability and survival until PND 4 were not affected in any dose group. The offspring NOAEL of 3000 ppm (equal to 212 mg/kg bw per day) was based on a significantly lower mean pup body weight (sexes combined) at the top dose level. In two high-dose pups and one from the 750 ppm group, there was evidence of inadequate nursing since milk spots in stomach were missing (Lewis, 2017).

In a two-generation study in Crl:CD(SD) rats, IN-QEK31 was administered at dietary doses of 0, 800, 2000 or 10 000 ppm. During lactation of F0 and F1 dams and for the first three weeks after weaning in F1 offspring these dose levels were reduced to 0, 480, 1200 or 6000 ppm, to ensure a comparable mean daily intake over the entire course of the study. Thirty animals per sex and dose were included to produce one litter per generation. The study design was very similar to that of the multigeneration study with parent fluazaindolizine (Munley, 2020) that is reported in detail in section 2.5(a). However, in addition, urine samples were taken during the study with IN-QEK31 for determination of downstream metabolites. The results of this analysis were reported by Swain & Ryan (2018, see above) but are of no relevance for assessment of reproductive toxicity.

Typical in studies of this type, the actual mean daily intake differed greatly between sexes, generations and reproductive phases. For risk assessment, the lowest calculated intake figures should be used and these were consistently the doses as received by F0 generation males in the pre-mating period. Rounded mean intakes of 0, 48, 117, and 596 mg/kg bw per day were calculated for the low-, mid- and high-dose groups of such males, and it is these that are relevant to risk assessment.

No unscheduled deaths or clinical signs were attributed to treatment. Indeed, the cause of death could not be determined in two females (F0) and one male (F1) receiving the highest dose, but the same was true also for one male and one female from the control group. Mean body weight in high-dose F0 generation males was slightly but significantly lower (by 4% to 7%) than in the control group. This difference was apparently due to lower body weight gain (by 12%–16%) during the first three weeks of treatment and over the whole premating period of 71 days (by ca 11%) but persisted then throughout most of the remainder of the study. In contrast, no adverse effects on body weight or its gain were observed in the F1 generation males or more generally in females. Likewise, food consumption and efficiency were not affected in any consistent way in these animals, in addition to which the occasional differences seen in these groups were not large enough to explain body weight effects in high-dose F0 males.

Gross changes at necropsy comprised calculi in the kidney and urinary bladder, at least in high-dose males, as well as dilation of renal pelvis the incidence of which was increased in all treated groups. Upon microscopic examination the incidence and severity of chronic progressive nephropathy and pelvis dilation were seen to increase in a dose-related manner, from the lowest dose level of 800 ppm upwards (Table 39). It is remarkable that kidneys were more frequently affected in F1 males than in the parental generation, but it must be also acknowledged that the duration of treatment was longer when potential exposure in utero and during lactation are included. Also, histopathological examination of the F1 generation was more comprehensive. However, the human relevance of chronic progressive nephropathy in rats is generally considered low. Also, it was mainly of minimal severity, there was no dose–response relationship for this finding (similarly for pelvic dilatation) at the low- and mid-dose levels, and statistical significance was apparently not achieved. Therefore, setting this nephropathy aside, the parental NOAEL of 2000 ppm (equal to 117 mg/kg bw per day) was based on hyperplasia of transitional epithelium of the bladder in high-dose F1 males, supported by the very frequent occurrence of pelvic dilation in this group. It must be emphasized that the effects resembled very much those noted in the two-generation study with the fluazaindolizine parent (Munley, 2020; see above).

Table 39. Incidence and severity of histopathological kidney and urinary bladder findings in F0 and F1 males in the two-generation study with IN-QEK31 in rats; critical findings in bold

	Generation and dietary dose (ppm)							
	F0 generation				F1 generation			
Organ/finding	**0**	**800**	**2000**	**10 000**	**0**	**800**	**2000**	**10 000**
Kidney								
Number examined	30	1	0	30	30	30	30	30
Chronic progressive nephropathy (CPN):								
total	7	0	-	**11**	7	11	11	**21**
minimal	7	0	-	9	7	9	11	**12**
mild	0	0	-	**2**	0	2	0	**7**
moderate	0	0	-	0	0	0	0	**2**
Dilatation of renal pelvis, unilateral and bilateral) total	2	1	-	2	7	13	12	**18**
Urinary bladder	Not examined				30	30	30	30
Hyperplasia of transitional ephithelium, total					0	0	1	**25**
minimal					0	0	0	**8**
mild					0	0	1	**16**
moderate					0	0	0	**1**

Source: Munley, 2019

Fertility and reproductive performance were not affected at any dose level in either the F0 or F1 generation, nor were sperm or estrous cycle parameters or ovarian follicle counts. The reproductive NOAEL was therefore the highest tested dose of 10 000 ppm, corresponding to a mean daily intake of 596 mg/kg bw.

No clinical signs, effects on body weight or body weight gain, or on organ weights were seen, and for the pups no between-group differences in the time to reach the landmarks of sexual maturity was apparent. However, since the pathological findings in the urinary tract in adult F1 males were more pronounced than in F0 generation males, the idea cannot be excluded that prenatal or lactational exposure, along with dietary intake after weaning, might have contributed to the higher incidence and severity in the F1 generation. Based on these considerations the NOAEL for offspring toxicity should be the same as the parental NOAEL, that is 2000 ppm (equal to 117 mg/kg bw per day) (Munley, 2019).

A developmental toxicity study was run in Crl:CD(SD) rats. Metabolite IN-QEK31 was dissolved in 0.5% aqueous methylcellulose containing 0.1% Tween 80 and administered by oral gavage to groups of 22 time-mated dams from GD 6 to GD 20 at dose levels of 0, 37, 110 or 330 mg/kg bw. These dosages had been selected on the basis of a preceding range-finding experiment in which mortality occurred among dams receiving the limit dose of 1000 mg/kg bw per day. This observation agrees entirely with the LD_{50} estimate in the acute oral toxicity study reported above. Significant reductions in body weight and food consumption were observed at 500 mg/kg bw per day. Accordingly, dose selection for the main study seems reasonable. The dams were killed on GD 21 and all maternal and fetal examinations as specified in OECD test guideline 414 were performed.

No unscheduled deaths and no clinical signs of toxicity were observed. Body weight gain was lower by 7% in the high-dose dams than the control group, but this difference was not statistically significant and did not result in a meaningful reduction in final body weight. A marginal decrease in food consumption was observed in the same group towards the end of the treatment period. Because of small size, these nutritional effects were not considered adverse. There were no gross postmortem findings in the dams and reproductive and litter parameters were unaltered. Accordingly, the highest dose of 330 mg/kg bw per day was the maternal NOAEL in this study.

The only fetal effect that may be attributed to treatment was a 4.7% reduction in mean fetal weight (sexes combined) in the high-dose group compared with controls. Since this difference was small and not accompanied by any increase in malformations or variations it was not considered adverse and the developmental NOAEL was also 330 mg/kg bw per day (Munley, 2018a)

(b) Metabolite IN-F4106: 2-Chloro-5-methoxybenzenesulfonamide

This metabolite was found in crops, livestock, water and soil but also in rodents. It is a hydrolysis product of fluazaindolizine and contains only the phenyl ring with a chlorine atom and additional functional groups (see Fig. 2). According to Munley (2017c) it is also a process intermediate in the synthesis of fluazaindolizine. In the rat, IN-F4106 was one of the few metabolites accounting for more than 1% of AD in excreta. More remarkably, it was the main metabolite in plasma, liver and kidney, representing 2.5%–3.1% of TRR in plasma, and 17.4%–26.5% of TRR in liver and kidney (Punler & Green, 2017). In the mouse, this metabolite was found only in traces in cagewash (Punler & Green, 2016b). To a large extent, IN-F4106 is further metabolized to metabolite IN-A5760 (see below) as shown in the rat by Himmelstein (2018a), and this may explain the low concentrations observed.

In routine toxicological studies, IN-F4106 was found in plasma in low concentrations similar to those of IN-QEK31 (see section 1.3). In vitro, it was detected following incubation of human, rat, and mouse hepatocytes with phenyl-labelled fluazaindolizine, present in a similar amounts to IN-QEK31 of around 3% of AD (Novo, 2018).

In the same way as IN-QEK31, metabolite IN-F106 was examined for various toxicological end-points in a sufficient number of studies to enable a separate risk assessment. These studies are summarized below in Table 40 and then briefly reported.

Table 40. Fluazaindolizine metabolite IN-F4106: overview of the available toxicological and genotoxicity studies

End-point, study type, test system	Information and remarks on study design	Results	Reference
Acute oral toxicity, rat	Up-and-down procedure was followed	LD_{50} > 5000 mg/kg bw but mortality was observed at this dose; 100% survival at 1750 mg/kg bw and below	Merrill, 2016b
Acute dermal toxicity, rat	Limit test	LD_{50} > 5000 mg/kg bw, no systemic toxicity, transient erythema in one male	Merrill, 2014b
Acute inhalation toxicity,	Sighting study and limit test at same single concentration, 4-hour nose-only exposure to aerosol	LC_{50} < 5 mg/L, no mortality and no clinical signs but transient body weight losses	Rajsekhar, 2014
Skin irritation, rabbit		Not irritant	Merrill, 2015c
Eye irritation, rabbit		Very mild and transient irritation not resulting in classification	Merrill, 2015d
Skin sensitization, Guinea pig	Magnusson & Kligman maximization test	Not sensitizing	Merrill, 2015e
Mutagenicity in bacteria	Ames test in four strains of *S. typhimurium* and one *E. coli* strain, ± metabolic activation	Negative	Myhre, 2015a
Mutagenicity in mammalian cells	HPRT assay in CHO cells, ± metabolic activation	Negative	Dutta, 2015
Chromosome aberration in vitro	Cytogenetic study in human peripheral lymphocytes, ± metabolic activation	Positive; structural aberrations with and without S9 mix after four-hour exposure	Kellum, 2015
Genotoxicity in vivo	Mouse micronucleus assay with single oral administration of up to 2000 mg/kg bw	Negative	Myhre, 2015b
Short-term toxicity, rat	14-day feeding study, dietary dose levels from 250 to 7500 ppm	NOAEL 800 ppm (c 70 mg/kg bw/day) based on lower body weight gain and on effects on liver and (males) urinary bladder, anestrus in high-dose females	Hoban, 2015
Short-term toxicity, rat	90-day feeding study, dietary dose levels from 50 to 2500 ppm	NOAEL 625 ppm (c 36 mg/kg bw/day), based on lower body weight (gain) and on histopathology (liver, urinary bladder, and perhaps thyroid)	Hoban, 2016b
Reproductive toxicity, rat	One-generation study with comprehensive evaluation of parental animals but limited evaluation of pups; dietary dose levels 0, 50, 150, 625 and 2500 ppm	Parental and offspring NOAEL 625 ppm (equal to 43 mg/kg bw/day), based on lower body weight and nutritional parameters in adults and pups and on higher organ weights of liver and kidney in adults; no evidence of reproductive toxicity up to highest dose (equal to 168 mg/kg bw/day)	Munley, 2017c
Reproductive toxicity, rat	Two-generation study with one litter per generation, dietary dose levels 0, 50, 150, 625 and 2000 ppm	Parental and offspring NOAEL 625 ppm (equal to 35 mg/kg bw/day), based on lower body weight in adults and weanlings; no evidence of reproductive toxicity up to highest dose of 2000 ppm (equal to 111 mg/kg bw/day)	Lewis, 2018

End-point, study type, test system	Information and remarks on study design	Results	Reference
Developmental toxicity, rat	Dose levels 0, 22, 67 and 200 mg/kg bw per day; administration from GD 6 to GD 20	Maternal NOAEL 22 mg/kg bw/day, based on effects on body weight (gain) and food intake; Developmental NOAEL 67 mg/kg bw/day based on reduced fetal weight and (non-significant) increase in short supernumerary ribs	Munley, 2018b

Acute toxicity

The acute oral toxicity of IN-F4106 was investigated in female Sprague Dawley-derived rats by means of an up-and-down procedure. In the first step, the limit dose of 5000 mg/kg bw was administered by oral gavage to a single animal that died on day 2 post dosing having exhibited hypoactivity, hunched posture, irregular breathing and reduced faecal volume. Gross necropsy revealed distension of the stomach and intestines. Because of this death, in the main test, one female rat each received a dose of 175, 550 or 1750 mg/kg bw, and three additional animals were administered the maximum dose of 5000 mg/kg bw. Treatment was performed in a stepwise manner with the decision to proceed with the next animal based on the survival of the previous one that had received the lower or same dose. In this experiment, all rats survived until the end of the 14-day postobervation period. Clinical signs as described above were observed in rats receiving 1750 or 5000 mg/kg bw, but disappeared within two days. One high dose female lost some weight during the first week. No gross abnormalities were noted in any of the surviving rats. On balance, the LD_{50} in this study was greater than 5000 mg/kg bw since three out of four animals survived this dose (Merrill, 2016b).

The acute dermal toxicity of this metabolite was tested in five male and five female Sprague Dawley-derived rats at the limit dose of 5000 mg/kg bw. All animals survived and there were no findings that would suggest systemic toxicity. In one male, erythema was noted on the application site on day 1. The acute dermal LD_{50} was greater than 5000 mg/kg bw (Merrill, 2014b).

The low acute toxicity of inhalation for IN-F4106 was demonstrated in male and female Wistar rats which were apparently exposed in two subsequent limit tests (the first originally intended to serve as what was described as a "sighting study") for four hours (nose only) to a mean actual aerosol concentration of 5 mg/L. In each experiment, three male and three female rats were employed. The nominal concentrations in the first trial was 8.75 mg/L (MMAD 2.35 µm) and in the second 9.2 mg/L (MMAD 2.96 µm). No mortality and no clinical signs of toxicity were observed. All animals lost weight but mostly only on day 1 following exposure, gaining weight later. Necropsy revealed no gross lesions. The LC_{50} was therefore greater than 5 mg/L and metabolite IN-F4106 can be considered of low inhalation toxicity (Rajsekhar, 2014).

Metabolite IN-F4106 proved negative in a skin irritation study in three female New Zealand White rabbits. All animals appeared active and healthy and gained weight over the course of the study. No dermal irritation was observed at any of the treated sites on the body (Merrill, 2015c).

Metabolite IN-F4106 was tested for eye irritation in three female New Zealand White rabbits. The test was performed, in a stepwise fashion starting with one animal. Under ocular anaesthesia 0.1 mL (c 66 mg) of the test substance was instilled into the conjunctival sac of the right eye. The left eye remained untreated and served as control. All animals appeared active and healthy and gained weight over the course of the study. Signs of eye irritation were mild. One hour after instillation, conjunctival redness of low severity (score 1 or 2) was observed in the treated eyes of all animals, slight iritis (score 1) was noted in two rabbits, and chemosis (score 1) in just one animal. All signs of irritation disappeared within 48 hours of instillation. To conclude, IN-F4106 caused only transient and very mild eye irritation that would not result in a need for classification according to any known regulatory system (Merrill, 2015d).

Metabolite IN-F4106 was tested for skin sensitizing potential in 20 male Hartley strain Guinea pigs according to the method of Magnusson & Kligman, (by intradermal and topical induction, followed by topical challenge). For challenge, the animals were treated with both a 75% (w/w) and a 25% (w/w)

mixture of the test substance in propylene glycol. Previously, primary irritation testing had been performed in 13 animals to select the appropriate concentrations for the different steps. Ten additional animals were used as the vehicle control group. A concurrent positive control group was not included but appropriate historical control data was provided with the report. Very faint erythema was noted in one animal at the 75% mixture application site at 24 hours following challenge. This minimal irritation sign then disappeared. It may be concluded that metabolite IN-F4106 is devoid of skin sensitizing potential (Merrill, 2015e).

Genotoxicity

An Ames test using the plate-incorporation method was performed with *S. typhimurium* strains TA98, TA100, TA1535 and TA1537, as well as in *E. coli* strain WP2 *uvr*A. Based on the results of a preceding toxicity-mutation test, IN-F4106 was applied to all tester strains at concentrations ranging from 333 to 5000 µg/plate in the presence and absence of S9 metabolic activation. No increase in revertant colonies over the vehicle (DMSO) control values was observed with any strain. Appropriate positive control substances gave the expected clear responses. No precipitation was seen, but TA1537 caused more than 50% reduction in revertant colonies at the maximum concentration with metabolic activation. IN-F4106 proved negative for bacterial mutagenicity under the conditions of this study (Myhre, 2015a).

Metabolite IN-F4106 was tested in CHO cells for its ability to cause forward gene mutation at the hypoxanthine–guanine phosphoribosyl transferase locus (HPRT assay). The test substance was dissolved in DMSO. The experiments were performed at concentrations of up to 2200 µg/mL, both in the presence and absence of metabolically activating S9 mix. No increase in mutant frequency was observed with the metabolite, while the positive control substances induced just such a response. Some precipitation was observed at the highest concentration but survival was sufficient (c 51% with and 86% without S9 mix) for evaluation, suggesting that the test compound was not strongly cytotoxic. In this in vitro assay, metabolite IN-F4106 proved negative for mutagenicity towards mammalian cells (Dutta, 2015).

In an in vitro chromosome aberration study in cultured human peripheral lymphocytes (obtained from a healthy male subject 32 years old), IN-F4106 was applied for four hours and for 22 hours in the absence of metabolic activation, or for four hours in the presence of S9 mix. A wide range of concentrations (0, 5, 10, 100, 250, 500, 750, 1250, 1500 and 1750 up to the limit of 2000 µg/mL) was tested, both with and without metabolic activation. However, based on precipitation and cytotoxicity which both differed greatly (particularly with regard to length of exposure), the concentrations selected for microscopic evaluation of at least 300 metaphase cells (150 from each duplicate culture) were as follows: 0, 500, 1000 and 1750 µg/ml for cytogenetic evaluation following four-hour exposure with and without activation, and 0, 30, 100 and 250 µg/mL for long-term exposure. Appropriate positive control substances were employed.

A statistically significant increase in structural chromosome aberrations was noted after a four-hour treatment period both with and without metabolic activation at 1750 µg/mL. Also, there was a positive trend with dose (see Table 41). In contrast, no such increase was seen following the 22-hour exposure, and numerical chromosome aberrations were not increased at any concentration, with or without activation. Nevertheless, on balance, IN-F4106 must be considered positive in this assay (Kellum, 2015)

Groups of five to seven Crl:CD1(ICR) mice per sex were administered single oral doses of IN-F4106 at concentrations of 0 (vehicle control; 0.1% Tween 80 in 0.5% aqueous methylcellulose), 500, 1000 or 2000 mg/kg bw to investigate micronucleus frequency in peripheral reticulocytes by flow cytometric analysis. A positive control group of five animals per sex received 30 mg/kg bw of cyclophosphamide. Blood sampling was performed approximately 48 and 72 hours post dose and 20 000 reticulocytes per blood sample were analyzed.

There were signs of neither mortality nor clinical toxicity in this study. Body weight and body weight gain were not affected apart from an initial, very small weight loss in males receiving the two upper doses and in females given the highest dose. In high-dose males and females this minimal loss was followed on the second day by a weight gain that was clearly higher than in the controls. The administration of the highest dose of 2000 mg/kg bw caused a depression in the percentage of

reticulocytes among red blood cells in male mice compared with the vehicle control group, suggesting that bone marrow had been reached. In females, however, such an effect was not noted. No increase in micronucleated reticulocytes in the peripheral blood of mice was seen after administration of IN-F4106 at any dose level, neither in males nor females. Cyclophosphamide administration (30 mg/kg bw)in both sexes resulted in a large decrease in reticulocytes in general, but also an increase in micronucleated reticulocytes. It may be concluded that IN-F4106 was negative in this micronucleus assay and that the positive findings in the in vitro chromosome aberration study (Kellum, 2015) could not be reproduced in vivo (Myhre, 2015b).

Short-term studies of toxicity

In a two-week feeding study in Crl:CD(SD) rats (five per sex and dose), IN-F4106 was administered at dietary concentrations of 0, 250, 800, 2500 or 7500 ppm (equal to mean daily intakes of 0, 22, 72, 216 and 561 mg/kg bw per day for males, 0, 22, 70, 203 and 546 mg/kg bw per day for females). All rats survived until scheduled termination and no clinical signs of toxicity were observed. However, body weight gain and food consumption were depressed at the two upper dose levels. In the male and female groups receiving 7500 ppm, final body weight was significantly reduced compared to controls, by 20% and 19% respectively. Food efficiency was also compromised. Haematology, blood and urine clinical chemistry did not reveal adverse effects that could be attributed to treatment, but mean liver weights were increased in males at 2500 and 7500 ppm and in high-dose females. This liver weight increase was accompanied by dark discolouration of the liver at the top dose, and by hepatocellular hypertrophy at 800 ppm and above in males and at 2500 ppm in females. Incidence and severity at 800 ppm in males and at 2500 ppm in females were very low and cannot be considered adverse, whereas the effect was more pronounced at higher doses. In addition in males, a higher incidence and increasing severity of mucosal hyperplasia of the urinary bladder was observed in the groups receiving 2500 and 7500 ppm. In females, anestrus was noted in the two high-dose animals. This finding could be due to lower body weight but a direct, and perhaps hormone-mediated effect of treatment cannot be excluded. This last effect was not confirmed in studies of longer duration and thus it is either an artefact or results from the administration of a very high dose. The NOAEL in this study was 800 ppm, corresponding to a mean daily dose of 70 mg/kg bw per day (Hoban, 2015).

Groups of young adult Crl:CD(SD) rats (10 per sex and dose), were fed IN-F4106 at dietary concentrations of 0, 50, 150, 625 or 2500 ppm for approximately 90 days. These nominal dietary levels were equal to mean daily intakes of 0, 2.8, 8.5, 36, and 149 mg/kg bw per day for males, 0, 3.3, 10, 42, and 165 mg/kg bw per day for females. All rats survived until scheduled termination and no clinical signs of toxicity were observed at any dose level. Neurobehavioural (abbreviated FOB and measurements of motor activity) and opthalmoscopic examinations did not reveal any findings that could be attributed to treatment. Administration of the maximum dose resulted in lower body weight gain in both sexes compared to controls (by 21% and 19% in males and females respectively) and in a reduction in final body weight (–13%) in males. These effects could be, at least partly explained, by significantly decreased food efficiency in both males and females at the top dose since mean food intake was not affected. Haematology, blood and urine clinical chemistry did not reveal adverse effects at any dose. The most prominent histopathological finding was hyperplasia of transitional epithelium of the bladder mucosa in five out of ten high-dose males. In females the same finding was confined to a single animal. On balance this effect was considered adverse and treatment-related. In addition, liver cell hypertrophy was observed in males at the two upper dose levels and in high-dose females; it was accompanied by a higher liver weight at 2500 ppm in males (by 21% against controls) and females (by 18%). In males thyroid hypertrophy was seen in four high-dose animals compared to only two control males. The relevance of this finding and its relation to treatment are equivocal. On one hand, it could be secondary to liver toxicity, but on the other the incidence of this finding in male rats is known to be highly variable. Since hepatocellular hypertrophy as an isolated finding is considered adaptive rather than adverse, the upper intermediate dose of 625 ppm (equal to 36 mg/kg bw per day) was the NOAEL in this study, and this would also cover a possible thyroid effect. It is worth noting that plasma and urine samples were taken around day 70 to investigate the kinetics and metabolism of IN-F4160. This separate study (Himmelstein, 2018a) is reported below in the section on metabolite IN-A5760 (Hoban, 2016b).

Multigeneration and developmental studies

In a combined systemic and reproductive toxicity screening assay, groups of 12 Crl:CD(SD) rats per sex were administered IN-F4106 at dietary dose levels of 0, 50, 150, 625 or 2500 ppm; the same as applied in the 90-day study by Hoban (2016b). The study design was virtually identical to the one described in detail above for a similar very study with IN-QEK31 (Lewis, 2017), and is therefore not repeated here.

Mean daily intakes of the test substance differed with regard to sex and reproductive phase. The lowest values were consistently calculated in this study for females during gestation and are given here to support risk assessment. In the various groups, the nominal dose levels mentioned above corresponded to mean daily intakes of 0, 3.3, 10.5, 43 and 168 mg/kg bw per day.

During the in-life phase of the study there were no deaths and no clinical signs that could be attributed to treatment. Body weight and body weight gain were reduced in high-dose males by more than 10% and 20% respectively; effects at 150 and 625 ppm were less severe and, even though probably treatment-related, not regarded as adverse (see Table 42). In high-dose females, body weight and body weight gain were also reduced throughout the study even though the differences were not always statistically significant. The most outstanding finding was a large reduction in body weight gain during the very short lactation period (PNDs 0–4 only). Adverse effects on body weight were accompanied in high-dose males by a lower food intake and decreased food efficiency. Similar findings in high-dose females were less severe and confined to lower food consumption during the pre-mating period, and reduced food efficiency during lactation.

Table 42. Mean body weight and body weight gain in male rats (F0 generation) fed IN-F4106

	Dose level (ppm)				
Parameter	**0 (control)**	**50**	**150**	**625**	**2500**
Body weight (g)					
Day 1	294.2	295.2	292.3	292.2	290.0
Day 7	342.1	346.3	335.7	333.0	318.6*
Day 15	391.0	398.3	385.8	380.6	364.2*
Day 22	416.4	418.4	405.5	398.2	383.5*
Day 29	450.3	452.4	433.1	426.9	413.4*
Day 36	482.2	487.0	463.7	456.2	436.2*
Body weight gain (g)					
Days 1–7	47.9	51.1	43.5	40.8*	27.7*
Days 7–15	48.8	52.0	50.1	47.7	45.6
Days 15–22	25.4	20.2	19.7	17.5	19.2
Days 22–29	34.0	33.9	27.7	28.7	30.0
Days 29–36	31.9	34.6	30.6	29.3	22.7
Total gain days 1–36	187.9	191.8	171.4	164.1*	145.2*

* Statistically significant using ANOVA and Dunnett's test at $p < 0.05$; Source: Munley, 2017c

Pathology revealed a higher relative organ weight for the liver in high-dose males (by 16.5% compared to the control mean) and females, and of the kidneys in the high-dose female group (by 14% against controls). There were no remarkable gross lesions but the higher liver weight was accompanied by minimal or mild hepatocellular hypertrophy in all male rats and in nine out of 12 females receiving 2500 ppm and in four males at the next lower dose level of 625 ppm. The latter isolated finding is not usually considered adverse but rather adaptive. No microscopic kidney findings were seen.

Based on effects on body weight, body weight gain, food consumption and food efficiency ,and supported by the higher weights of liver and kidney, partly with a morphological correlate, the parental NOAEL in this study was 625 ppm, equal to a mean daily intake of 43 mg/kg bw.

There were no adverse effects on fertility or reproductive performance up to the highest dose of 2500 ppm (equal to a mean daily intake of 168 mg/kg bw) which therefore is the reproductive NOAEL in this study.

Pup viability and survival until PND 4 were not affected in any dose group. Pathological evaluation of the pups on day 4 was apparently not performed. The offspring NOAEL of 625 ppm (equal to 43 mg/kg bw per day) was based on the significantly lower mean body weight of pups at the top dose. Mean birth weight was by 8% lower than in the control group, and by PND 4 this difference had risen to 14%, suggesting a lower body weight gain. All these findings were in line with the altered body weight, body weight gain and lower food efficiency in dams, particularly during lactation (Munley, 2017c).

In a two-generation study in Crl:CD(SD) rats, with one litter per generation, groups of 30 males and 30 females received IN-F4106 via their diet at nominal dose levels of 0, 50, 150, 625 or 2000 ppm. During lactation in F0 and F1 dams, and up to PND 42 in F1 offspring, these dose levels were reduced to 0, 30, 90, 375 and 1500 ppm to maintain a comparable mean daily intake over the entire course of the study. Typically for studies of this type, the actual mean daily intake differed greatly with regard to sex, generation and reproductive phase. For risk assessment, the lowest calculated intake figures should be used and these were consistently the doses received by F0 generation males in the premating period. For these F0 male groups rounded mean intakes of 0, 2.8, 8.3, 35 and 111 mg/kg bw per day were calculated for the low-, low intermediate-, high intermediate-, and high-dose groups respectively. The study design was similar to that in the multigeneration study with parent fluazaindolizine (Munley, 2020) that is reported in detail in section 2.5(a). It must be emphasized, however, that histopathological evaluation in the metabolite study was limited to reproductive organs and gross lesions.

No unscheduled deaths or clinical signs were attributed to treatment, but body weight and to some extent food consumption were affected in F0 and F1 parental animals receiving the highest dose. In males, body weight gain in the top-dose F0 generation was reduced by 15%, compared to the control group, resulting in lower final mean body weight by up to 9% compared to controls . In F1 males, similar reductions by 12% in body weight and 11% in weight gain, were noted. In F0 generation females the strongest effect was seen in the premating period during which the mean body weight gain was lower than controls by 22%, resulting in a mean body weight that was lower by 6%. During gestation and lactation the magnitude of difference was less. In the F1 generation, the most pronounced effect on body weight gain (17% below controls) and final body weight (up to 12%less than controls) was observed during gestation, whereas the impact on these parameters during the premating period and lactation was less severe. A reduction to some extent in food consumption was confined to F1 generation males and females (up to 13%) whereas a slight decrease in food efficiency was observed in F0 generation males.

In apparent agreement with the effects on nutritional and body weight parameters in the adult rats, mean body weights were reduced by 7% and 5% respectively in F1 and F2 litters at weaning on PND 21. These difference from the control group values were slight but statistically significant. By contrast, birth weight was not affected. Reproductive parameters and indices were not altered at any dose level, the study covering sperm and estrous parameters, ovarian follicle count in F1 dams and landmarks of sexual maturity in F1 offspring of both sexes.

Pathology revealed no remarkable gross findings that could be attributed to treatment. In contrast, compared with controls, higher relative liver weights (by up to 16% in F0 generation males) and kidney weight (maximum 11% higher than controls in F1 males) were noted in F0 and F1 adults of both sexes at the top dose. An increased relative liver weight (by 8% compared to controls) was also observed in F0 generation males at 625 ppm. According to the study author, these organ weight changes might reflect enzyme induction in both organs and should not be considered indications of an adverse effect. However, in the absence of histopathological examinations and clinical chemistry, this hypothesis remains unproven.

There was no effect on litter size, sex ratio in pups or pup survival and no gross pathological or organ weight changes were noted in F1 or F2 weanlings. On balance, under conditions of this study, the NOAEL for parental toxicity was 625 ppm (equal to 35 mg/kg bw per day) based on lower body weight and food intake (the weight increases in liver and kidney were at best equivocal). The same NOAEL was established for offspring toxicity, based on a lower mean pup weight at weaning in both generations seen at the maximum dose level of 2000 ppm. This highest tested dose of 111 mg/kg bw per day can be considered the NOAEL for reproductive toxicity since no adverse effects on fertility, reproductive performance or any other related parameter were observed at any dose level (Lewis, 2018).

A developmental toxicity study was run in Crl:CD(SD) rats. Metabolite IN-F4106 was dissolved

in 0.5% aqueous methylcellulose containing 0.1% Tween 80 and administered by oral gavage to groups of 22 time-mated dams from GD 6 to GD 20 at dose levels of 0, 22, 67 or 200 mg/kg bw. These dosages had been selected on the basis of a preceding range-finding experiment in which maternal body weight and food consumption were severely affected and salivation occurred at the top dose level of 400 mg/kg bw per day. In the main study, dams were killed on GD 21 and all maternal and fetal examinations, as specified in OECD TG 414, were performed.

All dams survived until scheduled termination and no clinical signs of toxicity were observed. In the high-dose group, mean body weight gain from GDs 6 to 8 was lower by 64% than in the control group, resulting in a significantly lower body weight on GDs 7 and 8. Thereafter body weight gain tended to normalize, but was occasionally reduced again. Body weight remained lower in high-dose dams for the rest of the study even though the difference from the control group was only statistically significant again from GDs 17 to 20. A significant reduction in body weight gain was also apparent in mid- and low-dose dams on the first three days of treatment, but further weight development was not affected at the low dose and the data obtained for the group receiving 67 mg/kg bw per day were equivocal. In the mid-dose group total body weight gain over the treatment period was low, similar to what was seen in the high-dose group (see Table 43). However, the initially lower body weight gain and low and transient reductions in this parameter at mid-dose level as the study progressed, did not result in a significantly lower absolute body weight in these groups compared with controls.

Table 43. Mean body weight gain and mean fetal weight in the rat developmental study with IN-F4106

	Dose (mg/kg bw per day)			
Parameter: time period	**0 (control)**	**22**	**67**	**200**
Body weight gain (g/rat):				
GDs 6–8	11.3	8.6*	7.5*	4.1**
GDs 8–10	11.1	13.6	12.7	13.4
GDs 10–12	13.2	13.2	13.3	11.9
GDs 12–14	11.5	12.1	9.7	9.8
GDs 14–16	17.0	16.0	17.2	16.5
GDs 16–18	31.9	34.4	27.8*	27.5*
GDs 18–20	30.9	33.9	28.7	28.0
GDs 20–21	18.2	17.7	18.2	20.8
Total GDs 6–21	145.0	149.3	135.1	132.0
Fetal weight (g/fetus)	5.91	5.81	5.61*	5.34**

GD: Gestation day; Source: Munley, 2018b

Statistically significant using Dunnett's test, two-tailed test: * $p < 0.05$, ** $p < 0.01$

Lower body weight gain on the first three days of treatment was paralleled by a significantly lower food consumption in high- and mid-dose dams. For the rest of the study, food intake in the high-dose group remained lower than in the controls and the difference was statistically significant from GDs 14 to 20, as well as being lower for the whole treatment period. Occasional reductions in food consumption were also noted in mid-dose females, and these achieved statistical significance from GD 18 to GD 20. At the low dose, food intake was similar to that in the controls (see Table 44). There were no grossly visible postmortem findings in the dams, and reproductive and litter parameters were not altered.

Table 44. Mean maternal food intake (g/rat per day) in the rat developmental study with IN-F4106

	Dose (mg/kg bw per day)			
Time period	**0 (control)**	**22**	**67**	**200**
Gestation days 6–8	22.1	21.4	20.5*	16.0*
8–10	22.2	23.1	22.0	21.5
10–12	22.3	23.2	23.2	21.5

		Dose (mg/kg bw per day)			
Time period		**0 (control)**	**22**	**67**	**200**
	12–14	24.0	24.2	23.6	22.8
	14–16	24.7	25.4	24.1	22.9*
	16–18	28.0	28.0	26.7	24.6*
	18–20	27.7	27.3	25.7*	24.9*
	20–21	25.8	26.6	24.9	24.8
Total	GDs 6–21	24.5	24.8	23.8	22.2*

* Statistically significant using ANOVA and Dunnett's test at $p < 0.05$; Source: Munley, 2018b

On balance, the intermediate dose of 67 mg/kg bw per day was the maternal NOAEL in this study, based on large adverse effects on body weight gain and food consumption at the highest dose. Effects were most pronounced in the initial phase of treatment, suggesting an acute effect. At the low- and mid-dose levels effects were considered too minor and transient to constitute adversity.

The only fetal effect that was presumed to be treatment-related was a significant reduction in mean fetal weight (sexes combined) in the mid- and high-dose groups. Since the difference at the mid dose was small and not accompanied by an increase in fetal malformations or variations, only the highly significant decrease in fetal weight at 200 mg/kg bw per day was considered adverse, corresponding with the lower body weight of the dams. There was in addition, at this high dose level, an increase in short supernumerary ribs, that is, a skeletal variation. Both the fetal (6.6% compared to 2.9% in controls) and the litter incidence (35% compared to 19% in controls) were higher, but although a vague dose response was perhaps visible, statistical significance was not achieved. The developmental NOAEL was 67 mg/kg bw per day (Munley, 2018b).

(c) Metabolite IN-A5760; 2-Chloro-5-hydroxybenzenesulfonamide

This is also an animal metabolite representing up to 5.4% of AD in the excreta of female mice (Punler & Green, 2016b). The metabolite can be formed via two different biochemical pathways and is itself subject to subsequent conjugation (see Fig. 2 and Himmelstein, 2018a). Its sulfate conjugate is a significant urinary metabolite in the rat, accounting for up to 7% of AD (Punler & Green, 2017), but is seen at much lower levels (up to 0.6% of AD) in the mouse (Punler & Green, 2016b). Plasma concentrations of IN-A5760 in routine toxicological studies (see for example Tables 12 and 13) were extremely low, which agrees completely with its being an intermediate. In addition to its occurrence in animal metabolism, IN-A5760 was found in treated crops, livestock, water/sediments and soil.

Metabolite IN-A5760 is a downstream metabolite of IN-F4106, formed by *O*-demethylation, at least this is so in the rat. Plasma and urine samples obtained on days 69 and 70 during a 90-day feeding study with IN-F4106 (Hoban, 2016b) were analyzed for both metabolites. Dietary doses in that study were 0, 50, 150, 625 or 2500 ppm, corresponding to (rounded) mean daily intakes of 0, 2, 6, 26 and 108 mg/kg bw per day of IN-F4106 in males. For females, mean daily intakes of 0, 2.5, 7.5, 31 and 124 mg/kg bw per day were calculated. These figures reflect the mean daily intakes in the week of sampling and may be different from overall intakes in this study as reported above. Plasma analysis revealed dose-related increases of IN-F4106, with concentrations ranging from 0.5 to 71 nmol/mL, and similar between the sexes. Lower concentrations of IN-A5760 were determined, ranging from 0.4 to 17.4 nmol/mL in males and 0.5 to 21.5 nmol/mL in females. When dietary dosing had been interrupted two days before sampling in a subgroup of animals, the plasma levels of both metabolites declined greatly, suggesting rapid oral absorption and distribution and early onset of metabolism. Urine analysis confirmed quantitative absorption, rapid elimination, predominant renal excretion and an extensive metabolism of IN-F4106 since in males IN-A5760 (including conjugates) in urine accounted for ca 75% of the total dietary dose whereas only 1.9% was excreted as IN-F4106. These figures were calculated across all dose levels. In females IN-A5760 was also the main radioactive fraction in urine, accounting for 80% of total excreted radioactivity, whereas only 1.6% was recovered as IN-F4106 (Himmelstein, 2018a). These data suggest that IN-A5760 might be expected to have contributed significantly to the toxicity of IN-F4160.

Toxicological studies with IN-A5760 were confined to its examination for genotoxicity in vitro and in vivo. The respective studies are briefly summarized and evaluated below.

Genotoxicity

An Ames test using the plate-incorporation method was performed with *S. typhimurium* strains TA1535, TA1537, TA98, and TA100 and *E. coli* strain WP2 *uvr*A, both with and without metabolic activation. First a toxicity-mutation test was conducted. An increase in revertant colonies was observed with TA1537 in the presence of S9 mix but this was neither dose-related nor reproducible. Therefore, the finding was considered to have occurred by chance and in the main test IN-A5760 was applied to all tester strains in the presence and absence of S9 mix at concentrations up to 5000 µg/plate. No increase in revertant colonies over that seen with the vehicle control (DMSO) was observed in any strain, while appropriate positive control substances gave the expected responses. No precipitation or cytotoxicity became apparent. Thus IN-A5760 proved negative for bacterial mutagenicity under the conditions of this study (Myhre, 2016a).

Metabolite IN-A5760 was tested in CHO cells for its ability to induce forward gene mutations at the hypoxanthine–guanine phosphoribosyl transferase locus (HPRT assay). The test substance was dissolved in DMSO. The experiments were performed at concentrations ranging from 250 to 2000 µg/mL, both in the presence and absence of metabolically activating S9 mix. No increase in mutant frequency was observed with the metabolite at any concentration without activation. With S9 mix, by contrast, significant increases in mutant frequency were observed at 250 and 500 µg/mL but not at higher concentrations. In a repeat experiment this apparently increase, apparently not dose-related, was not reproduced and so the assay was also considered negative in the presence of activation. It is worth noting that mutant frequency in this repeat experiment was higher at all concentrations than in the previous trial, including with the vehicle alone, suggesting a certain degree of natural background variability. The positive control substances induced the expected response. It can be concluded that IN-A5760 proved negative under the conditions of this in vitro HPRT assay (Wells, 2016).

In an in vitro chromosome aberration study, cultured peripheral lymphocytes obtained from a healthy woman 33 years old were exposed to IN-A5760 for four hours (harvested after 22 hours) both in the presence and absence of metabolic activation. Based on preliminary testing for precipitation and cytotoxicity, definitive testing was conducted at concentrations of 250 to 1750 µg/mL, both with and without S9 mix. Additional cultures were exposed for 22 hours with immediate harvest, but only in the absence of metabolic activation. In this experiment, only lower concentrations between 50 and 750 µg/mL could be tested.

Cytogenetic analyses were performed on test concentrations of 0, 500, 750 and 1650 µg/mL following the four-hour treatment, and 0, 100, 250, and 500 µg/mL following the 22-hour exposure. Slides, coded for anonymity, were scored for both structural and numerical chromosomal anomalies. At least 300 metaphases per concentration (150 from each duplicate culture) were analyzed. For the four-hour treatment without activation an additional set of prepared slides were scored resulting in a total of 600 metaphases per dose. Appropriate positive control substances were applied and gave the expected responses.

No statistically significant increase in structural or numerical chromosome aberrations was observed at any concentration in the experiments with four-hour treatment under activated conditions or in those with 22-hour treatment without S9 mix. In contrast, a significant increase of structural and numerical chromosome aberrations over the control was noted at 1650 µg/mL following four-hour treatment without activation and this was confirmed by a positive trend in the Cochran–Armitage test. Accordingly, IN-A5760 proved positive in this in vitro assay (Kellum, 2016b) (details are summarized in Table 45 below).

Table 45. Summary of numerical and structural chromosome aberrations in peripheral human lymphocytes after four-hour treatment (without activation) with IN-A5760 and a positive control substance

Treatment	Cells scored	Mitotic index (%)	Cells with aberrations (%)	
			Numerical	Structural
Vehicle (DMSO)	600	12.7	0.0	0.5
500 µg/mL	600	11.2	0.3	1.3
750 µg/mL	600	10.4	0.0	0.7
1650 µg/mL	600	5.6	5.3*	2.7*
Mitomycin C, 0.4 µg/mL	300	7.1	0.0	22.0*

* Statistically significant difference at $p < 0.05$% Fisher's test, (for test substance), and Cochran–Armitage test for trend

Source: Kellum, 2016b

Male and female Crl:CD1 (ICR) mice were administered single oral doses of 0 (vehicle control), 500, 1000 or 2000 mg/kg bw of IN-A5760 to investigate the micronucleus frequency in peripheral reticulocytes by flow cytometric analysis. In the main study, the vehicle control, low and intermediate dose and positive control groups contained five animals per sex; seven mice of each sex received the high dose. Blood sampling was performed approximately 48 and 72 hours post dosing and 20 000 reticulocytes per blood sample were analyzed. Following the 72-hour sampling, analysis was confined to control and top-dose animals. In addition, one male and one female mouse received the vehicle (Tween 80 in aqueous methylcellulose) and four per sex were administered the low dose of 500 mg/kg bw. These animals were killed four hours after dosing and blood was sampled to use in plasma analysis for IN-A5760 and its glucuronide and sulfate conjugates.

No mortality and no clinical signs of toxicity were observed. There was no increase in micronucleated reticulocytes in the peripheral blood of mice after administration of this metabolite up to the maximum dose of 2000 mg/kg bw, either in males or females. Cyclophosphamide administration (30 mg/kg bw) resulted in a strong decrease in reticulocytes in general, but an increase in micronucleated reticulocytes. Analysis of pooled plasma samples from low-dose animals revealed evidence of target tissue (bone marrow) exposure. Most of the test substance present in plasma was in a conjugated from. It may be concluded that IN-A5760 proved negative in this in vivo assay for clastogenic properties (Myhre, 2017c).

(d) Metabolite IN-REG72; 8-Chloro-*N*-[2-chloro-5-hydroxyphenyl)sulfonyl]-6-(trifluoromethyl)imidazole[1,2-α]pyridine-2-carboxamide

This is the fourth animal metabolite from the list of potentially relevant biotransformation products of fluazaindolizine. It results from demethylation of the parent compound but, apart from that, the chemical structures are identical. In rodent metabolism it is further processed to IN-A5760, or will undergo sulfate conjugation (see Fig. 2). From a quantitative point of view, IN-REG72 is a minor metabolite in the rat occurring at less than 2% of AD but was found to account for 5.4% of radioactivity in excreta of female mice (Punler & Green, 2016b). In routine toxicological studies it was detected at similar or even slightly higher concentrations than metabolites QEK31 or F4106. It worth noting that IN-REG72 was the main in vitro metabolite following incubation with human hepatocytes, but that it was found in much lower amounts in tests with liver cells from other species (Novo, 2018). The possibility cannot be excluded that IN-REG72 is a major human metabolite of fluazaindolizine. Metabolite IN-REG72 was also found in crops, livestock, water and soil.

The available toxicological studies for this metabolite were confined to genotoxicity end-point. The respective studies are briefly summarized and evaluated below.

Genotoxicity

An Ames test using the plate-incorporation method was performed in *S. typhimurium* strains TA1535, TA1537, TA98, and TA100, and *E. coli* strain WP2 *uvr*A, both with and without metabolic activation. Based on the results of a preceding toxicity-mutation test, IN-REG72 was applied to all tester strains, in both the presence and absence of S9 mix, at concentrations up to 5000 µg/plate. No increase in revertant colonies over that seen with the vehicle control (DMSO) was observed in any strain. Appropriate positive control substances gave the expected responses. No precipitation was seen and cytotoxicity (reduction in background lawn) was confined to strains TA1537 and WP2 *uvr*A when the test article was applied at the maximum concentration. Thus metabolite IN-REG72 proved negative for bacterial mutagenicity under the conditions of this study (Myhre, 2016b).

In an in vitro chromosome aberration study, cultured peripheral lymphocytes obtained from a healthy man, 32 years old, were exposed to IN-REG72 for four hours (harvest after 22 hours) and for 22 hours in the absence of metabolic activation, or for four hours in the presence of S9 mix. Based on preliminary testing for precipitation and cytotoxicity, the definitive test was conducted at concentrations of 100–900 µg/mL for the four-hour activated and non-activated test conditions, and from 100–500 µg/mL for the 22-hour treatment without activation. Cytogenetic analyses were performed at concentrations of 0, 500, 700 and 900 µg/mL following the four-hour treatments, and 0,100, 300, and 450 µg/mL following the 22-hour exposure. Slides, coded for anonymity, were scored for both structural and numerical chromosomal anomalies. At least 300 metaphases per concentration (150 from each duplicate culture) were analyzed. No statistically significant increase in structural or numerical chromosome aberrations compared with the negative control was observed at any concentration, either with or without metabolic activation. Appropriate positive control substances were applied and gave the expected responses. Metabolite IN-REG72 proved negative in this in vitro assay for clastogenic or aneugenic activity (Kellum, 2016c).

Male and female Crl:CD1(ICR) mice were administered single oral doses of 0 (vehicle control), 500, 1000 or 2000 mg/kg bw of metabolite IN-REG72 to investigate micronucleus frequency in peripheral reticulocytes by flow cytometric analysis. In the main study, the vehicle control, low and intermediate dose and positive control groups contained five mice per sex; seven mice of each sex received the high dose. Blood sampling was performed approximately 48 and 72 hours post dosing and 20 000 reticulocytes per blood sample were analyzed. Following the 72-hour sampling, analysis was confined to control and top-dose animals. In addition, one male and one female mouse received the vehicle (Tween 80 in aqueous methylcellulose) and four mice per sex were administered the low dose, 500 mg/kg bw of metabolite IN-REG72. These animals were killed four hours after dosing and blood taken with which to carry out plasma analysis for IN-A5760 and its glucuronide and sulfate conjugates.

No mortality or clinical signs of toxicity were observed. There was no increase in micronucleated reticulocytes in the peripheral blood of female mice after administration of IN-REG72 up to the maximum dose of 2000 mg/kg bw. In males a higher frequency of micronuclei was observed in the group receiving 1000 mg/kg bw but not in the low- or high-dose groups. This increase over the control group mean was statistically significant at the 72-hour sampling and was outside the historical control range in two out of five males. Similarly, at 48 hours the mean value in this group was more than double that of controls. For clarification, a follow-up test was performed in which five males per group received either the vehicle or the test substance at dose levels of 500, 750, 1000, 1500 or 2000 mg/kg bw. Again, blood was sampled at 48 and 72 hours post dosing. In this follow-up investigation no increase in micronucleus frequency was noted at any dose level or time point, so the evidence from the initial test was not found to be reproducible. Cyclophosphamide administration (30 mg/kg bw) resulted in a strong decrease in reticulocytes in general and an increase in micronucleated reticulocytes.

Analysis of pooled plasma samples from low-dose animals revealed levels of 3800 ng/mL in males and 10 100 ng/mL in females providing evidence of likely target tissue (bone marrow) exposure. On balance, metabolite IN-REG72 proved negative in this in vivo assay (Myhre, 2017d).

The following metabolites were not found to occur in animal metabolism but were detected in other matrices. Available toxicological and genotoxicity studies are reported in brief below.

(e) Metabolite IN-QZY47 (1*S*)-1-Carboxy-2-[(2-chloro-5-methoxy-phenyl)sulfonylamino]ethyl]ammonium chloride

This crop metabolite was tested for acute oral toxicity in the rat and for genotoxicity. Short-term feeding studies (7 and 28 day) were available and, as part of these, kinetics and metabolism were investigated at least to some extent.

Acute toxicity

A single oral dose of metabolite IN-QZY47 suspended in 0.1% Tween 80 (v/v) in 0.5% aqueous methylcellulose was administered by oral gavage to four fasted female Crl:CD(SD) rats at dose levels of 1750 or 5000 mg/kg bw. The animals were dosed one at a time with a minimum of 48-hour intervals following the up-and-down procedure and using 1750 mg/kg bw as the initial dose, given to a single female only. Rats were observed for clinical signs of toxicity, body weight effects, and mortality for up to 14 days after dosing before they were killed and gross examination carried out to detect any evidence of organ or tissue damage. No unscheduled deaths occurred and no clinical signs of toxicity, body weight effects or gross lesions were observed. Accordingly the LD_{50} was greater than 5000 mg/kg bw proving the low toxicity of this metabolite (Fallers, 2016a).

Genotoxicity

The genotoxic potential of IN-QZY47 was examined in a number of studies. In a bacterial mutagenicity (Ames) test with and without metabolic activation, the metabolite was tested in *S. typhimurium* strains TA1535, TA1537, TA98 and TA100 and *E. coli* strain WP2 *uvr*A by means of the plate-incorporation method. Based on the results of a preceding toxicity-mutation test, IN-QZY47 was applied in the main experiment to all tester strains at concentrations ranging from 333–5000 µg/plate in the absence and presence of S9 mix. An original study was run in 2015, and at that time the test material was of low purity containing only 89.7% active component. With the low-purity test article a reproducible increase in revertant colonies over the vehicle control (DMSO) values was observed with strain TA1535 both with and without activation. The highest increase was a 2.7-fold higher mutation rate observed at 3333 µg/plate with S9, and at 5000 µg/plate without S9 mix. No such increase was seen in any other tester strain. Appropriate positive control substances gave the expected clear responses. No precipitation or cytotoxicity were seen.

To help clarify this rather equivocal effect the tests with TA1535 were repeated in 2017 and 2018, using test article of higher purity (99.2%). This repeat experiment was the reason for the author revising the original report in 2018. In the repeat studies no increase in revertant mutants was seen with TA1535 up to the maximum concentration of 5000 µg/plate, either with or without metabolic activation. The conclusion was drawn that metabolite IN-QZY47 was negative for bacterial mutagenicity in this study, at least when material of sufficiently high purity was employed (Myhre, 2018c).

Metabolite IN-QZY47 was tested in CHO cells for its ability to cause forward gene mutation at the hypoxanthine–guanine phosphoribosyl transferase locus (HPRT assay). The test substance was dissolved in DMSO. Since a pretest did not reveal precipitation or cytotoxicity, the definitive experiments were performed at concentrations of 0, 89.4, 179, 358, 715 and 1430 µg/mL, both in the presence and absence of metabolically activating S9 mix. The absence of an impact on cell survival was confirmed. Without activation the mutant frequencies at all concentrations of IN-QZY 47 were markedly higher (by up to seven times) than in the vehicle control, but there was no dose-related increase. In the presence of S9 mix no increase in mutant frequency was observed with the metabolite at all. The positive control substances induced the expected responses. Metabolite IN-QZY47 proved negative for genotoxicity towards mammalian cells in this in vitro assay (Dutta, 2016b).

In an in vitro chromosome aberration study, cultured peripheral lymphocytes obtained from a healthy woman, 29 years old, were exposed to IN-QZY47 for four hours and 22 hours in the absence of metabolic activation, or for four hours only in the presence of S9 mix. Cells were harvested after 22 hours. Based on preliminary testing for precipitation and cytotoxicity, cytogenetic evaluations were conducted at concentrations of 0, 50, 100 and 250 µg/mL under the four-hour, non-activated test

conditions because of strong reduction in mitotic index at higher concentrations. Due to precipitation and not cytotoxicity, concentrations of 0, 250, 500 and 750 µg/mL were selected for analysis under the four-hour treatment with activation. For the 22-hour, non-activated treatment conditions only the low concentrations of 5, 10 and 25 µg/mL could be evaluated, again because of severe cytotoxicity. Anonymized slides were scored for both structural and numerical chromosomal anomalies. At least 300 metaphases per concentration (150 from each duplicate culture) were analyzed. No statistically significant increases in structural chromosome aberrations compared to vehicle (DMSO) control values were observed. Appropriate positive control substances were applied and gave the expected responses. There was also no evidence of an increase in numeric aberrations in this study. Accordingly, IN-QZY47 was devoid of a potential to cause chromosome aberrations under the conditions of this study (Kellum, 2016d).

Presumably as a follow-up study to the Ames test that was positive in TA1535 (see Myhre, 2018c above), a production batch of IN-QZY47 of rather low purity (89.7%) was tested in vivo in a UDS assay in rats. The test item was dissolved in 0.1% Tween 80 in 0.5% aqueous methylcellulose and administered as a single dose by oral gavage to Sprague Dawley rats. The dose levels of 0 (vehicle control), 500, 1000 and 2000 mg/kg bw had been selected on the basis of a preliminary range-finding experiment. The positive control substance dimethylnitrosamine was administered at a dose level of 35 mg/kg bw. Animals were killed either 2–4 or 12–16 hours post dosing. For each dose level and each time point of termination, the groups always comprised four rats of each sex.

Hepatocytes were isolated and cultured. At least six cultures per animal were set up. Three to six hours after plating on culture dishes cells were washed and exposed for four hours to a medium containing [^{3}H]thymidine. Afterwards, cultures were incubated for further 17 to 20 hours before slides were prepared. At least three slides per animals were scored under the microscope (interfaced with an automated colony counter) for silver grains in the nucleus and surrounding cytoplasm in 50 cells per slide. The net nuclear grain count for each nucleus was obtained by subtracting the mean cytoplasmic area count from the nuclear area count. A marked, dose-related and significant increase in this net grain count is considered indicative of unscheduled DNA synthesis. In addition, nuclei with an increased grain count may indicate cells described as in repair.

All treated rats survived until scheduled termination and piloerection was the only clinical sign attributed to treatment. There was no increase in net nuclear grain count for the treated animals at any dose level or time point. Nor was there any increase in the percentage of cells in repair, which accounted for 4% in each of the three dose groups, values absolutely comparable to 3% found in the vehicle control. The positive control substance revealed a clear positive response with 96% cells in repair following exposure for 2–4 hours. Following the longer treatment period of 12–16 hours, the number of cells in repair in the three dose groups were 1%, 3%, and 2% at 500, 1000 and 2000 mg/kg bw, respectively. In the vehicle control group 3% of cells were in repair, while dimethylnitrosamine had caused a clear increase to 94%.

Metabolite IN-QZY47 proved clearly negative in the UDS assay in primary rat hepatocytes following in vivo exposure up to the limit dose of 2000 mg/kg bw. There was no evidence of direct interaction of the test compound with rat liver DNA. However, it must be taken into consideration that the scientific value of this assay, and in particular its suitability as a follow-up to positive results from in vitro mutagenicity tests (see for example Kirkland & Speit, 2008; EFSA, 2017), is strongly contested (Bruce, 2016).

Short-term studies of toxicity

In a what is described as seven-day feeding study, IN-QZY47 was administered to male and female Crl:CD(SD) rats (five animals per sex and dose group) at dietary concentrations of 0, 800, 3200 or 12 000 ppm for not seven but nine days. The mean daily intakes for males were 0, 73, 292 and 1077 mg/kg bw per day; the mean daily intakes for females were 0, 69, 267, and 899 mg/kg bw per day. Parameters evaluated included body weight, body weight gain, food consumption, food efficiency, clinical signs, haematology, clinical chemistry, urinalysis, gross pathology, organ weights, and histopathology. In addition, blood and urine samples were collected on days 4 or 5 for examination of plasma concentrations, urinary excretion and analysis for metabolites. Plasma analysis was based on four time points over a 24-hour period.

There were no unscheduled deaths in this study and no clinical signs of toxicity were observed.

Exposure to 12 000 ppm, however, produced adverse and test substance-related effects on body weight, body weight gain, food consumption, and food efficiency in females. Final body weight was lower by more than 10% than for controls and this findings agreed well with a mean body weight gain that was by as much as 79% lower than for controls. Mean food intake was by 25% lower and food efficiency in high-dose females was severely compromised (71% below controls). In contrast no adverse effects on these parameters were observed in female rats at 3200 ppm or below, or in males at any dietary concentration. No adverse changes in haematological, clinical chemistry and urinalysis parameters were noted. Liver weights (relative to body weight) were increased by more than 15% compared to controls, and minimal centrilobular hepatocellular hypertrophy was observed in male and female rats at the top dose. These liver findings were not associated with microscopic or clinical pathology changes indicative of liver toxicity and were therefore considered adaptive rather than adverse.

Retinal degeneration or atrophy was present in two male rats at the mid-dose level and in three males and one female receiving the highest dose. Such changes are known to occur sporadically in rats of this strain but, in this study the effects were confined to the upper two dose levels and not seen in the controls of either sex. Based on the severity of this finding (moderate at 3200 ppm but only minimal to mild at 12 000 ppm), it was it was seen as not strictly dose-related, and not to warrant further evaluation in the 28-day study. The subsequent study (Hoban, 2017b, see below) did not provide further evidence of retinal damage even though more animals were employed. Ophthalmoscopy did not reveal any adverse changes, but histopathology revealed retinal degeneration or atrophy in 8/10 control males, and 4/10 females but not in the groups receiving 10 000 ppm. The mean daily intake of 735 mg/kg bw per day in male rats in the 28-day study was far below the maximum dose level in the seven-day study, but much higher than the intermediate dose of 292 mg/kg bw at which the increase in retinal damage had been observed in the first study. Also, duration of treatment in the second study was longer. Thus, the retinal changes in the seven-day study were considered chance findings.

The NOAEL in this study was 3200 ppm (equal to 267 mg/kg bw per day) and was based on adverse changes in body weight and nutritional parameters which were observed at the maximum dose level of 12 000 ppm (equal to 899 mg/kg bw per day) but only in female rats. In males, in contrast, there were no adverse findings up to the highest dose tested.

Analysis of plasma concentrations and determination of AUC revealed a linear increase with dose up to the intermediate dose of 3200 ppm, but above this a non-linear relationship which suggests some saturation in both sexes at 12 000 ppm. Limited urinary excretion was observed with an acetylated derivative of IN-QZY47 as the main metabolite. In addition, a number of further downstream biotransformation products were detected including IN-F4106 which is also a significant rat metabolite, IN-TMQ01, acetylated or sulfate-conjugated forms of IN-UJV12 (Hoban, 2018c).

In a 28-day feeding study (apparently performed after the 7-day study but reported earlier), IN-QZY47 was fed to male and female Crl:CD(SD) rats (10 animals per sex and dose) at nominal dietary concentrations of 0, 200, 800, 3000 or 10 000 ppm. The mean daily intakes for males were 0, 15, 59, 220 and 735 mg/kg bw per day; for females 0, 16, 64, 235 and 749 mg/kg bw per day. Parameters evaluated included mortality and the occurrence of clinical signs, body weight, body weight gain, food consumption, food efficiency, haematology, clinical chemistry, urinalysis, gross pathology, organ weights, and histopathology. Blood sampling for haematology and clinical chemistry was performed in five animals from each group prior to scheduled termination. Ophthalmoscopy and neurobehavioural examinations ,including abbreviated FOB and motor activity measurements, took place pretest and near the cessation of the study.

The toxicokinetics in plasma and urine of IN-QZY47 and its metabolites IN-F4106 and INA5760 were investigated in samples taken during the third week of treatment. For this purpose, blood was taken four times on test day 21 from all animals. Urine was collected from five animals per sex and group on test day 22 after they had been placed for 17 hours in metabolism cages. Analysis of urine was performed on pooled samples for each treatment group. Results of this metabolism investigation are reported below in *Study 8* (Himmelstein, 2018b).

There were no test substance-related deaths and no adverse clinical signs or neurobehavioural or ophthalmologic observations that could be attributed to test substance administration.

The only effects on body weight in male rats comprised a slightly lower food consumption and

body weight gain during the first week of treatment, perhaps suggesting reduced palatability. Afterwards these parameters normalized and final body weight and total body weight gain were only marginally below the control values, with the differences not achieving statistical significance. On balance these minimal effects on nutritional parameters may well be treatment-related but should not be regarded as adverse.

In females, body weight was significantly lower than in the control group throughout the study, with differences of 5% to 9%. Total body weight gain was significantly lower by 33% and was reduced to an even greater extent week 1. These findings concur with decreased food consumption, and also food efficiency, which was 23% lower than in controls over the whole study period. Despite the conclusions of the study author, the Meeting concluded that continuing impact on nutritional parameters in high-dose female rats should be considered adverse.

Increases in mean absolute and relative liver weights were seen in high-dose males and females. They were accompanied by minimal liver cell hypertrophy in both sexes. Usually, liver weight changes in the absence of related clinical chemistry findings and or any histopathological lesions other than hypertrophy, are not considered adverse. However in this case the magnitude of the effects should be taken into account. Based on a higher absolute liver weight in male rats by 29.1% and by more than 20% in females compared to controls, these findings should be considered potentially adverse. In addition, higher mean relative kidney weights were recorded in high-dose males and females. This increase could reflect the slightly lower terminal body weights but was interpreted by the study author to indicate induced renal metabolism; this remains, however, only a hypothesis. There were no test substance-related anatomical pathology findings in males or females fed dietary concentrations of 3000 ppm or less of the test substance.

The NOAEL in this study was the intermediate dose of 3000 ppm (equal to 220 mg/kg bw per day) based on an impact on nutritional parameters in females and on a strong increase in liver weight in both sexes at the maximum dose level of 10 000 ppm (Hoban, 2017b).

Plasma and urine samples obtained in the third week of the feeding study with IN-QZY47 (Hoban, 2017b, see above) were analyzed by means of LC-MS/MS for this compound and its metabolites IN-F4106 and IN-A5760. As outlined above, dietary doses in this study were 0, 200, 800, 3000 or 10 000 ppm. In the week of sampling, the corresponding (rounded) mean daily intakes in males were 0, 14, 57, 208 and 699 mg/kg bw per day in males; for females mean daily intakes were calculated as 0, 16, 60, 225 and 736 mg/kg bw per day.

Plasma analysis revealed a dose-related increase in IN-QZY47 that was, however, less than proportional to dose in both sexes. In contrast, a supra-linear increase with dose was observed for IN-F4106, and similarly for IN-A5760, but this last metabolite occurred only in small amounts. It was detected at dose levels greater than 200 ppm in males but was hardly quantifiable in females at 200 and 800 ppm. These data suggest some saturation of IN-QZY47 uptake and metabolism to IN-F106 and IN-5760.

All three metabolites were detected in rat urine but only in very small amounts. Even combined, IN-QZY47, IN-F4106 and IN-A5760 in urine accounted, in the individual dose groups, for not more than 1.7%–2.3% of the applied dose in males and 1.9%–3.6% in females, suggesting that urinary excretion was only a minor route of elimination. However, oral absorption of IN-QZY47 has been demonstrated but not quantified since mass balance data were not available. Therefore the kinetics and metabolism of IN-QZY47 cannot be regarded as sufficiently elucidated (Himmelstein, 2018b).

(f) Metabolite IN-TMQ01 (No IUPAC name available)

This crop metabolite was tested for acute oral toxicity in the rat and for genotoxicity. Also, short-term feeding studies over 7 and 28 days were available, including investigations on kinetics and further biotransformation.

Acute toxicity

A single oral dose of IN-TMQ01, suspended in 0.1% Tween 80 (v/v) in 0.5% aqueous methylcellulose, was administered by oral gavage to fasted female Crl:CD(SD) rats (nine in total) at dose levels of 0, 550, 1750 or 5000 mg/kg bw. The animals were dosed one at a time at a minimum of 48-hour intervals following the up-and-down procedure, starting from the lowest dose, which was given to a single female

only. Rats were observed for clinical signs of toxicity, body weight effects, and mortality for up to 14 days after dosing before they were killed and examined to detect grossly observable evidence of organ or tissue damage. One of the high-dose animals died within one day of dosing. The five survivors that had also been given 5000 mg/kg bw showed no signs of toxicity. No mortality, clinical abnormalities or body weight effects were observed in the one or two rats per dose that had been administered the lower doses. Consistent across the groups, body weight losses were noted on the day after dosing, but this effect was due to fasting and all surviving animals gained weight again thereafter. No gross lesions were noted at any dose level, except thymus discolouration in the early decedent, which was considered an equivocal finding. Accordingly, the LD_{50} was greater than 5000 mg/kg bw demonstrating the low toxicity of this metabolite (Fallers, 2016b).

Genotoxicity

An Ames test using the plate-incorporation method was performed with *S. typhimurium* strains TA98, TA100, TA1535 and TA1537, and *E. coli* strain WP2 *uvr*A. Based on the results of a preceding toxicity-mutation test, IN-TMQ01 was applied to all tester strains at concentrations ranging from 333 to 5000 µg/plate, both in the presence and absence of S9 metabolic activation. No increase in revertant colonies over the vehicle control (DMSO) values was observed in any strain. Appropriate positive control substances clearly gave the expected responses. No precipitation or cytotoxicity were observed in any strain up to the limit concentration (Myhre, 2015c).

In an in vitro chromosome aberration study, cultured peripheral lymphocytes obtained from a healthy woman, 32 years old, were exposed to IN-TMQ01 for four hours or for 22 hours in the absence of metabolic activation, or for four hours only in the presence of S9 mix. Cells were harvested after 22 hours. Based on preliminary testing for precipitation and cytotoxicity, cytogenetic evaluations were conducted at concentrations of 0, 500, 1000, and 2000 µg/mL for all test conditions. Anonymized slides were scored for both structural and numerical chromosomal anomalies. At least 300 metaphases per concentration (150 from each duplicate culture) were analyzed. No statistically significant increase in structural chromosome aberrations compared with vehicle (sterile water) control values was observed. Appropriate positive control substances were applied and gave the expected responses. There was no evidence either of an increase in numeric aberrations in this study. Accordingly IN-TMQ01 did not induce chromosome aberrations under the conditions of this study (Kellum, 2016e).

Short-term studies of toxicity

Metabolite IN-TMQ01was fed to male and female Crl:CD(SD) rats (five animals per sex and dose) at dietary concentrations of 0, 800, 3200 or 12 000 ppm for approximately seven days. The mean daily intakes as calculated for males were 0, 75, 307 and 1179 mg/kg bw per day; for females 0, 73, 287 and 1075 mg/kg bw per day. Parameters evaluated included body weight, body weight gain, food consumption, food efficiency, clinical signs, haematology, clinical chemistry, urinalysis, gross pathology, organ weights, and histopathology. In addition, blood and urine samples were collected for examination of plasma concentrations, urinary excretion and analysis for metabolites. Plasma analysis was based on four time points over a 24-hour period on exposure days 5 and 6, urine was collected overnight on days 4 to 5.

There were no unscheduled deaths in this study and no clinical signs of toxicity were observed. No meaningful alterations in body weight or other nutritional parameters were observed. Body weight gain tended to be suppressed in high-dose males and food efficiency was slightly lower in both sexes at the top dose, but the differences from the control group did not achieve statistical significance. No adverse changes in haematological, clinical chemistry or urinalysis parameters were noted. Organ weights were not affected and neither gross pathology nor histopathology revealed any remarkable findings that could be attributed to treatment. Like in a similar study with IN-QZY47 (Hoban, 2018c), retinal degeneration was noted in three high-dose males but not in the control group, whereas the eyes of low- and mid-dose animals were not examined microscopically. In females no such finding was apparent, neither in the control nor the high-dose group. Even though the observation in males might suggest a treatment-related effect it was not considered relevant because this finding was not found to be reproducible in the 28-day *study 5*, reported below (Sayers, 2017). This pattern also resembled what was observed with IN-QZY47 (Hoban, 2018c) as discussed in detail above.

Accordingly, the NOAEL in this study was 12 000 ppm (equal to 1075 mg/kg bw per day) the highest dose tested.

Analysis of plasma concentrations of IN-TMQ01 revealed a proportional increase with dose over the whole dose range in both sexes. In urine IN-TMQ01 was the major excreted metabolite in the urine. Metabolite IN-4106 and IN-TQD54, IN-A5760 or their sulfate conjugates were detected in much smaller amounts. It would appear that IN-TMQ01 is, to some extent, subject to further biotransformation (Hoban, 2018d).

In a 28-day feeding study (apparently performed after the seven-day study but reported earlier), IN-TMQ01 was fed to male and female Crl:CD(SD) rats (10 animals per sex and dose group) at nominal dietary concentrations of 0, 500, 3000 or 12 000 ppm. Mean daily intakes of 0, 34, 210 and 847 mg/kg bw per day were calculated for male rats; mean daily intakes in females being 0, 36, 219, and 902 mg/kg bw per day. Parameters evaluated included mortality and the occurrence of clinical signs, body weight, body weight gain, food consumption, food efficiency, haematology, clinical chemistry, urinalysis, gross pathology, organ weights and histopathology. Blood sampling for haematology and clinical chemistry was performed on five animals from each group prior to scheduled termination. Ophthalmoscopy and neurobehavioural examinations, including abbreviated FOB and motor activity measurements, took place pretest and near the cessation of the study. Histopathological examination was confined to high-dose and control animals, except for gross lesions which were rare and not treatment-related.

In addition to toxic effects, the toxicokinetics and metabolism of IN-TMQ01 were investigated. For this purpose blood was taken from all animals four times on test day 21; urine was collected from five animals per sex and group on test day 22 after animals had been placed for 17 hours in metabolism cages. Analysis of urine was performed on pooled samples for each treatment group. The results of these metabolic investigations are reported below in *study 6* (Chan, 2018).

There were no unscheduled deaths during the study and no adverse clinical signs, neurobehavioural or ophthalmological observations that could be attributed to test substance administration. Body weight, body weight gain and food consumption were unaffected at any dose level.

Haematological and clinical chemistry parameters were not altered. No effects on organ weights were noted and no gross or microscopic lesions could be attributed to treatment. Retinal degeneration or atrophy were of particular interest, but in contrast to the seven-day study (Hoban, 2018d), such changes were observed only in very few males and females in the control groups and not at all at the top dose level.

Accordingly the NOAEL in this study was 12 000 ppm (equal to 847 ~~or 902~~ mg/kg bw per day) the highest tested dose, which points to very low toxicity due to metabolite IN-TMQ01 (Sayers, 2017).

Plasma and urine samples obtained in the third week of the feeding study with IN-TMQ01 (see Sayers, 2017 above) were analyzed by means of LC-MS/MS for this compound and its metabolites IN-F4106 and IN-A5760. As outlined above, dietary doses in this study were 0, 500, 3000 and 12 000 ppm. In the week of sampling, the corresponding (rounded) mean daily intakes for males were calculated as 0, 33, 200 and 819 mg/kg bw per day; corresponding values for females were 0, 34, 205 and 867 mg/kg bw per day.

Plasma analysis in both sexes revealed dose-related increases in IN-TMQ01 that were, however, slightly less than proportional to dose. A slightly supralinear increase with dose was observed for IN-F4106, suggesting limited biotransformation of IN-TMQ01 to IN-F4106. Nonetheless IN-TMQ01 was much more abundant in plasma than IN-F4106. Levels of IN-A5760 were below the limit of quantification at all dose levels.

In urine, 12%–18% of AD of IN-TMQ01 was excreted virtually unchanged chemically. IN-F4106 and IN-A5760 were detected only in traces, even when combined, accounting for no more than 0.015% of AD. On balance, oral absorption of IN-TMQ01 was demonstrated, but its metabolism appears from these results to be very limited (Chan, 2018).

(g) Metabolite IN-UJV12 (No IUPAC name available)

The crop metabolite IN-UJV12 was tested for acute oral toxicity in the rat and for genotoxicity in various test systems.

Acute toxicity

A single oral dose of IN-UJV12, suspended in 0.1% Tween 80 (v/v) in aqueous 0.5% methylcellulose, was administered by oral gavage to four fasted female Crl:CD(SD) rats at dose levels of 0, 1750 or 5000 mg/kg bw. The animals were dosed one at a time at a minimum of 48-hour intervals following the up-and-down procedure, and using 1750 mg/kg bw as the initial dose given to a single female. Rats were observed for clinical signs of toxicity, body weight effects, and mortality for up to 14 days after dosing before they were killed and examined for any grossly observable evidence of organ or tissue damage. No unscheduled deaths occurred and no clinical signs of toxicity, body weight effects or gross lesions were observed at any dose level. The LD_{50} was greater than 5000 mg/kg bw, demonstrating the low toxicity of IN-UJV12 (Fallers, 2016c).

Genotoxicity

A first Ames test using the plate-incorporation method was performed in *S. typhimurium* strains TA98, TA100, TA1535 and TA1537, and *E. coli* strain WP2 *uvr*A. The batch used was IN-UJV12-003 with a purity of 97.0%. As in all the other studies for bacterial mutagenicity with fluazaindolizine metabolites, this assay was performed in two phases. In a toxicity-mutation test (in duplicate), IN-UJV12 was applied to all tester strains at concentrations ranging from 33.3 to 5000 µg/plate, both in the presence and absence of S9 metabolic activation. No precipitation and no cytotoxicity were observed. Evidence of a mutagenic response was obtained for TA1535. Without metabolic activation, mutant frequency was increased at the two upper concentrations of 3333 µg/plate (a 3.1-fold increase over DMSO vehicle controls) and 5000 µg/plate (a 4.0-fold increase). In the presence of S9 mix, 2.0- and 2.4-fold increases respectively were noted at these same concentrations. In contrast, no increase in revertant colonies over the vehicle control values was observed in any other strain. Appropriate positive control substances clearly gave the expected responses (Myhre, 2016c).

In the definitive mutagenicity test employing three plates per concentration, IN-UJV12 was applied to all tester strains in concentrations of 0 (vehicle control), 333, 667, 1000, 3333 and 5000 µg/plate. Again, a positive mutagenic response was observed with TA1535 in the absence of S9 mix as demonstrated by a 3.2-fold increase in mutant frequency over the concurrent control at the maximum concentration; a 2.8-fold increase was noted at the same top concentration with activation. No such effects were observed in any other strain. The results obtained with TA1535 in both phases of the study are summarized in Table 46.

Table 46. Mutagenicity testing of IN-UJV12 in S. typhimurium strain TA1535 (mean number of revertant colonies per plate at selected concentrations, findings presumed critical shown in bold)

	Test system			
Test item: concentration	**Pretest, −S9**	**Pretest, +S9**	**Main test, −S9**	**Main test, +S9**
DMSO (vehicle control)	9	15	13	14
IN-UJV12: 33.3 µg/plate	12	8	-	-
333 µg/plate	15	14	11	16
667 µg/plate	15	15	23	16
1000 µg/plate	15	15	19	17
3333 µg/plate	**28**	**29**	**29**	**35**
5000 µg/plate	**36**	**35**	**40**	**38**
Sodium azide: 2.0 µg/plate (+ control)	898	-	908	-
2-aminoanthracene: 2.5 µg/plate (+ control)	-	241	-	300

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*; Source: Myhre, 2016c

Under the conditions of this study IN-UJV12 proved positive for bacterial mutagenicity because of a reproducible positive response in *S. typhimurium* strain TA1535 (Myhre, 2016c).

Another Ames test was performed by means of the plate-incorporation method in which IN-UJV12 was applied, once again, to *S. typhimurium* strains TA98, TA100, TA1535 and TA1537, and *E. coli* strain WP2 *uvr*A. This time Batch IN-UJV12 (purity 95.8%) was used, (different to *study 3* by Myhre, 2016c). Based on the results of a preceding toxicity-mutation test, all tester strains were exposed to concentrations ranging from 333 to 5000 µg/plate, both in the presence and absence of S9 metabolic activation. No increase in revertant colonies over the vehicle control (DMSO) values was observed in any strain, including TA1535 this time. Appropriate positive control substances clearly gave the expected responses. No precipitation and no cytotoxicity were observed in any strain up to the limit concentration. To conclude, this study revealed no evidence of a mutagenic potential of IN-UJV12 in bacteria. Under the conditions of this study the positive findings observed previously with TA1535 (Myhre, 2016c) were not confirmed even though the study conditions were nearly identical. It is worth noting, however, that the purity of the test substance (95.8%) in this second experiment was still lower than in the study revealing the positive result (97.0%) suggesting that purity might not be an appropriate explanation for the positive outcome of the preceding study (Faranda, 2017).

A third Ames test with IN-UJV12 was performed by means of the plate-incorporation method on *S. typhimurium* strains TA98, TA100, TA1535 and TA1537, and *E. coli* strain WP2 *uvr*A, with and without metabolic activation. A batch of very high purity (99%) test article was used, different to that employed by Myhre (2016c) and Faranda (2017) and reported above. Since there was no evidence of toxicity in a preceding toxicity-mutation test, all tester strains were exposed to concentrations ranging from 333 to 5000 µg/plate. No increase in revertant colonies over the vehicle control (DMSO) values was observed in any strain, neither in the presence nor absence of S9 mix. Appropriate positive control substances gave the expected responses. No precipitation and no cytotoxicity were observed in any strain up to the limit concentration. Under the conditions of this study, no evidence of a mutagenic potential in bacteria for IN-UJV12 of high purity was obtained. Since humans will be exposed, via their diet to the pure metabolite, not to material from direct chemical synthesis that would contain unknown impurities, the Meeting agreed that this negative test was given more weight in risk assessment than the two contradictory studies that were performed previously, and in which IN-UJV12 of lower purity was tested (Myhre, 2021).

Two batches of IN-UJV12 of different purities were tested in an HPRT assay in CHO cells. Purity of batch IN-UJV12-003 was 97.0% as confirmed by a laboratory in India. Batch IN-UJV12-004 had a purity of 97.4%, analysis being undertaken this time in the Czech Republic. Both batches were dissolved in DMSO, which was also employed as negative (vehicle) control. Based on the outcome of a pretest for cytotoxicity and precipitation, the definitive mutagenicity assays were performed at concentrations of 0, 125, 250, 500, 1000 and 2000 µg/mL, both with and without metabolically activating S9 mix. When IN-UJV12-003 was tested in the presence of S9 mix, no increase in mutant frequency was observed at any concentration. Without activation however, a positive trend was observed and at the maximum concentration the difference from the vehicle control value nearly achieved statistical significance, just falling short of cut-off for $p < 0.01$.

Because of this borderline finding, the assay without activation was repeated at the same concentrations but this time with batch IN-UJV12-004. In this experiment, mutant frequency at all concentrations exceeded that of the vehicle control by at least four times, and mostly by more than ten times, but in the absence of any clear dose–response relationship, no positive trend could be discerned as would be expected if the test item were in fact genotoxic. Historical control data as provided in a very small table in the study report are of not much value because this HCD was obtained between 2012 and 2014, that is before the conduct of this study and, more importantly, includes a number of solvents such as culture medium, distilled water, or acetone, so is not based on DMSO alone as a vehicle.

In this study, no cytotoxicity became apparent up to the limit concentration of 2000 µg/mL in any of the experiments. The positive control substances consistently gave the expected mutagenic responses. An overview of the mutant frequencies is given in Table 47.

It can be concluded that IN-UJV12-003 was devoid of any potential to cause gene mutation in CHO cells at the hypoxanthine–guanine phosphoribosyl transferase locus in the presence of metabolic activation, whereas the outcome of the non-activation experiment was positive. On the other hand, the observed increase was moderate if compared to the positive control ethylmethanesulfonate, suggesting low or even equivocal mutagenic potency for Batch IN-UJV12-003. Furthermore, negative control data

suggest a large intrinsic variability in this study. The minimally purer Batch IN-UJV12-004 did not show a positive trend in a repeat test without activation, even though markedly higher mutant frequency were observed at all tested concentrations compared to the vehicle controls, a result that should not be ignored. On balance, no final conclusion with regard to the mutagenicity of IN-UJV12 towards mammalian cells can be drawn but, similar to the Ames tests above, the impact of impurities in the tested batches appears likely. It is notable that the same batch gave positive results in both the Ames test and in the HPRT assay which were not found reproducible when other batches were tested (Dutta, 2017b).

Table 47. Average mutant frequencies in the HPRT assays with two batches of IN-UJV12 (critical findings shown in bold)

Test item: concentration		IN-UVJ12-003, +S9	IN-UVJ12-003, −S9	IN-UVJ12-004, −S9
DMSO (vehicle, negative control)		5.41	0.77	0.26
IN-UJV12:	125 µg/mL	2.82	1.63	2.17
	250 µg/mL	0.59	1.55	3.17
	500 µg/mL	0.85	1.27	2.96
	1000 µg/mL	2.27	**2.61**	1.09
	2000 µg/mL	1.02	**5.30**	2.23
Ethylmethanesulfonate, (positive control):	0.2 µL/mL	-	309.69	338.27
Benzo(*a*)pyrene, (positive control):	4 µg/mL	118.48	-	-

Source: Dutta, 2017b

In an in vitro chromosome aberration study, cultured peripheral lymphocytes were exposed to IN-UJV12 (dissolved in DMSO) for four hours and for 22 hours in the absence of metabolic activation, or for four hours only in the presence of S9 mix. The lymphocytes had been obtained from a healthy man, 33 years old, for preliminary toxicity testing and from a healthy woman of same age for the chromosomal aberration assay. Cells were harvested after 22 hours. Based on the pretest, cytogenetic evaluations were conducted at concentrations of 500, 1000 and 2000 µg/mL for the four-hour test conditions with and without activation. Because of some cytotoxicity, cytogenetic analysis was confined to 250, 500 and 1000 µg/mL following the 22-hour exposure without S9 mix. Anonymized slides were scored for both structural and numerical chromosomal anomalies. At least 300 metaphases per concentration (150 from each duplicate culture) were analyzed.

Following a four-hour exposure, no statistically significant increase in structural chromosome aberrations were observed compared to vehicle (DMSO) control values, either with or without metabolic activation. In contrast, a significant increase and a positive trend for chromosome aberrations was observed when the lymphocytes had been treated for 22 hours (see Table 48). The increase at the maximum concentration of 1000 µg/mL was accompanied by cytotoxicity as demonstrated by a lower mitotic index. Appropriate positive control substances were applied and gave the expected responses proving the test system was functional. A small increase in numeric chromosome aberrations was also observed at the two upper dose levels, but this finding did not gain statistical significance.

Table 48. Summary of structural chromosome aberration in peripheral human lymphocytes after 22-hour treatment with IN-UJV12 or with the positive control substance

Treatment: concentration	Activation	Mitotic index (percentage)	Cells scored	Structural aberrations	Numerical aberrations
Vehicle control (DMSO)	−S9	5.7	300	1.3%	0.0%
IN-UJV12: 250 µg/mL	−S9	3.5	300	1.3%	0.0%
500 µg/mL	−S9	3.4	300	2.7%	0.3%
1000 µg/mL	−S9	2.4	300	5.0%*[#]	0.7%
Mitomycin C: 0.2 µg/mL	−S9	3.6	100	23.0%*	0.0%

* Statistically significant difference using Fisher's test, $p < 0.05\%$; Source: Kellum, 2017b

[#] Statistically significant dose response using Cochran–Armitage test, $p < 0.05$

On balance, metabolite IN-UJV12 was considered clastogenic under the conditions of this in vitro study. No robust conclusion with regard to aneugenic potential could be drawn (Kellum, 2017b).

To examine the potential clastogenic or aneugenic activity of IN-UJV12 in vivo, a micronucleus assay was run in Crl:CD(SD) rats. Five or six (high-dose group only) animals per sex received the test item (mixture of two batches of IN-UJV12 with similar purities of 97.4% and 98.2%, due to limited substance availability) once by oral gavage at dose levels of 500, 1000 or 2000 mg/kg bw. The concurrent negative and positive control groups also comprised five rats per sex. The negative control group received the vehicle, (0.1% Tween 80 in 0.5% aqueous methylcellulose) and cyclophosphamide at a dose of 10 mg/kg bw, dissolved in deionized water served as the positive control.

The analysis was performed by means of flow cytometric examination of red blood cells in samples of blood taken from the tail vein at 48 hours, except for the vehicle control group and five high-dose animals per sex that were sampled at 72 hours. The frequency of reticulocytes (immature erythrocytes) among all RBCs was determined, and 20 000 reticulocytes per blood sample were scored for the abundance of micronuclei. Plasma analysis for the test compound was also performed to investigate target tissue exposure. For this latter purpose blood was collected two hours after dosing from four male and four female rats receiving the low dose of 500 mg/kg bw. Pooled plasma samples were analyzed by ultra-high performance liquid chromatography coupled with LC-MS/MS).

No mortality occurred during the study. No clinical signs of toxicity were observed and body weight and its gain were unaffected. There was no significant or biologically meaningful increase in micronucleated reticulocytes compared to the vehicle control values in any dose group, either in males or females. Likewise, no decrease was observed in reticulocytes as a percentage of all RBCs. The positive control substance caused both an increase in micronucleus frequency and a depression in total reticulocytes. Plasma concentrations in the low-dose groups were 2740 and 1740 ng/mL in males and females, respectively. The downstream metabolite IN-A5760 was found in traces, below 10 ng/mL.

To conclude, metabolite IN-UJV12 proved negative in the in vivo micronucleus assay in rats. Based on plasma analysis it can be reasonably assumed that the target organ (bone marrow) was exposed even though the distribution and elimination rate of this metabolite in the rat are not known and, therefore, duration of exposure cannot even be estimated (Myhre, 2017e; Himmelstein, 2018c).

(h) Metabolite IN-TQD54 (No IUPAC name available)

The available toxicological information for this metabolite, which occurs in small amounts in some crops, is limited. It was tested for acute oral toxicity in the rat and for two genotoxic end-points in vitro.

Acute toxicity

A single oral dose of metabolite IN-TQD54, suspended in 0.1% Tween 80 (v/v) in 0.5% aqueous methylcellulose, was administered by oral gavage to four fasted female Crl:CD(SD) rats at dose levels of 0, 1750 or 5000 mg/kg bw. The animals were dosed one at a time at a minimum of 48-hour intervals following the up-and-down procedure, using 1750 mg/kg bw as the initial dose which was given to a single female only. Rats were observed for clinical signs of toxicity, body weight effects and mortality for up to 14 days after dosing before they were killed and examined to detect observable evidence of gross organ or tissue damage. No unscheduled deaths occurred and no clinical signs of toxicity or gross lesions were observed. Body weight loss was noted in the rat receiving 1750 mg/kg bw from day 8 to day 15 post dosing, but this finding cannot be regarded as an effect of treatment since all three animals at the high-dose level gained weight during this period. Accordingly, the LD_{50} was greater than 5000 mg/kg bw demonstrating the low toxicity of metabolite IN-TQD54 (Fallers, 2016d).

Genotoxicity

An Ames test using the plate-incorporation method was performed with *S. typhimurium* strains TA1535, TA1537, TA98, and TA100, and *E. coli* strain WP2 *uvr*A. Based on the results of a preceding toxicity-mutation test, IN-TQD54 was applied to all tester strains at concentrations ranging from 333 to 5000 µg/plate in the absence and presence of S9 metabolic activation. No increase in revertant colonies over the vehicle control (sterile water) values was observed in any strain. Appropriate positive control substances gave the expected responses. No precipitation or cytotoxicity were observed. Under the conditions of this assay metabolite IN-TQD54 proved negative for bacterial mutagenicity (Myhre, 2016d).

In an in vitro chromosome aberration study, cultured peripheral lymphocytes obtained from a healthy woman, 30 years old, were exposed to metabolite IN-TQD54, dissolved in sterile water, for four hours or 22 hours in the absence of metabolic activation, or for four hours only in the presence of S9 mix. Cells were harvested after 22 hours. Based on preliminary testing for precipitation and cytotoxicity, cytogenetic evaluations were conducted at concentrations of 0, 500, 1000 and 2000 µg/mL for all three test conditions. Anonymized slides were scored for both structural and numerical chromosomal anomalies. At least 300 metaphases per concentration (150 from each duplicate culture) were analyzed. No statistically significant increase in structural chromosome aberrations was observed compared to vehicle control values. Appropriate positive control substances were applied and gave the expected responses. There was also no evidence of an increase in numeric aberrations in this study. Accordingly, metabolite IN-TQD54 was found to be devoid of any clastogenic potential under the conditions of this study (Kellum, 2017c).

(i) Metabolite IN-VM862: 2-Amino-3-chloro-5-trifluoromethyl pyridine

The metabolite IN-VM862 occurs in soil and to a very minor extent in rotational crops, but has not been detected in rat metabolism. A number of studies were submitted by the sponsor for this metabolite, which differs from all the previously described biotransformation products in that it had already been subject to comprehensive toxicological assessment as a "new industrial chemical" (CAS no. 79456-26-1) under the code name P-007 in the 1980s and 1990s. Also, it is an intermediate in the synthesis of the fungicide fluazinam.

Acute toxicity

Compound P-007 was tested for acute oral toxicity in male and female CD rats at doses ranging from 102 to 800 mg/kg bw. Following administration of the lowest dose, a transient decrease in motor activity was observed. The first dose causing mortality (1/5 males and 2/5 females) was 202 mg/kg bw; after administration of 402 and 800 mg/kg bw 9 out of 10 or even all animals died. Principal signs of toxicity in these dose groups, either preceding death or, in survivors resolving by day 4 after dosing, included lethargy, ataxia, laboured breathing and unconsciousness. Gross pathological findings in the decedents were stained fur and skin, abnormal gut contents and dark serous fluid in the bladder. For the sexes combined, an LD_{50} of 249 mg/kg bw was calculated, with single sex values of 229 mg/kg bw for males and 251 mg/kg bw for females.

In a limit test, the acute dermal toxicity of this compound in the rat was above 2000 mg/kg bw since no deaths and no clinical signs, systemic or local, were observed. Consistent with this, P-007 was not irritating to the skin when tested on rabbits. In an eye irritation test, only very slight and transient signs were observed and no classification was warranted. Also, P-007 proved negative in a Magnusson & Kligman maximization test for skin sensitization in Guinea pigs.

Short-term studies of toxicity

Short-term toxicity of P-007 was investigated in a four-week study with gavage administration to CD rats which received the compound at daily doses of 0, 2, 10 or 50 mg/kg bw. No mortality occurred and no clinical signs were attributed to treatment. A NOEL of 2 mg/kg bw per day was proposed by the original study author, based on slight reductions in body weight, a higher liver weight without concomitant morphological findings, and an increase in plasma urea at 10 mg/kg bw. Because of the limited nature of the changes, the Meeting considered 10 mg/kg bw to be the NOAEL. At the top dose, reductions in body weight and food consumption were noted. Some alterations in haematological and clinical chemistry parameters were observed and liver weight increase was accompanied by hepatocellular hypertrophy.

Genotoxicity

Compound P-007 proved negative for bacterial mutagenicity in the Ames test with *S. typhimurium* strains TA1535, TA1537, TA98 and TA100 up to the highest tested concentration of 2500 µg/plate. In an in vitro study for chromosome aberrations in peripheral human lymphocytes, the result was negative with metabolic activation up to the highest concentration of 100 µg/mL, but was equivocal for the test under non-activation conditions. However, the assay did not comply with current standards and no final conclusion can be drawn (Cummins et al., 1987).

A 90-day study with gavage administration of P-007 was run in Sprague Dawley rats. Groups of

ten male and ten female rats were given daily doses of 0 (vehicle control, 0.5% aqueous methylcellulose), 2, 10, or 50 mg/kg bw. The rats were monitored daily for mortality and clinical signs of toxicity. Ophthalmoscopy was performed prior to treatment and during week 13. Samples for haematological examination, clinical chemistry and urinalysis were also obtained during the last week of treatment. At termination, all animals were killed and gross examination carried out. Selected organs were weighed and subjected to histopathology.

No unscheduled deaths occurred and the only clinical sign was excess salivation at the top dose level. Body weight and food intake were not affected. Haematology revealed an increase in neutrophils at the top dose and, in some animals, at 10 mg/kg bw per day, but in the absence of further blood changes, this finding could not be regarded as adverse. An increase in total protein was noted in both sexes at the two upper dose levels. Cholesterol and, in female rats, calcium were elevated at 50 mg/kg bw per day. At the same dose level, a higher urine volume and lower specific gravity was noted. Proteinuria was observed in both sexes at the top dose level and also in males receiving the mid dose of 10 mg/kg bw per day.

Livers, kidneys and mandibular lymph nodes were enlarged in many or all animals from the highest dose group. An increase in liver size was also apparent in some mid-dose males. This finding was accompanied by hepatocellular hypertrophy. Microscopic kidney findings at the two upper dose levels comprised tubular dilatation and basophilia, also acidophilic globules in the cortical epithelium and eosinophilic casts. Incidence and severity appeared to increase with dose. In the mandibular lymph nodes, hyperplasia, histiocytosis, plasmocytosis and epitheloid cell aggregation were noted in the mid-dose group and, to a greater degree in the high-dose group as well. In a few females given 10 mg/kg bw per day and in most females receiving the highest dose, the number of endometrial glands was increased. The NOAEL in this study was the lowest dose of 2 mg/kg bw per day (Sauvez, 1994).

In addition, a small series of more recent studies with IN-VM862 was performed on behalf of the sponsor for fluazaindolizine.

In an acute oral study in Wistar rats, a single dose of 300 mg/kg bw was administered to three males and three females. All animals survived and transient lethargy was the only clinical sign of toxicity. Apparently this study was intended as a follow-up to the previous experiment by Cummings et al. (1987, see *study 1* above) to clarify on the appropriate classification for oral toxicity in which 300 mg/kg bw is an important threshold (Verma, 2020).

An Ames test using the plate-incorporation method was performed with *S. typhimurium* strains TA1535, TA1537, TA98 and TA100 and *E. coli* strain WP2 *uvr*A. Based on the results of a preceding toxicity-mutation test, IN-VM862 was applied to all tester strains at concentrations ranging from 333 to 5000 µg/plate in the presence of S9 metabolic activation and at concentration between 100 and 3333 µg/plate without. No increase in revertant colonies over the vehicle control (DMSO) values was observed with any strain. Appropriate positive control substances clearly gave the expected responses. No precipitation was seen, but some cytotoxicity (reduction in background lawn and greater than 50% reduction in revertant colonies) was observed from 3333 µg/plate upwards, depending on strain and metabolic activation conditions. The test item proved negative for bacterial mutagenicity in this in vitro assay (Myhre, 2016e).

Metabolite IN-VM862 was tested in CHO cells for its ability to cause forward gene mutations at the hypoxanthine–guanine phosphoribosyl transferase locus (HPRT assay). The test substance was dissolved in DMSO. Experiments were performed at concentrations of up to 500 µg/mL in the presence of metabolically activating S9 mix, and at concentrations ranging from 25 to 1000 µg/mL without activation. Some precipitation was observed at higher concentrations but evaluation for mutagenicity was still possible. No increase in mutant frequency was observed with metabolite IN-VM862, whereas the positive control substances induced the expected response. The metabolite therefore proved negative for mutagenicity under conditions of the HPRT assay (Dutta, 2017c).

In an in vitro chromosome aberration study, cultured peripheral lymphocytes obtained from a healthy woman, 32 years old, were exposed to IN-VM862 for four hours and 22 hours in the absence of metabolic activation, or for four hours only in the presence of S9 mix. Cells were harvested after 22 hours. Based on preliminary testing for precipitation and cytotoxicity, cytogenetic evaluations were conducted at concentrations of 0, 25, 50, and 100 µg/mL under the four-hour, non-activated test conditions because

of a serious reduction in the mitotic index at higher concentrations. For the same reason, concentrations of 0, 50, 100 and 150 µg/mL were selected for analysis following the four-hour treatment with activation and the 22-hour non-activated treatment conditions. Anonymized slides were scored for both structural and numerical chromosomal anomalies. At least 300 metaphases per concentration (150 from each duplicate culture) were analyzed. Appropriate positive control substances were applied and gave the expected responses. No statistically significant increase in structural chromosome aberrations, compared to the vehicle (DMSO) control values was observed. There was also no evidence of numerical aberrations in this study. Accordingly, IN-VM862 was found to be devoid of any clastogenic or aneugenic potential in this in vitro assay (Kellum, 2016f).

(j) Metabolite IN-RYC33; 8-Chloro-6-(trifluoromethyl)imidazo[1,2-a]pyridine-2-carboxamide

Metabolite IN-RYC33 is a minor rat metabolite that is structurally very similar to the well investigated major rat metabolite IN-QEK31, but apparently derives directly from the parent substance (see Fig. 2). It was found in the urine of female rats and in bile in very small amounts of up to 0.2% of AD. It occurs in plant metabolism and was found also in animal matrices. No toxicological or genotoxicity data was submitted. During the Meeting a QSAR analysis (OASIS; ISS) was run and did not return any alerts, additional to those for the parent compound, for in vitro or in vivo genotoxicity, or for the other tested toxicological end-points.

(k) Impurities

No studies on impurities were submitted even though the apparent differences between various batches of fluazaindolizine with regard to their potential to cause irritation and skin sensitization suggest a possible impact. At least, five impurities (R2Y16, QJA58, RXZ20, TKE32 and WCW73) were predicted negative for bacterial (Ames test) mutagenicity in two independent in silico systems (Zhang, 2019; see section 2.4 for details).

3. Observations in humans

No adverse health effects on manufacturing plant personnel were noted. At the time when this monograph was prepared no reports on poisoning incidents were available. It must be acknowledged however, that fluazaindolizine is a new compound therefore the number of people exposed to this substance or its metabolites is as yet very limited.

4. Microbiological aspects

There was no information available in the public domain, and no experimental data were submitted which addressed the possible impact of fluazaindolizine residues on the human intestinal microbiome.

Comments

Biochemical aspects

Fluazaindolizine was subject to extensive investigations of its toxicokinetic behaviour and metabolism in the rat and to a much lesser extent in other species and in vitro. For the absorption, distribution, metabolism and excretion (ADME) studies in rats and mice, the test substance was ^{14}C-radiolabelled in the imidazopyridine moiety or in the phenyl ring.

Following oral administration of a single low dose of 10 mg/kg body weight (bw) to rats, rapid absorption was observed, the time to reach maximum concentration (T_{max}) being 0.25–0.625 hours after administration: this was slower (T_{max} at 3–6 hours) after a single high dose of 200 mg/kg bw. Based on urinary and biliary excretion, an oral absorption of 52–60% was estimated for the low dose whereas absorption of the high dose was slightly lower at 45–50%. The applied radioactivity was widely distributed throughout the body but total organ and tissue residues after seven days accounted for less than 0.5% of the applied dose, irrespective of dose or repeated administration of unlabelled compound. Highest concentrations were consistently found in plasma, followed by liver and kidneys. There was no evidence of accumulation.

Elimination was virtually complete after seven days with the major part of radioactivity excreted within the first 24 hours. At the low dose, elimination via urine and faeces was more or less equal, accounting for 41–54% for the two routes in the different groups and experiments. Repeated low dose administration did not alter this pattern. Excretion via bile was variable, ranging from 5–18% in the various groups, Excretion via exhalation was negligible.

Metabolism in the rat was limited and the major part of the administered dose (AD) excreted chemically unchanged. However several metabolites were identified resulting from *O*-demethylation, hydroxylation of the phenyl ring, and hydrolysis of the amide bond. A few metabolites were further conjugated. The only metabolite excreted in appreciable quantities was IN-QEK31. This metabolite amounted to 8–10% of AD in urine and bile when combined (Punler & Green, 2016a, 2017).

In the mouse, elimination was similar in rapidity to that seen in the rat and virtually complete, but faecal elimination was by far the predominant route. Biliary excretion was not investigated in the mouse. Similar to the rat, metabolism was limited and metabolic pathways and resulting metabolites were the same in both species (Punler & Green, 2016b).

A qualitatively similar metabolism to that seen in vivo was confirmed by a comparative in vitro study in which hepatocytes of mouse, rat, rabbit, dog and human origin were used. In all these species, metabolism was similar even though the most rapid biotransformation took place in human cells with dog liver cells showing the lowest metabolic rate. Again, unchanged parent was the most abundant analyte. Metabolites were the same as in vivo but there were quantitative differences between species. No unique human metabolite was found (Novo, 2018).

Analysis of blood samples obtained from routine toxicological studies of different duration (14 days to approximately one year) revealed an increase in plasma concentrations of fluazaindolizine with dose. This increase, however, was partly sublinear suggesting a saturation of absorption at doses above 44 mg/kg bw per day (300 ppm) in mice and greater than 29 mg/kg bw per day (500 ppm) in female rats, but the effect was not seen in male rats or in dogs. The same metabolites as in the ADME studies were found in plasma but in very much lower amounts than the parent which was consistently the predominant compound (Han, 2014, 2016, 2017, 2018; MacKenzie, 2013; Moon, 2018).

Toxicological data

The acute oral median lethal dose (LD_{50}) of fluazaindolizine in rats ranged from 940 to 1248 mg/kg bw for different batches (Fallers, 2017a, 2018, 2019).

Dermal LD_{50} values of greater than 2000 mg/kg bw or even greater than 5000 mg/kg bw were reported (Lowe, 2013a; Slonina, 2018a). Inhalation median lethal concentrations (LC_{50}) of greater than 5.3 mg/L, and greater than 5.8 mg/L were reported (Kegelman, 2020; Ng, 2013).

The compound was either not, or only marginally, irritating to the skin (Lowe, 2013b; Slonina, 2018b) or to the eyes of rabbits (Lowe, 2017; Slonina, 2020). Studies for skin sensitization revealed contradictory results depending on the batch tested. The current technical fluazaindolizine was not sensitizing, though some older batches showed evidence of sensitizing potential (Hoban, 2017a, 2018a; Lowe, 2013c, Merrill, 2017).

In repeat-dose studies target organs were the kidney in rodents and the liver in dogs.

In a 90-day study in mice, dietary doses of 0, 200, 1000, 3000 or 7000 ppm (equal to 0, 44, 146, 444 and 1101 mg/kg bw per day for males, 0, 50, 157, 511 and 1177 mg/kg bw per day for females) were administered. The NOAEL was 1000 ppm (equal to 146 mg/kg bw per day), based on histopathological findings in the gallbladder and kidneys in both sexes at 3000 ppm (equal to 444 mg/kg bw per day) (Han, 2018).

In a combined 90-day study of general toxicity and neurotoxicity (see also below) in rats, fluazaindolizine was fed at dietary doses of 0, 500, 1500, 3000 or 6000 ppm (equal to 0, 28, 84, 166 and 348 mg/kg bw per day for males, 0, 31, 97, 189 and 376 mg/kg bw per day for females). The NOAEL was 1500 ppm (equal to 84 mg/kg bw per day) based on histopathological findings in the kidney at 3000 ppm (equal to 166 mg/kg bw per day) (MacKenzie, 2013).

In a 90-day toxicity feeding study in dogs, the initial dose levels were 0, 125, 500, 1500 or 4000 ppm. The maximum dose was reduced because of lower food intake, first to 3000 ppm and then, because of one female animal's death, to 2500 ppm. The corresponding mean daily intakes were approximately 0, 5.5, 20, 59 and 68 mg/kg bw for males, and for females 0, 5.1, 21, 61, and 93 mg/kg bw. The NOAEL was 500 ppm (equal to 20 mg/kg bw per day) based on mild histopathological and clinical chemistry findings of liver toxicity, and a lower body weight gain in males at 1500 ppm (equal to 59 mg/kg bw per day) (Han, 2014).

In a one-year study in dogs, doses of 0, 125, 500, 1000 or 2000 ppm (equal to 0, 4.4, 20, 36 and 66 mg/kg bw per day for males, 0, 4.6, 17, 37 and 70 mg/kg bw per day for females) were applied. The NOAEL of 1000 ppm (equal to 36 mg/kg bw per day) was based on transient body weight losses, lower food consumption and food efficiency in both sexes as well as on mild anaemia and one death in females at 2000 ppm (equal to 66 mg/kg bw per day) (Han, 2016).

The overall NOAEL in dogs was 36 mg/kg bw per day with a LOAEL of 59 mg/kg bw per day.

In an 18-month carcinogenicity study in mice, fluazaindolizine was administered at dietary doses of 0, 100, 300, 1000 or 3000 ppm (equal to 0, 15, 43, 142 and 427 mg/kg bw per day for males, 0, 17, 54, 177, and 525 mg/kg bw per day for females). The NOAEL was 1000 ppm (equal to 142 mg/kg bw per day), based on increased incidences of amyloidosis in numerous organs in males and females at 3000 ppm (equal to 427 mg/kg bw per day). The NOAEL for carcinogenicity was 3000 ppm (equal to 427 mg/kg bw per day), the highest dose tested (Han, 2017, 2021).

In a combined chronic toxicity and carcinogenicity study in rats, fluazaindolizine was administered over 12 or 24 months. Over 12 months the nominal concentrations were 0, 150, 500, 1500 or 4500 ppm (equal to 0, 6.5, 25, 76 and 241 mg/kg bw per day for males, 0, 6.8, 27, 78 or 254 mg/kg bw per day for females). The NOAEL for long-term toxicity was 1500 ppm (equal to 76 mg/kg bw per day) based on histopathological findings and a few gross lesions of the kidneys in both sexes, an increase in relative kidney weight in females and marginal urinalysis findings in males at 4500 ppm (equal to 241 mg/kg bw per day). The NOAEL for carcinogenicity was 4500 ppm (equal to 241 mg/kg bw per day), the highest dose tested (Moon, 2018).

The meeting concluded that fluazaindolizine is not carcinogenic in mice or rats.

Fluazaindolizine was tested for genotoxicity in an adequate range of studies in vitro and in vivo. The in vitro studies for gene mutation in bacteria (Faranda, 2018: Myhre, 2012, 2017a, 2019; Wagner, 2013) and mammalian cells (Dutta, 2017a; Dutta & VanDyke, 2018) were all negative. Various batches of fluazaindolizine were tested. By contrast, the compound proved positive for chromosome aberrations in peripheral human lymphocytes (Kellum, 2017a; Roy & Jois, 2013). However, when the same (and also other) batches of fluazaindolizine were tested in the in vivo micronucleus assay in mice with examination either of bone marrow cells or peripheral reticulocytes, the results from these studies

were consistently negative (Myhre, 2014, 2017b, 2018a).

The Meeting concluded that fluazaindolizine is unlikely to be genotoxic in vivo.

In view of the lack of in vivo genotoxicity and the absence of carcinogenicity in mice and rats, the Meeting concluded that fluazaindolizine is unlikely to pose a carcinogenic risk to humans.

In a two-generation study, fluazaindolizine was administered to rats at dietary concentrations of 0, 150, 500, 1500 or 4500 ppm. During certain study intervals, namely the lactation periods in F0 and F1 females and up to postnatal day (PND) 42 for the F1 offspring, these dietary doses were reduced to 90, 300, 900 and 2700 ppm to adjust for increased food consumption and compound intake. The overall mean daily intakes in the parental generation were lowest in males during the premating phase and were calculated as 0, 9, 30, 88 and 265 mg/kg bw. The parental NOAEL was 1500/900 ppm (equal to 88 mg/kg bw per day) based on lower body weight gain and reduced food consumption, gross and histopathological lesions in the kidneys and the urinary tract, supported by urinalysis findings at 4500 ppm (equal to 265 mg/kg bw per day). The reproductive NOAEL was the highest dose tested, 4500/2700 ppm (equal to 265 mg/kg bw per day). Since microscopic lesions in the kidney and urinary bladder were observed in weanlings at the two upper dose levels, an offspring NOAEL of 500/300 ppm (equal to 30 mg/kg bw per day) was identified (Munley, 2020).

In a developmental study in rats, fluazaindolizine was administered by oral gavage from GD 6 to GD 20 at dose levels of 0, 35, 100, 200 or 400 mg/kg bw per day. The maternal NOAEL was 200 mg/kg bw per day based on lower food consumption throughout the treatment period and initial body weight losses followed by lower body weight gain for the rest of the study at 400 mg/kg bw per day. The developmental NOAEL was 200 mg/kg bw per day based on lower mean fetal weight at 400 mg/kg bw per day (Munley 2017b).

In a developmental study in rabbits, fluazaindolizine was administered by oral gavage at doses of 0, 10, 30 or 120 mg/kg bw per day from days 7–28 of presumed gestation. The maternal NOAEL was 30 mg/kg bw per day, based on poor condition in a number of does at 120 mg/kg bw per day that was characterized by lower food intake, a decrease in body weight gain and transient body weight losses, followed by abortion or premature killing for humane reasons. There was histopathological evidence of nephrotoxicity and liver toxicity, along with haemorrhages in the stomach and urinary tract. In the absence of any adverse effects the NOAEL for developmental toxicity was 120 mg/kg bw per day, the highest dose tested (Wirbisky, 2017).

The Meeting concluded that fluazaindolizine is not teratogenic.

In an acute neurotoxicity study in rats, doses of 0, 30, 125, 450 or 1750 mg/kg bw were administered by oral gavage. The systemic NOAEL in this study was 125 mg/kg bw based on effects on body weight and feed consumption, lower body temperature on day of dosing and a transient decrease in motor activity at 450 mg/kg bw per day. The NOAEL for acute neurotoxicity was 1750 mg/kg bw, the highest dose tested (Mukerji, 2017).

In a combined general toxicity and neurotoxicity study over 90 days (see above) no evidence of neurotoxicity was observed up to 6000 ppm (equal to 348 mg/kg bw per day), the highest dose tested (MacKenzie, 2013).

The Meeting concluded that fluazaindolizine is not neurotoxic.

In a four-week dietary immunotoxicity study, fluazaindolizine was fed to male rats at dietary concentrations of 0, 500, 1500 or 5500 ppm (equal to 0, 35.5, 106 and 393 mg/kg bw per day). The NOAEL for immunotoxicity was 5500 ppm (equal to 393 mg/kg bw per day), the highest dose tested (Hoban, 2018b).

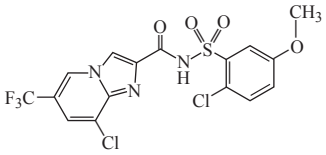
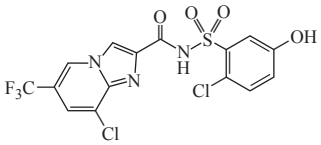
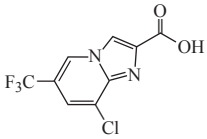
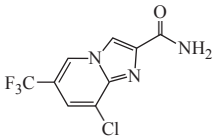
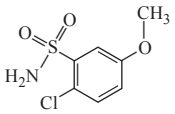
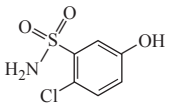
The Meeting concluded that fluazaindolizine is not immunotoxic.

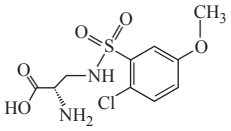
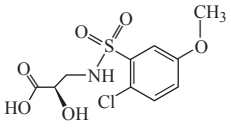
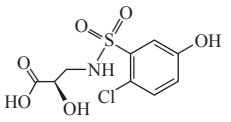
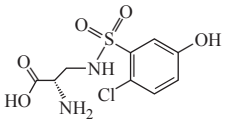
A small number of mechanistic studies were performed to investigate the potential effects of fluazaindolizine on the endocrine system even though no such evidence was obtained in the many routine toxicological studies carried out. These studies were not informative for risk assessment.

Toxicological data on metabolites and/or degradates

A number of plant and animal metabolites were examined for various toxicological end-points.

Overview of toxicological characterization of plant/livestock metabolites of fluazaindolizine (parent compound included for comparison)

Compound/codes and chemical structure	Major rat metabolite (≥ 10% of AD)?	Genotoxicity assessment	General toxicity	Conclusion on toxicological reference values
Fluazaindolizine (parent) 	Parent	Not genotoxic in vivo	Full dataset	ADI: 0.3 mg/kg bw ARfD: 1.0 mg/kg bw
IN-REG72 	No; Minor rat and food animal metabolite	Not genotoxic (studies in vitro and in vivo)	No data, but covered by parent (structural similarity)	Covered by parent ADI and ARfD
IN-QEK31 	Yes; (up to 8–10% in urine and bile if combined); Food animal, crop, soil and water metabolite	Not genotoxic in vivo (studies)	Comprehensive data set; not more toxic than parent	Covered by parent ADI and ARfD
IN-RYC33 	No; Animal (hen) metabolite	No data; negative QSAR prediction (OASIS, ISS)	No data, but covered by IN-QEK31 (structural similarity) and consequently by parent	Covered by parent ADI and ARfD
IN-F4106 	No; Rat and food animal, soil and water metabolite; Also process intermediate in manufacture of parent	Not genotoxic in vivo (studies)	Comprehensive data set; not more toxic than parent	Covered by parent ADI and ARfD
IN-A5760 	No; Rat and food animal, crop, water and soil metabolite	Not genotoxic in vivo (studies)	No data, but covered by IN-F4106 (structural similarity and further biotransformation) and consequently by parent	Covered by parent ADI and ARfD

Compound/codes and chemical structure	Major rat metabolite (≥ 10% of AD)?	Genotoxicity assessment	General toxicity	Conclusion on toxicological reference values
IN-QZY47 	No; Rodent intermediate but not present intact in detectable quantities; Crop metabolite	Not genotoxic (in vitro data)	Limited database suggesting lower toxicity than parent	Covered by parent ADI and ARfD
IN-TMQ01 	No; Rat intermediate but not present intact in detectable quantities; Crop metabolite	Not genotoxic (in vitro data)	Limited database suggesting lower toxicity than parent	Covered by parent ADI and ARfD
IN-TQD54 	No; Crop metabolite	Not genotoxic (in vitro data)	Low acute oral toxicity in the rat; no further data	TTC for nongenotoxic compounds (Cramer class III) should be used (1.5 µg/kg bw/day)
IN-UJV12 	No; Crop metabolite	Unlikely to be genotoxic (studies)	Low acute oral toxicity in the rat; no further data	TTC for nongenotoxic compounds (Cramer class III) should be used (1.5 µg/kg bw/day)

AD: Administered dose; ADI: Acceptable daily intake;
ARfD: Acute reference dose; TTC: Threshold of toxicological concern

Microbiological data

There was no information available in the public domain, and no experimental data were submitted which addressed the possible impact of fluazaindolizine residues on the human intestinal microbiome.

Human data

From health observations in manufacturing personnel, no adverse effects had been reported. No information on poisoning incidents were available.

The Meeting concluded that the existing database on fluazaindolizine was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

Toxicological evaluation

The Meeting established an ADI of 0–0.3 mg/kg bw for fluazaindolizine based on the NOAEL of 30 mg/kg bw per day for maternal toxicity in the developmental toxicity study in rabbits, and offspring toxicity in the two-generation study in rats and using a safety factor of 100. The ADI is supported by the NOAEL of 36 mg/kg bw per day from the one-year study in dogs.

The Meeting established an ARfD of 1.0 mg/kg bw for fluazaindolizine from the NOAEL of 125 mg/kg bw for systemic toxicity in the acute neurotoxicity study in rats and using a safety factor of 100.

Levels relevant to risk assessment of fluazaindolizine

Species	Study	Effect	NOAEL	LOAEL
Mouse	78-week study of toxicity and carcinogenicity [a]	Toxicity	1000 ppm, equal to 142 mg/kg bw per day	3000 ppm, equal to 427 mg/kg bw per day
		Carcinogenicity	3000 ppm, equal to 427 mg/kg bw per day [c]	-
Rat	Acute neurotoxicity study [b]	Systemic toxicity	125 mg/kg bw	450 mg/kg bw per day
	Two-year study of toxicity and carcinogenicity [a]	Toxicity	1500 ppm, equal to 76 mg/kg bw per day	4500 ppm, equal to 241 mg/kg bw per day
		Carcinogenicity	4500 ppm, equal to 241 mg/kg bw per day [c]	-
	Two-generation study of reproductive toxicity [a]	Reproductive toxicity	4500/2700 ppm equal to 265 mg/kg bw per day [c]	-
		Parental toxicity	1500/900 ppm equal to 88 mg/kg bw per day	4500/2700 ppm equal to 265 mg/kg bw per day
		Offspring toxicity	500/300 ppm equal to 30 mg/kg bw per day	1500/900 ppm equal to 88 mg/kg bw per day
	Developmental toxicity study [b]	Maternal toxicity	200 mg/kg bw per day	400 mg/kg bw per day
		Embryo/fetal toxicity	200 mg/kg bw per day	400 mg/kg bw per day
Rabbit	Developmental toxicity study [b]	Maternal toxicity	30 mg/kg bw per day	120 mg/kg bw per day
		Embryo/fetal toxicity	120 mg/kg bw per day [c]	-
Dog	90-day and one-year studies [a,d]	Toxicity	1000 ppm, equal to 36 mg/kg bw per day	1500 ppm, equal to 59 mg/kg bw per day

[a] Dietary administration [b] Gavage administration [c] Highest dose tested [d] Two studies combined

Acceptable daily intake (ADI), applies to fluazaindolizine, IN-QEK31, IN-REG72, IN-QZY47, IN-TMQ01, IN-F4106, IN-A5760 and IN-RYC33

0–0.3 mg/kg bw

Acute reference dose (ARfD), applies to fluazaindolizine, IN-QEK31, IN-REG72, IN-QZY47, IN-TMQ01, IN-F4106, IN-A5760 and IN-RYC33

1.0 mg/kg bw

Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure.

Critical end-points for setting guidance values for exposure to fluazaindolizine

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid (T_{max} 0.25–0.625 h at low dose of 10 mg/kg bw); Absorption 52–60% at low dose, 45–50% at high dose of 200 mg/kg bw
Dermal absorption	No data
Distribution	Widely distributed, highest residues in plasma and liver; Much of non-absorbed portion found in GIT and contents
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	Nearly complete within 7 days; mostly excreted within 24 h
Metabolism in animals	Limited; at least 7 metabolites present in small amounts; Mainly hydroxylation of the phenyl ring, *O*-methylation and cleavage of amide bond, sometimes followed by conjugations
Toxicologically significant compounds in animals and plants	Fluazaindolizine, IN-QEK31, IN-REG72, IN-QZY47, IN-TMQ01, IN-F4106, IN-A5760 and IN-RCY33
Acute toxicity	
Rat, LD_{50}, oral	940 mg/kg bw
Rat, LD_{50}, dermal	> 5000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.8 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Not irritating
Dermal sensitization	Not sensitizing (M & K test, LLNA) when batches from current manufacturing process were tested
Short-term studies of toxicity	
Target/critical effect	Body weight, feed consumption, anaemia, mortality, liver (dog); kidney (rat, mouse); gallbladder, (mouse)
Lowest relevant oral NOAEL	36 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	1000 mg/kg bw per day (rat)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Amyloidosis (mouse); nephrotoxicity (rat)
Lowest relevant NOAEL	76 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic in mice or rats
Genotoxicity	Unlikely to be genotoxic in vivo
Reproductive toxicity	
Target/critical effect	Food consumption and body weight (parental); microscopic lesions in kidney and urinary bladder (parental and offspring)
Lowest relevant parental NOAEL	88 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	30 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	265 mg/kg bw per day, highest dose tested (rat)
Developmental toxicity	
Target/critical effect	Body weight gain and food intake (rat) Reduced food intake and body weight, abortions and mortality (rabbit) Reduced fetal weight (rat)
Lowest relevant maternal NOAEL	30 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	200 mg/kg bw per day (rat), 120 mg/kg bw per day, highest dose tested (rabbit)

Neurotoxicity	
Acute neurotoxicity NOAEL	125 mg/kg bw, for systemic toxicity (rat) 1750 mg/kg bw, highest dose tested, for neurotoxicity (rat)
Subchronic neurotoxicity NOAEL	348 mg/kg bw per day, the highest dose tested (rat)
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity	393 mg/kg bw per day, the highest dose tested (rat)
Studies on toxicologically relevant metabolites	
IN-QEK31	Not genotoxic in vivo, toxicity similar to parent compound (experimental studies on acute, short-term, reproductive and developmental toxicity)
IN-REG72	Not genotoxic in vitro or in vivo
IN-F4106	Not genotoxic in vivo, toxicity similar to parent compound (experimental studies on acute, short-term, reproductive and developmental toxicity)
IN-A5760	Not genotoxic in vivo
IN-QZY47	Not genotoxic (in vitro data), very low acute oral toxicity, short-term toxicity less severe than with parent
IN-TMQ01	Not genotoxic (in vitro data), very low acute oral toxicity, no effects in short-term toxicity studies up to 847 mg/kg bw per day (less toxic than parent)
IN-TQD54	Not genotoxic (in vitro data), very low acute oral toxicity
IN-UJV12	Unlikely to be genotoxic
Microbiological data	No data available
Human data	Not available for this new compound; no evidence of health effects in manufacturing plant personnel

Summary

	Value	Study	Safety factor
ADI [a]	0–0.3 mg/kg bw	Developmental study in rabbits and two-generation study in rats, supported by one-year study in dogs	100
ARfD [a]	1.0 mg/kg bw	Acute neurotoxicity (rat)	100

a Applies to fluazaindolizine and the following metabolites: IN-QEK31, IN-REG72, IN-QZY47, IN-TMQ01, IN-F4106, IN-A5760 and IN-RYC33

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Fludioxonil (addendum)

First draft prepared by
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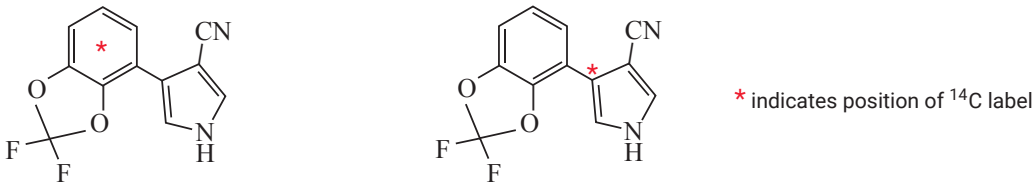
Explanation

Fludioxonil is the International Organization for Standardization (ISO)-approved name for 4-(2,2-difluoro-1,3-benzodioxol-4-yl)-*1H*-pyrrole-3-carbonitrile (IUPAC), for which the Chemical Abstracts Service number is 131341-86-1. Fludioxonil is a non-systemic, broad-spectrum fungicide with a long residual activity. It is a member of the phenylpyrrole group of substances. While originally developed as a seed storage protectant, fludioxonil is also used as a post-harvest, shelf-life extending treatment for fruit. Fludioxonil, being fat soluble, is absorbed into the outer wax layer of the plant and seeds resulting in mycelial growth inhibition and spore rupture. Within fungal cells, fludioxonil interferes with cell intracellular osmolality (Brandhorst et al., 2019).

Fludioxonil was first evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 2004 (JMPR, 2005), which established an acceptable daily intake (ADI) of 0–0.4 mg/kg body weight (bw) based on hepatotoxicity and reduced body weight gain at the next highest dose, in a two-year study in rats. An acute reference dose (ARfD) was considered unnecessary.

Figure 1. Chemical structure of fludioxonil (CGA 173506) and its radiolabelling sites



[*Phenyl-U*-^{14}C]fludioxonil [*4-pyrrole*^{14}C]fludioxonil

Fludioxonil was reviewed by the present Meeting because new information was supplied on metabolites that had not been previously evaluated by JMPR and following a request by the Codex Committee on Pesticide Residues (CCPR) for consideration of additional minimal risk levels (MRLs). All submitted studies were compliant with good laboratory practices (GLP) unless otherwise stated, were validated by the use of appropriate controls, and were generally conducted in accordance with current test guideline requirements. The purity of fludioxonil in all the test articles used was 96.6% or greater.

Evaluation for acceptable daily intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

(a) Oral route

The pharmacokinetics and excretion of radioactivity derived from [*phenyl-U*-^{14}C]fludioxonil was investigated in rats (four/sex per dose route) following a single oral dose of 1 mg/kg bw and following a single intravenous dose of 0.5 mg/kg bw. Samples of excreta were collected for four days following dosing. Blood samples were collected over three days following dosing. The estimated oral absorption (based on oral : intravenous urinary radioactivity excretion ratios) was about 90% in males and 73% in females. The time to reach maximum (T_{max}) concentration for orally administered radioactivity was 0.5 hours in females and one hour in males. The total area under the concentration–time curve ($AUC_{0-\infty}$) values were 0.693 and 0.805 µg equiv. × h/g (10–12% extrapolated) in males and females respectively. Elimination of radioactivity following oral dosing was multiphasic, with the terminal first-order elimination phase starting at about 24 hours post dose. Elimination was essentially complete by 96 hours; residual carcass radioactivity was equal or less than 0.3% of administered radioactivity (AR). Most of the orally administered radioactivity (96% in males, 90% in females) was excreted within 24 hours of dosing. The apparent radioactivity terminal phase half-life ($t_{½}$) was 23.7 and 29.6 hours in males and females respectively. The major route of elimination of radioactivity was in the faeces (86% of AR in males, 80% in females). Urinary elimination accounted for 12% and 11% of the administered radioactivity in males and females respectively.

Following intravenous (i.v.) dosing (0.5 mg/kg bw) elimination of radioactivity from the blood was largely complete by 24 hours post dose, with radioactivity levels below the limit of detection by 48–72 hours in males and females respectively. Elimination following i.v. dosing was multiphasic, the terminal first-order elimination phase starting at about 24 hours post dose. The $AUC_{0-\infty}$ for radioactivity was 0.658 and 0.595 µg equiv. × h/g (14% extrapolated in both sexes) in males and females respectively. The radioactivity terminal $t_{½}$ for elimination was 27.5 and 28.9 hours in males and females respectively. Radioactivity clearance (CL) was 715/832 g/h per kg bw (males/females) and renal clearance (CL_R) was 102/146 g/h per kg bw (males/females). The radioactivity volume of distribution at steady state (V_{SS}) was 15.4/20.3 L/kg (males/females) implying substantial extravascular distribution. The mean radioactivity residence time was 21.6 hours in males and 25.1 hours in females. The major route of elimination was via the faeces (84% of AD in males, 78% of AR in females). Urinary elimination accounted for 12% and 15% of AR in males and females respectively. Most of the administered radioactivity (97% of males, 93% in females) was excreted by 24 hours following dosing, and excretion

was essentially complete by 96 hours, with only 0.3% of AR retained in the carcass in both sexes (Hutton, 2017).

Overall, the findings of the newly submitted pharmacokinetic study of [*phenyl-U*-^{14}C]fludioxonil in rats are consistent with the conclusions of the 2004 JMPR evaluation (JMPR, 2005).

1.2 Biotransformation

(a) In vivo

In data available for the 2004 JMPR evaluation showed that the metabolism of fludioxonil following oral dosing of Han Wistar rats with 100 mg/kg bw of [*4-pyrrole*-^{14}C]fludioxonil occurred primarily via oxidation of position 2 of its pyrrole ring. This results in the formation of one major (56% of AR) oxo-pyrrole metabolite: 2-hydroxy-4(2,2-difluoro-1,3-benzdioxol-4-yl)-*1H*-pyrrole-3-carbonitrile. Oxidation at position 5 of the pyrrole ring results in the formation of one minor (4% of AR) oxo-pyrrole metabolite: 4-(2,2-difluoro-1,3-benzdioxol-4-yl)-5-hydroxy-*1H*-pyrrole-3-carbonitrile. Hydroxylation of the phenyl ring at position 4 results in a phenol metabolite: 4-(2,2-difluoro-7-hydroxy-1,3-benzdioxol-4yl)-*1H*-pyrrole-3-carbonitrile) which accounted for about 2% of AR. These metabolites were excreted in bile as polar β-glucuronyl and sulfuric acid conjugates, and subsequently underwent deconjugation in the faeces. Dimerization of the deconjugated hydroxypyrrole metabolite produces an intensely blue coloured pigment. Excretion of [*4-pyrrole*-^{14}C]fludioxonil-derived radioactivity primarily occurred in the bile (67.5% of AR). In bile duct-cannulated rats, elimination via the urine was a minor route of excretion (10.0% of AR). In uncannulated rats urinary excretion accounted for 18% of AR. No significant biliary re-absorption occurred (Thanei, 1992).

(b) In vitro

In a new study, in vitro metabolic profiling of [*phenyl-U*-^{14}C]fludioxonil was performed by incubating the chemical at a concentration of 7 µM, with human (100 male and 100 female pooled) or male (200 pooled) or female (100 pooled) Wistar rat liver microsomes and a NADPH-regenerating system (system validated by appropriate controls). Suitable positive and negative controls were included. Both human and rat liver microsomes were found to have metabolized fludioxonil extensively following 60 minutes incubation. The parent compound accounted for 28.5%, 12.5% and 9.1% of the dose in human, female rat and male rat preparations respectively. Incubation with human liver microsomes resulted in the formation of up to nine detectable metabolites, with P11 corresponding to unchanged fludioxonil. Metabolites P6 and P7 were the major human microsomal metabolites accounting for 23.4% and 24.9% of the dose respectively. Metabolite P2 accounted for 6.9% of the dose and the other metabolic peaks, (P1, P3, P4, P8, P9 and P10) each equalled 5% or less of the dose. The metabolic pattern for [*phenyl-U*-^{14}C]fludioxonil in male and female rat liver microsome preparations was similar to that observed with the human preparations. All the identified human liver microsomal metabolites were produced by the rat liver microsomal preparations. Metabolites P6 and P7 were also the major rat liver microsomal metabolites, accounting for 28.7%–35.6% of the dose in males and 21.9%–29.0% of the dose in females. The rat metabolite P5 (4% of dose) was not detected with human liver microsomes. The minor metabolite P8, produced by human liver microsomes, was detected only with female rat liver microsomes. Based on these data, there were no human-specific metabolites and the rat is an acceptable model for human metabolism. The individual microsomal (P450) enzymes responsible for the metabolism of fludioxonil were not identified (Thibaut, 2017).

2. Toxicological studies

2.1 Genotoxicity

(a) In vitro studies

All assays were validated by the use of positive and negative controls as appropriate and were conducted in a manner generally consistent with current test guideline requirements.

Table 1. Summary of in vitro genotoxicity studies

Study type	Test system	Dose levels (µg/plate)	Purity (%)	Result	Comments	Reference
Bacterial reverse mutation test	*S. typhimurium* strains TA 1535, TA 1537, TA 98 and TA100; *E. coli* strains WP2uvrA-pKM101 and WP2pKM101; All strains ± S9	0, 50, 150, 500, 1500, and 5000	96.6	Negative	Precipitation occurred at ≥1500 µg/plate	Bowles, 2009
	S. typhimurium strains TA 1535, TA 1537, TA 98 and TA100; *E. coli* strains WP2uvrA-pKM101 and WP2pKM101; All strains ± S9	0, 33, 100, 333, 1000, 2500 and 5000	98.4	Negative	Precipitation occurred at ≥2500 µg/plate Appropriate reference mutagens were used as positive controls; they showed a distinct increase of induced revertant colonies The historical range of positive controls was exceeded in *E. coli* WP2 pKM101 and WP2 uvrA pKM101 (Exp. I) without metabolic activation, and in *S. typhimurium* TA 98 and TA 100 (Exp. I) with metabolic activation; this effect indicates the sensitivity of the strains rather than compromising the assay	Chang, 2017a
	S. typhimurium strains TA 1535, TA 1537, TA 98 and TA100; *E. coli* strains WP2uvrA-pKM101 and WP2pKM101; All strains ± S9	0, 10, 33, 100, 333, 1000, 2500 and 5000	98.1%	Negative	Precipitation was observed at ≥1000 µg/plate. Cytotoxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), were observed in Exp. I in *E. coli* WP2 pKM101 from 2500 to 5000 µg/plate ± S9, and in Exp. II in *E. coli* WP2 pKM101 at 5000 µg/plate with S9	Chang, 2017b

2.2 Special studies

(a) Neurotoxicity

In an acute oral gavage neurotoxicity study using six-week-old SD rats, groups of 10 animals/sex per dose were administered a single gavage dose of fludioxonil at 0, 500, 1000 or 2000 mg/kg bw. Fludioxonil had no effect on clinical findings. Over the period study day 0–1 males dosed at 1000 mg/kg bw showed a body weight change of 0 g (significant at $p < 0.05$), and males dosed at 2000 mg/kg bw showed a body weight change of –7 g (significant at $p < 0.05$); this was compared to a body weight increase in the control group of 6 g. These body weight changes correlated with a significant ($p < 0.05$) reduction in food consumption (by 4 g or more) at doses of 1000 mg/kg bw and above. For females, a mean body weight loss (–5 g compared with controls; significant at $p < 0.05$) occurred in the 2000 mg/kg bw group in the period study day 0–1. This correlated with significantly ($p < 0.05$) reduced mean food consumption by –3 g or more compared to controls).

Functional observational battery (FOB) and locomotor activity assessments were performed six hours post dosing on study day 0 (the approximate time of peak effect of fludioxonil on total and ambulatory locomotor activity at doses of 1000 mg/kg bw and above), and on study days 7 and 14. Fludioxonil had no effect on FOB evaluations. Significantly reduced total and ambulatory locomotor activity counts occurred in males and females at 500 mg/kg bw and above (the reduction in total counts for females were not statistically significant at 500 mg/kg bw) at six hours post dose (see Table 2). The effects on females at 500 mg/kg bw were due largely to a single animal. Other than this, mean counts in this group were not notably different from controls and it was concluded that there were no substance-related effects on locomotor activity in females at 500 mg/kg bw. The magnitude of the effect in males at 1000 mg/kg bw and above was considered adverse.

The effects on locomotor activity were transitory, not observed on study days 7 and 14, and lacked anatomical pathology correlates. Fludioxonil had no effects on brain morphometry or nerve system micro-anatomical pathology in the high-dose cohorts (only dose group investigated). The NOAEL for acute neurotoxicity was 2000 mg/kg bw, the highest dose tested. The NOAEL for general toxicity was 500 mg/kg bw due to the effects on body weight parameters, food consumption on study days 0–1, and transient effects on locomotor activity on day 1 (Herberth, 2014).

Table 2. Mean overall (0–60 minutes) total and ambulatory locomotor activity counts for rats on study day 0 starting six hours after dosing

	Dose (mg/kg bw)							
	Males				Females			
	0	500	1000	2000	0	500	1000	2000
Mean total LMA counts [% of control]	2118	1667* [–21.3]	1285* [–39.3]	1271* [–40.0]	2317	1836 [–20.8]	1594* [–31.2]	1312* [–43.4]
Mean ambulatory LMA counts [% of control]	445	321* [–27.9]	288* [–35.3]	288* [–35.3]	648	428* [–34.0]	373* [–42.4]	320* [–50.6]

LMA: Locomotor activity ; Source: Herberth, 2014

* Significantly different from the control group at $p \leq 0.05$

In a repeat-dose, oral exposure, neurotoxicity study, groups of 12 SD rats per sex were fed fludioxonil for 13 weeks in their diet at concentrations of 0, 500, 3000 or 8000 ppm for females (equal to 0, 42.0, 258.8 and 610.9 mg/kg bw per day) and 0, 500, 3000 and 7000 ppm for males (equal to 0, 34.8, 206.6 and 493.0 mg/kg bw per day). Test substance-related blue material around the urogenital area was noted in the 8000 ppm males, and darkened and blue faeces and blue staining on the tail and cage papers were noted for the 3000, 7000/8000 ppm males and females. These findings were due to the presence of a blue-coloured dimer derived from fludioxonil and are not an indication of adversity. A test substance-related lower mean body weight gain compared with controls was noted during study days 0–21, and also a mean body weight loss during study days 84–91 for the 8000 ppm males. In 7000 ppm females, lower mean body weight gains compared with controls were noted to occur sporadically throughout the exposure period. As a result, significantly lower mean overall (study days 0–91) body weight gains were noted (at $p < 0.05$) for the 8000 ppm males (approximately 85% of

controls). In the 8000 ppm females, lower overall body weight gains (approximately 83% of control on study days 0–91; significant at $p > 0.05$) was noted. These body weight effects occurred in the absence of effects on food consumption. Test substance-related lower mean body weights were noted for the 8000 ppm males and 7000 ppm females during study weeks 3, 7, and 12 when FOB evaluations were being conducted; these lower mean body weights were consistent with the test substance-related effects observed during the weekly body weight evaluations. With the exception of these lower mean body weights, no test substance-related effects were apparent from the FOB evaluations at study weeks 1, 3, 7 and 12. No test substance-related effect on mean ambulatory or total motor activity counts was apparent at any exposure level from evaluations at study weeks 1, 3, 7 and 12. There were no test substance-related alterations in brain weight, brain measurements, macroscopic findings, or microscopic observations. Based on lower body weight gain at 7000/8000 ppm, the NOAEL for general toxicity was 3000 ppm (equal to 206.6 mg/kg per day). The NOAEL for neurotoxicity was 7000 ppm (equal to 493.0 mg/kg per day), the highest exposure level tested (Beck 2013)

(b) Immunotoxicity

The primary T-cell-dependent splenic IgM-forming cell response to intravenously administered sheep red blood cells (RBCs) was assessed in groups of 10 female CD1 (ICR) mice administered fludioxonil (purity 96.6%) in the diet at concentrations of 0, 1000, 2000 or 5000 ppm (equal to 0, 253.8, 454.0 and 1230.0 mg/kg bw per day) for 28 days. The assay was appropriately validated by the induction of immunosuppression using the positive control agent cyclophosphamide (CPA). Administration of fludioxonil in the diet had no adverse effects on survival, body weight, food consumption, macroscopic findings and no effects on spleen or thymus weights. Higher mean adjusted liver weight (12.9% greater than control; significant at $p > 0.05$) occurred in the high-dose cohort; this was not adverse and was consistent with the adaptive changes in the liver seen in other studies on fludioxonil at similar exposure levels. A small, but significant ($p > 0.05$) increase occurred in the high-dose group (201% of control), in total spleen activity, as measured by the number of splenic antibody-forming cells (AFCs) per spleen. Specific activity (AFCs per 106 spleen cells) was significantly ($p > 0.05$) increased in the low- and high-dose groups (199% and 209% of control respectively) but not in the mid-dose group. It was noted that there was a marked reduction in these parameters when treated with the positive control for immunosuppression, CPA. The overall increases were the result of a small number of high-responder animals, were not dose-related and were therefore considered not related to fludioxonil treatment. The NOAEL for immunotoxicity was 5000 ppm (equal to 1230 mg/kg bw per day) the highest dose tested (Crittenden, 2011).

(c) Phototoxicity

Fludioxonil was not phototoxic in the in vitro UVA 3T3 neutral red uptake phototoxicity test. Due to lack of phototoxicity a half-effective concentration value (EC_{50}) could not be determined and a photo-irritancy factor was therefore not calculated (Lehmeier, 2015)

2.3 Studies on metabolites

(a) Genotoxicity studies

A range of in vitro and in vivo genotoxicity studies not previously evaluated by JMPR was supplied for metabolites of fludioxonil. All assays were validated by the use of positive and negative controls as appropriate and were conducted in a manner generally consistent with current test guideline requirements. Where necessary QSAR evaluation using OECD ToolBox ver. 4.5 (including actual and simulated mammalian metabolism) was conducted for structural alerts relating to genotoxicity. It was noted that CGA 308565 is a tautomer of SYN 518579 (see Fig. 3). Accordingly, CGA 308565 and SYN 518579 are considered as a tautomeric pair for the purposes of this evaluation.

Figure 2. Chemical structures of fludioxonil metabolites

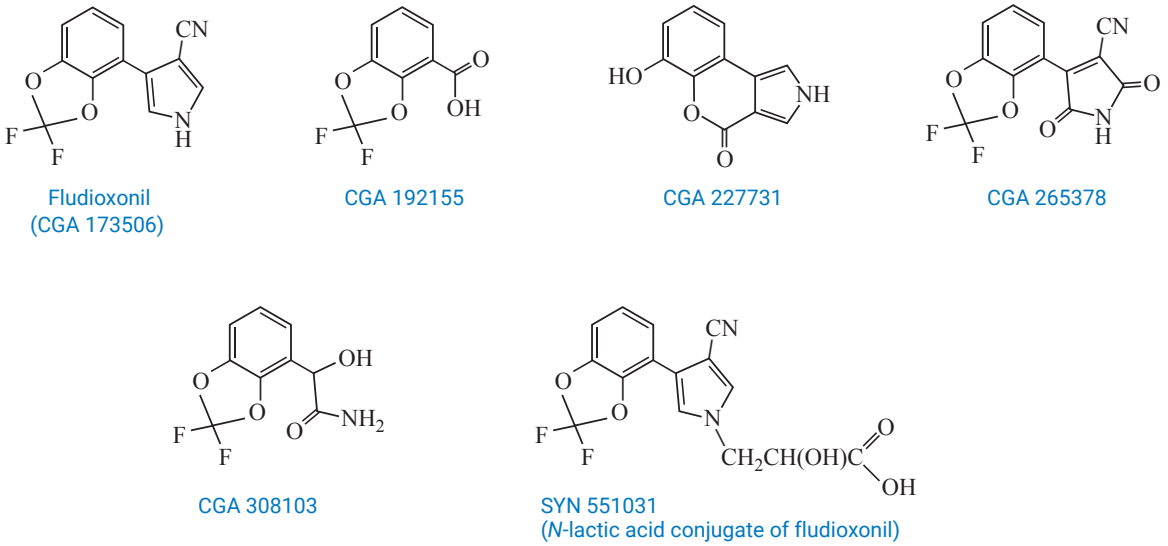


Figure 3. Tautomerism of CGA 308565 and SYN 51879

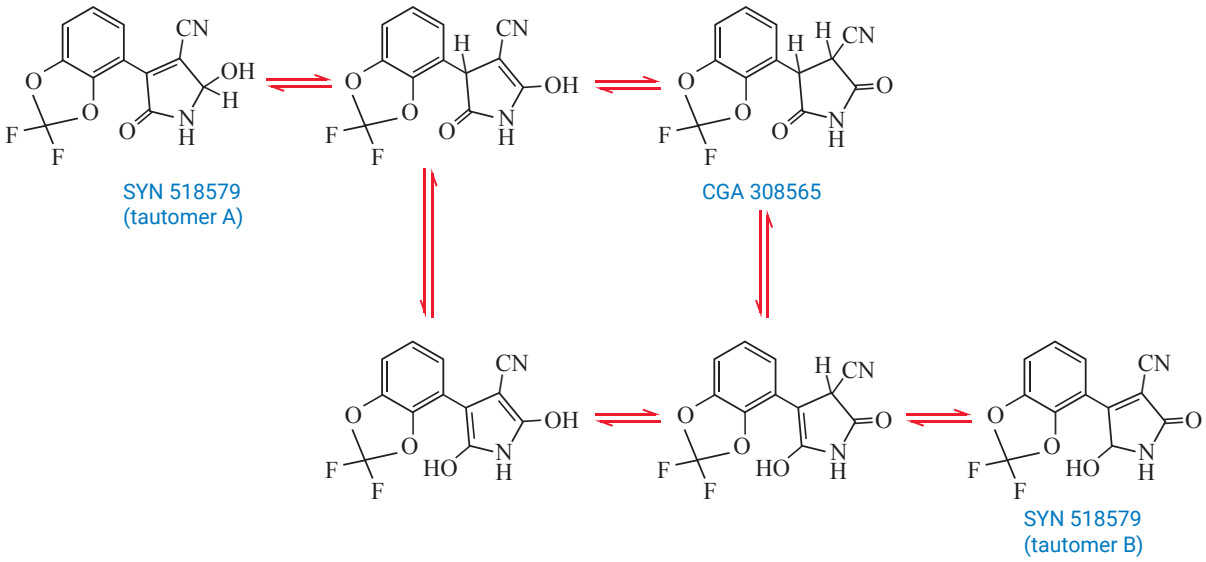


Table 3. Summary of genotoxicity studies on metabolites of fludioxonil

Study type	Test system	Dose levels	Purity	Result	Comments	Reference
			CGA 192155			
Bacterial reverse mutation assay	*S. typhimurium* and *E. coli*	78.1–1250 µg/plate (+S9 *S. typhimurium,* Experiment 1) 312.5–5000 µg/plate (all other experiments)	NR	Negative	As reported by JMPR 2004	JMPR, 2005
In vitro mouse lymphoma cell forward mutation assay	Mouse lymphoma L5178Y cell $TK^{+/-}$ forward mutation assay ± S9	Experiment 1: 0, 126.2, 252.2, 505, 1010 and 2020 µg/mL Experiment 2: 0, 250, 500, 1000, 1500, 2000 µg/mL	100%	Negative	Precipitation occurred at ≥ 1010 µg/mL	Volkner, 2007
In vitro chromosome aberration test	Human lymphocytes ± S9	0, 13.2, 23.1, 40.4, 70.7, 123.7, 216.4, 378.8, 662.9, 1160.0 and 2030.0 µg/mL	100%	Positive	Evaluated experimental points shown in bold; precipitation occurred at ≥ 1160.0 µg/mL; 4 hour or 22 hour exposure without S9; 4 hour exposure with S9	Bohnenberger, 2007
In vivo mouse bone marrow micronucleus test	NMRI mouse	24 hours post dose study: 0, 500, 1000 and 2000 mg/kg bw 48 hours post dose study: 2000 mg/kg bw	100%	Negative	Dosing by oral gavage; CGA 192155 did not exert bone marrow cytotoxicity; systemic exposure was confirmed following dosing at 2000 mg/kg bw	Bruce, 2018; Honarvar, 2007
			CGA 227731			
Bacterial reverse mutation test	*S. typhimurium* strains TA 1535, TA 1537, TA 98 and TA 100; E. coli WP2 uvrA pKM101; All strains ± S9	0, 5, 15, 50, 150, 500, 1500 and 5000 µg/plate	96%	Positive	See footnote [a]	Gilby, 2018a
In vitro micronucleus test	Human lymphocytes ± S9	0, 12.5, 125.0, 250.0, 275.0, 300.0, 325.0, 350.0, 375.0, 400.0, 450.0 and 500.0 µg/mL	≥ 95%	Negative	Evaluated experimental points shown in bold; precipitation occurred at ≥ 300 µg/mL	Gilby, 2018b
In vivo comet assay	SD rat liver and duodenum	0, 500, 1000 and 2000 mg/kg bw (oral gavage)	95%	Negative	Systemic exposure confirmed; hepatotoxicity observed at 2000 mg/kg bw	Herring, 2018
QSAR evaluation	OECD ToolBox ver. 4.5	NA	NA	Positive	Structural alerts for genotoxicity in the bacterial reverse mutation assay for predicted in vivo rat metabolites and rat S9 metabolites: 1 for α, β-unsaturated carbonyls, 1 for quinones	NA

Study type	Test system	Dose levels	Purity	Result	Comments	Reference
		CGA 265378				
Bacterial reverse mutation assay	*S. typhimurium* and *E. coli*	312.5–5000 µg/plate ±S9	NR	Negative	As reported in JMPR 2004	JMPR, 2005
In vitro mouse lymphoma cell forward mutation assay	Mouse lymphoma L5178Y cells $TK^{+/-}$ forward mutation assay ± S9	Experiment I –S9: 0, 1.4, 2.8, 5.5, 11.0 and 16.5 µg/mL + S9: 0, 5.5, 11.0, 16.5, 22.0 and 33.0 µg/mL Experiment II –S9: 0, 3.0, 6.0, 12.0, 14.0, 16.0 and 18.0 µg/mL +S9: 0, 6.0, 12.0, 20.0, 23.0 and 26.0 µg/mL	99%	Negative	Cytotoxicity occurred at ≥ 11 µg/mL	Wollny, 2015
In vitro chromosome aberration test	Human lymphocytes ± S9	10 concentrations from 0 to 2780 µg/mL	99%	Positive	Precipitation occurred at ≥ 907.8 µg/mL; Cytotoxicity occurred at ≥ 169.4 µg/mL	Sokolowski, 2015.
In vivo mouse bone marrow micronucleus test	CD-1 mice	0, 100, 200, 400, 600 and 800 mg/kg bw (gavage)	99%	Negative	Doses ≥ 600 mg/kg bw resulted in mortality and unscheduled euthanasia; the maximum tolerated dose was 400 mg/kg bw; Systemic exposure was confirmed.	Dunton, 2015
		CGA 308103				
Bacterial reverse mutation assay	*S. typhimurium* and *E. coli*	312.5–5000 µg/plate		Negative	As reported at JMPR 2004	JMPR, 2005
In vitro micronucleus test	Human TK6 lymphocytes ± S9	3 h with S9 mix, treatment schedule: 888.9 to 2000 µg/mL 3 h without S9 mix, treatment schedule 888.9 to 2000 µg/mL Continuous treatment schedule (24 h without S9 mix) 800.0 to 1905 µg/mL	99%	Negative		Clare, 2018a

Study type	Test system	Dose levels	Purity	Result	Comments	Reference
		CGA 308565/SYN 51879 tautomeric pair				
Bacterial reverse mutation assay	*S. typhimurium* and *E. coli*	312.5–5000 µg/plate		Negative	As reported at JMPR 2004	JMPR, 2005
In vitro micronucleus test	Human TK6 lymphocytes± S9	3 h with S9 mix, treatment schedule 436.3 to 644.6 µg/mL 3 h without S9 mix, treatment schedule, 175.6 to 395.1 µg/mL Continuous treatment schedule, (24 h without S9 mix) 156.6 to 242.9 µg/mL	95%	Positive		Clare, 2018b
In vivo mouse bone marrow micronucleus test	CD-1 mice	0, 125, 250 and 500 mg/kg bw	98%	Negative	Systemic exposure confirmed.	Dunton, 2018a
		CGA 339833				
Bacterial reverse mutation assay	*S. typhimurium* and *E. coli*	312.5–5000 µg/plate	NR	Negative	As reported at JMPR 2004	JMPR, 2005
In vitro gene mutation	Mouse lymphoma L5178Y cells	4 h without S9 mix, 400–3200 µg/mL; 4 h with S9 mix, 200–3400 µg/mL; 24 h without S9 mix, 400–3200 µg/mL	NR	Negative	As reported at JMPR 2004	JMPR, 2005
In vitro chromosomal aberration assay	Chinese hamster V79 cells	4 h + 14 h without S9, 800–2400 µg/mL; 4 h + 14 h with S9, 200–1600 µg/mL; 18 h without S9, 800µg/mL; 28 h with S9, 200–2400 µg/mL 4 h + 24 h with S9, 200–800 µg/mL	NR	Positive: 18 h without S9; All other tests negative	As reported in JMPR 2004	JMPR, 2005
In vivo bone marrow micro-nucleus assay	Rat	0, 500, 1000, 2000 mg/kg bw	NR	Negative	As reported at JMPR 2004	JMPR, 2005
In vivo comet assay	Wistar rat liver and duodenum	0, 350, 700 and 1400 mg/kg bw (gavage)	96%	Negative	Duodenal toxicity (villous atrophy) seen at ≥ 700 mg/kg bw; Hepatocyte glycogen depletion occurred at 1400 mg/kg bw; Systemic exposure was confirmed.	Penn, 2016; Waters, 2017

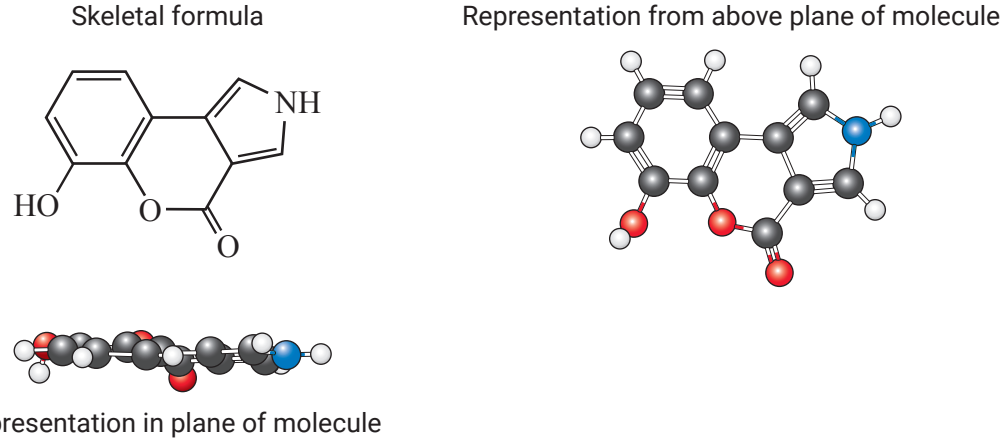
Study type	Test system	Dose levels	Purity	Result	Comments	Reference
			SYN 551031			
Bacterial reverse mutation test	S. typhimurium strains TA 1535, TA 1537, TA 98 and TA100 ; E. coli strains WP2 uvrA pKM101; all strains ± S9	0, 5, 15, 50, 150, 500, 1500, 5000 µg/plate	Equal to or greater than 99.5%	Negative	Cytotoxicity occurred at 5000 µg/plate	Gilby, 2018c
In vitro micronucleus test	Human TK6 lymphocytes ± S9	11 concentrations between 0 and 2000 µg/mL	95%	Positive	Cytotoxicity occurred at ≥ 1050.0 µg/mL	Gilby, 2018d
In vivo mouse bone marrow micronucleus test	CD-1 mice	0, 500, 1000, 2000 mg/kg bw	95%	Negative		Dunton, 2018b

[a] Evidence of mutagenic activity was seen towards the strain TA 1537 following exposure to CGA 227731. In the absence of S9 mix, increases in revertant colony counts together with some evidence of a positive concentration–response relationship, reaching 2.3 × and 4.9 × mean concurrent vehicle control values at 500 and 1500 µg/plate, respectively. In the presence of S9 mix, increases in revertant colony counts together with some evidence of a positive concentration–response relationship, reaching 2.5 × and 5.0 × mean concurrent vehicle control values at 500 and 1500 µg/plate, respectively. In the absence of S9 mix, toxicity was observed in strain TA 1535 at 5000 µg/plate, in strains TA 98, TA 100 and TA 1537 at ≥ 1500 µg/plate and in strain WP2 uvrA (pKM101) at ≥ 500 µg/plate. In the presence of S9 mix, toxicity was observed in strain TA 1537 at 5000 µg/plate, in strains TA 98, TA 100 and TA 1535 at ≥ 1500 µg/plate and in strain WP2 uvrA (pKM101) at ≥ 500 µg/plate. Precipitate was observed on all plates containing CGA 227731 at 5000 µg/plate in the absence of S9 mix.

NA; Not applicable; NR: Not reported S9 mix: Rat liver supernatant fraction obtained by centrifuging at 9000 g

Metabolite CGA 227731 induced reverse mutations in *S. typhimurium* strain TA 1537 (in the presence and absence of S9) which detects frameshift mutations caused by DNA intercalation by planar molecules. As can clearly be seen in Fig. 4, CGA 227731 is a planar molecule. These types of mutations cannot be detected by in vitro micronucleus assays or in vivo Comet assays (outside the applicability domain of these assays). Accordingly, a new transgenic rodent somatic and germ cell gene mutation assay was submitted for evaluation.

Figure 4. The planar nature of metabolite CGA 227731



Metabolite CGA 227731 was administered by oral gavage for 28 consecutive days to male transgenic Fischer 344 Big Blue® rats at 0, 250, 500 mg/kg bw per day (n = six per dose) and at 1000 mg/kg bw per day (n = seven per dose) based on the results of a preliminary 14-day dose-ranging study. The assay was appropriately validated by the use of positive and negative controls. No mortality or morbidity occurred and all animals gained weight during the study. Cumulative body weight losses were observed compared to the vehicle control for all test substance groups. Feed consumption was similar among all groups. Red or brown nasal discharge (vehicle, 250 and 500 mg/kg bw per day), ruffled fur (250, 500 and 1000 mg/kg bw per day), crusty eyes (500 mg/kg bw per day) and slight diarrhoea (1000 mg/kg bw per day) were exhibited during the study in either cageside or detailed hands-on observations. No significant differences in organ weights were observed. Treatment with CGA 227731 did not cause a statistically elevated mutant frequency at the cII gene in the liver, duodenum or bone marrow of male Big Blue® rats. Under the conditions of this study, the administration of CGA 227731 at doses up to and including 1000 mg/kg bw per day was not mutagenic in the liver, duodenum or bone marrow. (Bruce, 2021).

(b) Repeat dose toxicity studies

CGA 192155

In a 28-day repeated dietary oral exposure study in Wistar rats groups of five animals/sex per dose were fed diets containing 0, 1000, 5000 or 15 000 ppm CGA 192155 (equal to 0, 78, 382 and 1147 mg/kg bw per day in males, 0, 80, 389 and 1065 mg/kg bw per day in females). All animals survived the scheduled treatment period and no clinical signs of toxicological relevance were noted. At 15 000 ppm, body weight (ca 85% of controls by study day 26) and body weight gain (ca 57% of controls by study day 26) were significantly lower ($p < 0.05$) than controls throughout the treatment period in males and females. Food consumption was lower than controls during the early part of the study in both sexes at 15 000 ppm. Minor, but statistically significant, haematological changes were observed at 15 000 ppm, comprising a slight reduction in haematocrit (Ht) in females only and of distribution width in males. Glucose levels were slightly lower and serum creatinine levels slightly higher than for controls in males, while cholesterol, triglyceride and phospholipid levels were slightly higher in both sexes receiving 15 000 ppm. In addition, total bilirubin levels were reduced in high-dose females. Protein and globulin levels were slightly lower than for controls and the albumin:globulin ratio (A : G) slightly higher in both sexes receiving 15 000 ppm. Plasma albumin levels were also slightly higher in males treated with 15 000 ppm. Urinary pH was increased in high-dose females. Liver weights (adjusted for terminal body weight) were slightly higher than controls in males at 15 000 ppm. At 5000 ppm, there was a slight increase in haemoglobin (Hb) concentration distribution width in males only. There were no treatment-related effects on food consumption or body weight at this dose level and there were no other toxicologically significant effects on haematology or clinical chemistry parameters. Hence, the change in Hb concentration distribution width at 5000 ppm was not considered to be of toxicological significance. There were no effects at 1000 ppm CGA 192155. There were no treatment-related microscopic findings in the kidney or liver at any dose level. The NOAEL was 5000 ppm (equal to 382 mg/kg bw per day), based on reduced body weight correlated with reduced food consumption at 15 000 ppm (equal to 1147 mg/kg bw per day) (Harder, 2008a).

In a 13-week repeated dietary oral exposure study in Wistar rats groups of 10 animals/sex per dose were fed diets containing 0, 100, 1000 or 7000 ppm CGA 192155 (equal to 0, 5.9, 57.5 and 414.7 mg/kg bw per day in males, 0, 6.7, 66.2 and 461.2 mg/kg bw per day in females). There were no premature deaths and no clinical signs of toxicological relevance. Ophthalmoscopic examinations, FOB, analysis of locomotor activity, clinical pathology examinations, analysis of organ weights, macroscopic examinations at necropsy and histopathological examinations did not reveal any toxicologically relevant effect due to treatment with the test item. A slight decrease in food consumption compared to controls was recorded in males at 1000 and 7000 ppm and in females at 7000 ppm. There was a statistically significant reduction of body weight and body weight gain in males and females at 7000 ppm. Throughout the treatment period, a less pronounced effect on body weight and body weight gain was noted in males at 100 and 1000 ppm. This showed no dose–response relationship, and only occasionally reached statistical significance. No specific organ effects were observed and there was no evidence of neurotoxic potential. Due to the observed body weight effects at 7000 ppm (equal to 414.7 mg/kg bw per day), the NOAEL was 1000 ppm (equal to 57.5 mg/kg bw per day) (Harder, 2008b).

CGA 339833

A 90-day dietary toxicity study on CGA 339833 was evaluated by JMPR in 2004 as follows:

"Groups of 10 male and 10 female HanBrl:WIST(SPF) rats were given diets containing CGA 339833 (purity, 96%) at a concentration of 0, 10, 100, 800, 2500 or 7000 ppm for 90 days. Actual achieved doses of CGA 339833 were 0.7, 7.1, 58, 190, 510 mg/kg bw per day for males and 0.9, 8.7, 67, 210, 600 mg/kg bw per day [sic]. Stability and homogeneity of the test article in the diet were checked before treatment and at intervals during the study. Mortality was checked twice per day and clinical signs were checked daily. Body weight, food and water consumption were recorded before the start of treatment and once per week thereafter. Ophthalmological examinations were performed for all animals before treatment, and for animals in the control group and in the group receiving the highest dose in week 13. A functional observational battery (activity and overexcitation of the central nervous system, sensorimotor, autonomic and physiological functions and motor activity tests) was performed towards the end of treatment. At the end of treatment, haematology, clinical chemistry, urine analysis, organ weights, gross pathology and histopathology were performed.

There were no treatment-related effects on mortality, clinical signs, the functional observation battery, food and water consumption, haematology, ophthalmological parameters, or gross pathology. Body-weight gain was slightly reduced in animals at the highest dose, resulting in final body weights that were 10% and 5% lower than those of the controls for males and females respectively. Apparently lower body-weight gain in males at 100 ppm was at least partly due to single animals in the control group and in the group receiving fludioxonil at 10 ppm with cumulative weight gains that were >50% greater than the average for those groups. If body-weight gains are calculated without these two outliers, then values for groups of males treated at ≤100 ppm are similar to those of the controls. Weight gains for groups treated with fludioxonil at 800 to 7000 ppm remain approximately 10–13% below that of the control group, but with a flat dose–response relationship particularly at 800 and 2500 ppm. Consequently, lower body-weight gains in males treated at 100 ppm are considered to be incidental to treatment and those observed at 800 and 2500 ppm to be of equivocal relationship to treatment. Significantly lower triglyceride and higher phosphorus values were observed in males at 7000 ppm. Males at 7000 ppm excreted slightly larger volumes of a more dilute urine. A slightly increased absolute weight of the liver was noticed in females at 7000 ppm and relative weights were increased in both sexes at this dose. The weight changes correlated with centrilobular hepatocellular hypertrophy. Relative weight of the kidney was increased for males at 7000 ppm and slight non-significant increases were seen in males at 2500 ppm and in females at 7000 ppm. Histopathologically, a slightly increased incidence and severity of tubular casts was seen in males at 800 to 7000 ppm. The tubular casts were found in the proximal tubuli and consisted of eosinophilic formations in the tubular lumen and walls without causing tubular distension. As tubular casts were present in more than half of the control males, the slight increase in incidence and grading was not considered to be adverse—especially for males at 800 ppm where this finding was not accompanied by any change in kidney weight or other indications of kidney damage in clinical pathology parameters. Increased relative weights of the testes in males at 7000 ppm were considered to be secondary to reduced weight gains in this group, as absolute testes weights tended to be preserved. Increased weights of the ovaries in females treated at 100 ppm were attributable to the presence of watery cysts in one animal, and increased adrenal weight in this group was discounted as incidental owing to the lack of a dose–response relationship. Histopathology revealed an increased incidence of minimal to slight atrophy in the olfactory epithelium at 2500 and 7000 ppm, which was characterized by the disorderly arrangement of the olfactory epithelium, retention of secretion in the olfactory mucosal glands, and foamy appearance of submucosal supporting cells. A slightly increased incidence of follicular cell hypertrophy in the thyroid gland was seen in females at 7000 ppm.

The NOAEL for CGA 339833 was 800 ppm (equal to 58 and 67 mg/kg bw per day in males and females) on the basis of increased relative weight of the liver correlating with hepatocellular hypertrophy, increased relative weight of the kidney and tubular casts at ≥2500 ppm (males only), and minimal to slight atrophy of the olfactory epithelium at ≥2500 ppm (Sommer, 2001)."

(c) Conclusions on metabolites

CGA 192155

CGA 192155 is a plant and animal metabolite that is not found in rats. A comparison of the toxicity of CGA 192155 compared with that of fludioxonil is presented in Table 4.

Table 4. Comparative toxicity of CGA 192155 and fludioxonil

Study	CGA 192155	Fludioxonil
Acute oral LD_{50} (rat)	> 2000 mg/kg bw	> 5000 mg/kg bw
90-day repeated dietary toxicity study	NOAEL = 57.5 mg/kg bw per day LOAEL = 417.5 mg/kg bw per day	NOAEL = 64 mg/kg bw per day LOAEL = 430 mg/kg bw per day
Genotoxicity test battery	Negative	Negative

Overall, the toxicological properties of CGA 192155 resemble those of fludioxonil.

CGA 227731

CGA 227731 is a plant metabolite that is not found in rats. No repeat-dose toxicity was submitted or available for CGA 227731.

CGA 265378

CGA 265378 is an animal metabolite that is not found in rats. No repeat-dose toxicity was submitted or available for CGA 265378.

CGA 308103

CGA 308103 is a plant metabolite that is not found in rats. No repeat-dose toxicity data was submitted or available for CGA 308103.

CGA 308565/SYN 518579 tautomeric pair

The CGA 308565/SYN 518579 tautomeric pair are plant metabolites that are not found in rats. No repeat dose toxicity data was submitted or available for the CGA 308565/SYN 518579 tautomeric pair.

CGA 339833

CGA 339833 is a plant metabolite that is not found in rats. A comparison of the toxicity of CGA 339833 with that of fludioxonil is presented in Table 5.

Table 5. Comparative toxicity of CGA 339833 and fludioxonil

Study	CGA 339833	Fludioxonil
Acute oral LD50 (rat)	> 2000 mg/kg bw	> 5000 mg/kg bw
90-day repeated dietary toxicity study	NOAEL = 58 mg/kg bw per day LOAEL = 190 mg/kg bw per day	NOAEL = 64 mg/kg bw per day LOAEL = 430 mg/kg bw per day
Genotoxicity test battery	Negative	Negative

Overall, the toxicological properties of CGA 339833 resemble those of fludioxonil.

Metabolite SYN 551031 (N-lactic acid conjugate of fludioxonil)

SYN 551031 is a plant metabolite not found in rats. No data was submitted, however the metabolite is likely to hydrolyse to fludioxonil when ingested from the diet.

3. Microbial aspects

The possible impact of fludioxonil residues on the human intestinal microbiome was evaluated.

A search of the literature available in the public domain did not identify information describing any direct or indirect experimental evidence that addressed the impact of fludioxonil residues on the human intestinal microbiome. No experimental data was submitted by the sponsor in this regard.

Comments

Toxicological data on metabolites and/or degradates

CGA 192155

Metabolite CGA 192155 is a plant and animal metabolite that is not found in rats. The acute oral median lethal dose (LD_{50}) of CGA 192155 in rats is greater than 2000 mg/kg bw.

In a 28-day repeated dietary oral exposure study, rats were treated with CGA 192155 at 0, 1000, 5000 or 15 000 ppm (equal to 0, 78, 382 and 1147 mg/kg bw per day for males, 0, 80, 389 and 1065 mg/kg bw per day for females). The NOAEL was 5000 ppm (equal to 382 mg/kg bw per day) based on reductions in body weight and body weight gain (correlated with reduced food consumption) at 15 000 ppm (equal to 1065 mg/kg bw per day) (Harder, 2008a).

In a 13-week repeated dietary oral exposure study, rats were treated with CGA 192155 at 0, 100, 1000 or 7000 ppm (equal to 0, 5.9, 57.5 and 415 mg/kg bw per day for males, 0, 6.7, 66.2 and 461 mg/kg bw per day for females). The NOAEL was 1000 ppm (equal to 57.5 mg/kg bw per day) based on reductions in body weight and body weight gain (correlated with small reductions in food consumption) at 7000 ppm (equal to 415 mg/kg bw per day) (Harder, 2008b).

Metabolite CGA 192155 did not induce bacterial reverse mutations (JMPR, 2005), nor did CGA 192155 induce mutations in mammalian cells in vitro (Volkner, 2007). It did induce chromosomal aberrations in vitro (Bohnenberger, 2007) but was negative in an in vivo micronucleus test (Bruce, 2018; Honarvar, 2007).

In view of these studies the Meeting concluded that CGA 192155 is unlikely to be genotoxic in vivo.

Overall, the Meeting concluded that the toxicological properties of CGA 192155 resemble those of fludioxonil. Accordingly, human exposure to CGA 192155 should be covered by HBGVs for fludioxonil.

CGA 227731

This compound is a plant metabolite not found in rats. CGA 227731 is unlikely to be genotoxic based on findings in bacterial reverse mutation assays, an in vitro micronucleus assay, an in vivo comet assay and a transgenic rat somatic and germ cell gene mutation assay. No other toxicological data were available on this metabolite. Accordingly, the Meeting concluded that the Cramer class III threshold of toxicological concern (TTC) of 1.5 µg/kg bw per day should apply to CGA 227731.

CGA 265378

This compound is an animal metabolite not found in rats. No repeat-dose toxicity data was provided or available for CGA 265378. The Meeting in 2004 (JMPR, 2005) had previously concluded that metabolite CGA 265378 was unlikely to be genotoxic. Accordingly, the Meeting concluded that the Cramer class III TTC of 1.5 µg/kg bw per day should apply to CGA 227731.

CGA 308103

This compound is a plant metabolite not found in rats. No repeat-dose toxicity data were provided or available for CGA 308103. Metabolite CGA 308103 is unlikely to be genotoxic. Accordingly, the Meeting concluded that the Cramer class III TTC of 1.5 µg/kg bw per day should apply to CGA 308103.

CGA 308565/SYN 51879 tautomeric pair

The CGA 308565/SYN 51879 tautomeric pair are plant metabolites not found in rats. No repeat-dose toxicity data were provided or available for CGA 308565/SYN 51879. The metabolite pair CGA 308565/SYN 518579 is unlikely to be genotoxic. Accordingly, the Meeting concluded that the Cramer class III TTC of 1.5 µg/kg bw per day should apply to the CGA 308565/SYN 51879 tautomeric pair.

CGA 339833

This compound is a plant metabolite not found in rats. The acute oral LD_{50} of CGA 339833 in rats is greater than 2000 mg/kg bw. In a 90-day dietary toxicity study, rats were treated with CGA 339833 at 0, 10, 100, 800, 2500 or 7000 ppm (equal to 0, 0.7, 7.1, 58, 190 and 510 mg/kg bw per day for males, 0, 0.9, 8.7, 67, 210 and 600 mg/kg bw per day for females). The NOAEL for CGA 339833 was 800 ppm (equal to 58 mg/kg bw per day) based on increased relative liver weight correlating with hepatocellular hypertrophy, increased relative kidney weight correlating with urinary tubular casts in males, and minimal to slight olfactory epithelial atrophy at 2500 ppm (equal to 190 mg/kg bw per day) (Sommer, 2001).

Overall, the Meeting concluded that the toxicological properties of CGA 339833 resemble those of fludioxonil. Accordingly, human exposure to CGA 339833 should be covered by the HBGVs for fludioxonil.

SYN 551031 (N-lactic acid conjugate of fludioxonil)

This compound is a plant metabolite not found in rats. Metabolite SYN 551031 did not induce bacterial reverse mutations, was genotoxic in an in vitro micronucleus test, but was not genotoxic in an in vivo micronucleus test. Based on weight of evidence the Meeting concluded that SYN 551031 is unlikely to be genotoxic in vivo. The Meeting concluded that the toxicological potency of SYN 551031 (an *N*-lactic acid conjugate of fludioxonil) is likely to be less than, or equal to, that of fludioxonil given the structure and physicochemical properties of the compound. Overall the Meeting concluded that the toxicity of SYN 551031 should be covered by HBGVs for fludioxonil.

Toxicological evaluation (addendum)

The 2004 Meeting established an ADI of 0–0.4 mg/kg bw for fludioxonil based on a NOAEL of 40 mg/kg bw per day in the two-year combined carcinogenicity and toxicity study in rats, and using a safety factor of 100. The current meeting concluded that the parent ADI applies also to CGA 192155, CGA 339833 and SYN 551031.

The 2004 Meeting concluded that an ARfD for fludioxonil was not necessary and this conclusion applies to all of the metabolites assessed by the present Meeting.

Levels relevant to risk assessment of fludioxonil metabolites (addendum)

Species	Study	Effect	NOAEL	LOAEL
CGA 192155				
Rat	28-day study of toxicity [a]	Toxicity	5000 ppm, equal to 382 mg/kg bw/day	15 000 ppm, equal to 1065 mg/kg bw/day
	13-week study of toxicity [a]	Toxicity	1000 ppm, equal to 57.5 mg/kg bw/day	7000 ppm, equal to 414.7 mg/kg bw/day
CGA 339833				
Rat	90-day study of toxicity [a]	Toxicity	800 ppm, equal to 58 mg/kg bw/day	2500 ppm, equal to 190 mg/kg bw/day

[a] Dietary administration

*Acceptable daily intake (ADI)**

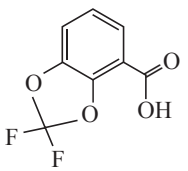
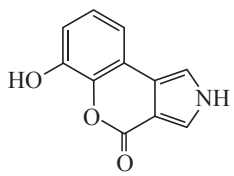
0–0.4 mg/kg bw

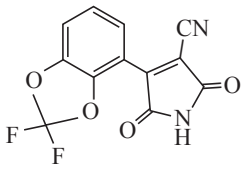
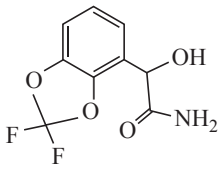
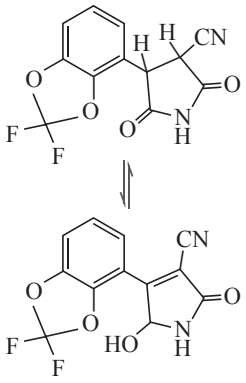
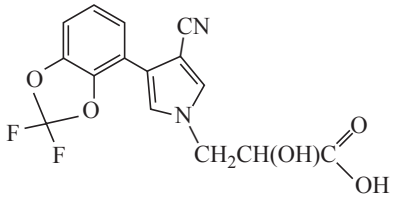
Acute reference dose (ARfD)

Not necessary

* Established by JMPR 2004. Applies to sum of fludioxonil, CGA 192155, CGA 339833 and SYN 551031

Critical end-points for setting guidance values for exposure to fludioxonil metabolites

Studies on toxicologically relevant metabolites		
CGA 192155		Acute oral LD_{50}: >2000 mg/kg bw (rat) 28-day NOAEL: 382 mg/kg bw per day (rat) 13-week NOAEL: 57.5 mg/kg bw per day (rat) Not genotoxic: negative Ames, negative forward mutation in mammalian cells, positive in vitro chromosomal aberration, negative in vivo micronucleus test
CGA 227731		Not genotoxic: positive Ames in *S. typhimurium* strain TA 1537; negative in other strains and species, negative in vitro micronucleus, negative in vivo comet assay, negative in vivo transgenic rat gene mutation assay

CGA 265378 [a]		Acute oral LD_{50}: >2000 mg/kg bw (rat) Not genotoxic: negative Ames, negative forward mutation in mammalian cells, positive in vitro chromosomal aberration, negative in vivo micronucleus
CGA 308103 [a]		Acute oral LD_{50}: > 1000 < 2000 mg/kg bw (rat) Not genotoxic: negative Ames, negative in vitro micronucleus
SYN 51879 [a] CGA 308565 (tautomeric pair)		Acute oral LD_{50}: >2000 mg/kg bw (rat) 90-day NOAEL: 58 mg/kg bw per day Not genotoxic: negative Ames, negative forward mutation in vitro, positive in vitro chromosomal aberration, negative in vitro micronucleus, positive in vivo micronucleus
SYN 551031		Not genotoxic: negative Ames, positive forward mutation in vitro, negative in vivo micronucleus Toxicological potency less than, or equal to that of fludioxonil

[a] Evaluated by JMPR in 2004

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Fluindapyr

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Explanation

Fluindapyr is the International Organization for Standardization-approved name for 3-(difluoromethyl)-*N*-(7-fluoro-1,1,3-trimethyl-2,3-dihydro-1*H*-inden-4-yl)-1-methyl-1*H*-pyrazole-4-carboxamide (IUPAC), with Chemical Abstracts Service number 1383809-87-7.

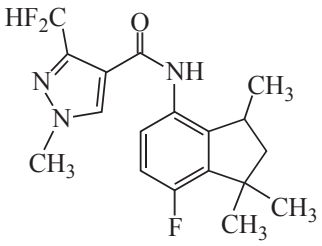
Fluindapyr is a systemic fungicide belonging to the chemical class of pyrazole4carboxamides and to the group of succinate dehydrogenase inhibitors (SDHI). It possesses protective, curative and eradicant properties, and has broad-spectrum activity against a wide range of fungal diseases in plants.

Fluindapyr is a racemic mixture containing two enantiomers *R* and *S* in the ratio 1:1. Fluindapyr has not previously been evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and was reviewed by the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR).

All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with current test guidelines unless otherwise specified. No additional information from a literature search was identified that complemented the toxicological information submitted for the current assessment.

The chemical structure of Fluindapyr is shown in Fig. 1.

Figure 1. Chemical structure of fluindapyr (F9990/IR9792)



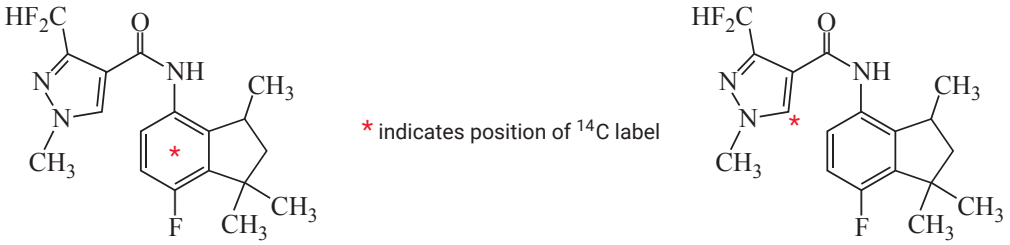
Evaluation for acceptable daily intake

1. Biochemical aspects

The absorption, distribution, metabolism and excretion (ADME) characteristics of fluindapyr were investigated in adult male and female Sprague Dawley (SD) rats following administration of [*phenyl*-^{14}C]fluindapyr or [*pyrazole*-^{14}C]fluindapyr

The positions of the radiolabels in fluindapyr used in the ADME studies are shown in Figure 2.

Figure 2. Position of radiolabels in fluindapyr used in ADME studies



[*Phenyl*-^{14}C]fluindapyr

[*Pyrazole*-^{14}C]fluindapyr

1.1 Absorption, distribution and excretion

(a) Oral route

In a study aimed at investigating excretion (urine and faeces) following administration of a single oral dose, radiolabelled test article, [*pyrazole*-^{14}C]fluindapyr (Radiochemical purity 99.1%; Lot no. 54706-18-20) was given by single oral (PO) administration of 50 mg/kg bw of test article formulated in aqueous 0.5% low viscosity carboxymethyl cellulose (CMC) containing Tween 80 at 0.1% (v/v). Four male and four female adult, albino Sprague Dawley rats were treated; at dose initiation these were 7–10 weeks old, weights for all males and females were in the range 270–300 g.

Urine was collected from each animal before administration (the predose sample) and over the periods 0–8, 8–24, 24–48, 48–72, 72–96, 96–120, 120–144 and 144–168 hours post dose. Faeces were collected from each animal before administration (the predose sample) and over the periods 0–24, 24–48, 48–72, 72–96, 96–120, 120–144 and 144–168 hours post dose. Cage washing was performed daily (time intervals of 24 hours) until 168 hours post dose. The carcass of each animal was collected after sacrifice at 168 hours post dose.

The radioactivity excreted via urine within 168 hours of dose administration accounted for about 27% of the dose , while about 65% of the dose was eliminated in faeces, with amounts excreted being similar in both sexes. The elimination of radioactivity was rapid with most of the radioactivity (more than 87% of administered dose) excreted in the first 48 hours following administration. Very small amounts of radioactivity were recovered from carcasses, amounting to less than 0.2% of the administered dose (AD). The total mean radioactivity recovered in excreta and carcass was for 93.89% and 91.09% of AD in male and female rats, respectively. Excretion profiles were similar for male and female rats (Ghiglieri, 2016a).

Table 1. Cumulative excretion balance (percentage of AD recovered) after 168 hours following oral dose of 50 mg/kg bw of [pyrazole-^{14}C]fluindapyr

	Percentage AD (pyrazole label) recovered	
Matrix	**Males**	**Females**
Urine plus cage wash	27.07	26.68
Faeces	66.67	64.25
Carcass (with skin)	0.14	0.15
Total	93.89	91.09

Source: Ghiglieri, 2016a

In a second study reported by Ghiglieri (2016b), excretory balance (urine, faeces, carbon dioxide), blood and plasma levels and tissue distribution of radioactivity were investigated after administration of [*pyrazole*-^{14}C]fluindapyr to albino Sprague Dawley rats (body weights at the time of first dosing 250–340 g for males, 214–291 g for females) following single and repeated oral dose administration. In addition, blood and plasma levels of ^{14}C products, and mass balance of excretion were determined following a single intravenous (i.v.) administration of [*pyrazole*-^{14}C]fluindapyr in order to evaluate bioavailability and absorption. Mean plasma and blood pharmacokinetic parameters of total radioactivity were evaluated after an i.v. dose of 4 mg/kg bw, and after oral doses of 50 and 1000 mg/kg bw of phenyl-labelled fluindapyr. The test article used throughout this study was Batch no. 54704-16-19, with a radiochemical purity of 99.0%.

The details of administration are described below in Table 2.

Table 2. Fluindapyr ADME studies in SD rats using [phenyl-C^{14}] fluindapyr

Purity	**Study type**	**Dose route and regimen**	**Dose (mg/kg bw)**	**Number of animals**	
				Males	**Females**
98.6%	Excretory balance	Single oral low dose	50	4	4
		Single oral high dose	1000	4	4
		Repeated oral low dose	50	4	4
	Toxicokinetics	Single oral low dose	50	4	4
		Single oral high dose	1000	4	4
		Repeated oral low dose	50	4	4
	Excretory balance and toxicokinetics	Single i.v. dose	4	4	4
	Tissue distribution at T_{max}	Single oral low dose	50	4	4
		Single oral high dose	1000	4	4
		Repeated oral low dose	50	4	4

T_{max} : Time at which maximum concentration is achieved; i.v. : Intravenous; From Ghiglieri, 2016b

Regarding total radioactivity, after i.v. and oral administration to male and female rats, blood systemic exposure was 20% to 30% lower than that in plasma. Values were calculated for C_{max} (maximum concentration achieved) and $AUC_{0–t(last)}$ (area under the concentration–time curve until last

sampling). After single oral doses, blood and plasma ratios of total radioactivity (dose-normalized) for 1000 mg/kg bw compared with 50 mg/kg bw were in the range 0.4–0.7 for male and female rats. This indicates that the extent of exposure increased less than in direct proportion to the dose. After repeated dosing at 50 mg/kg bw per day, no accumulation was observed in either sex. In both sexes blood and plasma concentrations of radioactivity declined with an apparent terminal half-life ($t_{½}$) of 4–6 hours after single or repeated 50 mg/kg bw doses, and with a slightly higher $t_{½}$ of 7–10 hours, with dosing at 1000 mg/kg bw. Absolute bioavailability of total radioactivity in blood and plasma in both sexes after single oral 50 and 1000 mg/kg bw doses was approximately 60% and 40%, respectively. Plasma pharmacokinetics of unchanged fluindapyr in male and female rats was characterized by moderate clearance, with a $t_{½}$ of 2–4 hours, approximately half that of total radioactivity. Following either route of administration, systemic exposure to fluindapyr was higher in females than males.

After intravenous administration, the plasma C_{max} of fluindapyr accounted for most of total radioactivity (66% and 100% in males and females respectively). In contrast, total unchanged parent radioactivity $AUC_{0–t(last)}$ ratios were 18% in the male and 31% in the female rat, suggesting extensive metabolism of the compound. Following oral administrations (50 and 1000 mg/kg), fluindapyr to total radioactivity C_{max} and $AUC_{0–t(last)}$ ratios ranged from 2% to 5% in males, and 3% to 10% in females. Furthermore, the bioavailability of unchanged fluindapyr was 9% and 4% respectively following administration of 50 mg/kg bw and 1000 mg/kg bw doses. These data were indicative of a relevant hepatic first pass effect and/or prehepatic metabolism. The mean pharmacokinetic parameters of the unchanged parent in plasma following i.v. and oral administration of [pyrazole-^{14}C]fluindapyr are shown in Table 3.

Table 3. Mean pharmacokinetic parameters, total radioactivity and unchanged fluindapyr in plasma following i.v. and oral administration of [pyrazole-^{14}C] fluindapyr to rats

Pharmacokinetic parameter	Dose regime and sex							
	Intravenous 4 mg/kg bw		Single oral low 50 mg/kg bw		Single oral high 1000 mg/kg bw		Repeated oral low 50 mg/kg bw, day 14	
	M	F	M	F	M	F	M	F
Total radioactivity								
C_{max} (µg equiv./g)	2.96	2.79	6.29	6.20	65.58	65.18	7.40	8.96
$AUC_{0–t(last)}$ (µg equiv. h/g)	8.45	9.20	59.85	68.16	866.99	709.91	61.07	81.37
$t_{½}$ (h)	5.74	6.50	5.55	5.24	6.80	8.72	3.85	5.67
T_{max} (h)	-	-	2	3	2	3	1	2
Fluindapyr								
C_{max} (µg equiv./g)	1.94	2.90	0.30	0.58	1.27	1.68	0.17	0.46
$AUC_{0–t(last)}$ (µg equiv. h/g)	1.54	2.95	1.45	3.50	17.10	29.82	1.06	3.94
$t_{½}$ (h)	1.45	2.25	1.83	2.60	3.50	3.99	2.71	2.01
T_{max} (h)	-	-	5	4	6	6	4	5

C_{max}: Maximum concentration achieved; M: Male; F: Female Source: Ghiglieri, 2016b

T_{max}: Time at which C_{max} is achieved; $AUC_{0–t(last)}$: Area under the concentration–time curve until last sampling

The excretory balance results (mean recovery of radioactivity dose) after i.v. and oral administration of [*phenyl*-^{14}C]fluindapyr to rats are presented below in Table 4.

Table 4. Cumulative excretion balance (mean percentage of radioactivity dose recovered) after 168 hours following various dosing regimes with [phenyl-^{14}C]fluindapyr

	Dose regime and sex							
	Intravenous 4 mg/kg bw		Single oral low 50 mg/kg bw		Repeated oral low 50 mg/kg bw		Single oral high 1000 mg/kg bw	
Matrix	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Urine plus cage wash	33.61	24.72	22.32	22.62	30.79	28.91	18.62	18.10
Faeces	58.09	69.28	70.37	70.74	68.12	68.79	76.18	74.95
Carcass with skin	0.20	0.16	0.14	0.09	0.15	0.09	0.12	0.08
Total	91.90	94.16	92.83	93.45	99.05	97.78	94.92	93.13

Source: Ghiglieri, 2016b

With regard to tissue distribution of radioactivity, no relevant differences were measured between single and repeated 50 mg/kg bw doses two hours after oral administration of [*phenyl*-^{14}C]fluindapyr; this indicates no accumulation of radioactive material in the analyzed tissues. Slightly lower levels of radioactivity were measured in male than female rats. The highest levels of radioactivity after single or repeated 50 mg/kg bw doses were measured in the gastrointestinal (GI) tract and liver. After oral administration of 1000 mg/kg bw, higher levels of radioactivity were measured in the GI tract, liver, kidney and other tissues. The levels measured in organs after a single oral dose of 1000 mg/kg bw dose were approximately 6–12 times higher than those measured after a single oral dose of 50 mg/kg bw, confirming that the extent of exposure increased less than in direct proportion to size of dose.

Overall, very low levels of radioactivity were measured in all analyzed organs seven days (168 hours) after administration of labelled fluindapyr, and no measurable radioactivity was found in brain, heart, lungs, spleen, thyroid, ovaries or testes. In terms of percentage of administered radioactivity, the cumulative amount recovered 168 hours after dosing from organs, including the whole carcass, averaged between 0.08% and 0.14% of the administered radioactive dose. One week after single and repeated oral doses at 50 mg/kg bw, very low levels of radioactivity were still measured in the skin and GI tract, liver, kidney, white fat and lungs. After a single oral dose of 1000 mg/kg bw, low levels of radioactivity were measured in skin, liver, kidney, GI tract and white fat.

The tissue distribution of radioactivity at T_{max} in various organs and tissues of male and female rats following single and repeated oral administration of [*phenyl*-^{14}C]fluindapyr is shown in Table 5.

Table 5. Distribution, at two hours (T_{max}) after dosing, of radioactivity in organs and tissues of male and female rats after single and repeated oral dosing (percentage of administered dose)

	Dose regime and sex					
	Oral, single 50 mg/kg bw		Oral, repeated 50 mg/kg bw		Oral, single 1000 mg/kg bw	
Matrix	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Brain	0.14	0.33	0.10	0.27	0.10	0.09
Heart	0.11	0.18	0.09	0.18	0.06	0.06
Lung	0.13	0.23	0.10	0.23	0.08	0.08
Kidney	0.56	0.52	0.51	0.57	0.26	0.17
Liver	4.75	5.02	4.78	5.27	2.40	1.36
Spleen	0.04	0.07	0.03	0.05	0.02	0.02
GI tract	5.56	5.50	5.95	5.55	7.13	3.22
Thyroid	0.00	0.00	0.00	0.00	0.00	0.00
Testes	0.14	0.02	0.10	0.02	0.09	0.01
Skin	3.16	4.09	1.96	2.61	2.12	1.24
Carcass	77.31	75.96	77.71	77.95	79.95	86.05
Total	91.91	91.91	91.33	92.71	92.21	92.30

Source: Ghiglieri, 2016b

Bile excretion of radioactivity in rat after repeated dosing

Unlabelled fluindapyr (purity 98.65% by HPLC; Lot no. P/13/013) was administered for 13 days, followed by a last administration of radiolabelled [*pyrazole*-^{14}C]fluindapyr in order to obtain information on the excretion profile of radioactive fluindapyr-related material in bile and excreta. The test article was administered at 50 mg/kg bw per day to male and female rats by oral gavage. At the start of dosing the albino rats were aged 7–10 weeks, males weighing 270–300 g, females 270–300 g Animals were surgically prepared to allow bile collection through bile duct catheterization. After dosing, the animals were housed in metabolism cages and the elimination of radioactive fluindapyr-related material was determined in bile, and all urine and faeces were collected in 24-hour periods up to 72 hours after final administration. The carcasses were analyzed for residual radioactivity. Information that provides a time profile of excretion is reproduced below in Table 6.

Table 6. Excreted percentage of administered radioactive dose following repeated oral administrations of [pyrazole-^{14}C] fluindapyr, (mean value ± standard deviation, for 3 rats/group)

		Sex and period of sampling (hours post dose)							
		Males				Females			
Sample		**0–24**	**24–48**	**48–72**	**Subtotal**	**0–24**	**24–48**	**48–72**	**Subtotal**
Bile	Mean	65.07	0.85	0.14	66.06	56.24	2.04	0.27	58.56
	SD ±	12.77	0.55	0.11	12.55	4.16	1.97	0.34	58.56
Urine	Mean	14.71	2.48	0.77	17.96	9.29	1.90	0.56	11.75
	SD ±	7.74	0.68	0.28	7.77	3.65	0.51	0.27	4.31
Faeces	Mean	5.11	0.85	0.40	6.36	7.40	0.43	0.06	7.90
	SD ±	2.35	0.59	0.11	1.84	5.73	0.17	0.06	5.58
		0–72 hours				**0–72 hours**			
Cage wash	Mean		1.84		1.84		5.41		5.41
	SD ±		0.80		0.80		1.88		1.88
Carcass	Mean		2.0		2.0		4.74		4.74
	SD ±		2.06		2.06		1.62		1.62
TOTAL	**Mean**				**94.23**				**88.36**
	SD ±				**4.85**				**3.14**

SD: Standard deviation; Source: Ghiglieri, 2017

After administration of [*pyrazole*-^{14}C]fluindapyr to albino rats, the radioactivity was mainly excreted in bile; 66.1% and 58.6% of AD in males and females respectively. Urinary excretion of radioactivity (including cage wash) was approximately 19.8% and 17.2% of AD in males and females respectively. The percentage of radioactive dose eliminated in faeces was about 6.4% and 7.9% in males and females respectively. At the end of the period of sample collection, that is at the termination of the study, very little radioactivity was recovered from the carcasses of the animals, less than 5% of AD. In view of the above it was concluded that, following repeated dose administration of fluindapyr to bile duct-cannulated male and female rats, the excretion pattern of total radioactivity was similar in male and female rats (see Table 7) indicating no gender specific differences in the elimination process. Excretion balance (mean recovered radioactive dose) after 14 days repeated oral administration at 50 mg/kg bw per day to male and female Sprague Dawley rats is reported below in Table 7.

Table 7. Excretion balance (mean percentage recovered of radioactivity dose) after 14 days repeated oral dose of 50 mg/kg to rats

Sample	Time period (hours post dose)	Males	Females
Bile	0–72	66.06	58.56
Urine	0–72	17.96	11.75
Faeces	0–72	6.36	7.90
Cage wash	0–72	1.84	5.41
Carcass	at 72 hours post dose	2.00	4.74
Total		94.23	88.36

Source: Ghiglieri, 2017

(b) Dermal route

No data were available regarding absorption, distribution and excretion following dermal application.

1.2. Biotransformation

The metabolite profile of fluindapyr in biological samples (urine, faeces, bile and plasma) from adult male and female Sprague Dawley rats collected during the excretion mass balance studies (Ghiglieri, 2016a, b; Mainolfi & Garau, 2017) was thoroughly investigated. The absorption, distribution, metabolism and excretion (ADME) studies involved oral administration of ^{14}C-labelled fluindapyr in a medium consisting of an aqueous solution of 0.5% low viscosity CMC containing Tween at 0.1% (v/v).

Overall the elimination of radioactivity was rapid, as about 90% of the administered radioactivity was excreted within 48 hours of dosing via faeces (about 70% of AD) and urine (about 20% of AD). Urine samples from rats of the same gender containing significant radioactivity were pooled for each time interval. Aliquots of the pooled urine samples were analyzed directly by HPLC for radioactivity distribution. The faeces samples from rats of the same gender containing significant radioactivity were pooled per time interval and extracted with mixtures of acetone-water. The extracts were analysed for radioactivity content by LSC and the profile of the metabolites was obtained by high-performance liquid chromatography (HPLC). Representative extracts were analyzed also by chiral HPLC to check the enantiomeric ratio of the active ingredient. The dried faeces remaining after extraction were combusted to determine the unextractable radioactivity content by liquid scintillation counting (LSC). Plasma samples collected two hours after dosing were pooled, extracted and analyzed to make a comparison with the profiles obtained for the excreta.

Chromatographic analyses established that fluindapyr was extensively metabolized. Several of the urinary metabolites were also present in faeces. Different doses and administration regimes affected the relative proportions of the detected metabolites, but the differences in metabolic profile were small. The same conclusions could be drawn by comparing the chromatographic profiles obtained from phenyl-labelled and pyrazole-labelled fluindapyr administrations, indicating that the parent molecule is not significantly cleaved. Metabolic profiles were similar in male and female rats, although differences in relative amounts of metabolites were observed. Unchanged fluindapyr was almost exclusively excreted via faeces. Slightly higher levels of unchanged parent were found in the faeces of male rats than females. The parent compound constituted 11.58% and 7.78% of AD in male and female rats, respectively after low-dose administration, 6.71% and 5.12% after high-dose administration, and 10.00% and 10.61% after repeat-dose administration (all these values being the mean of values obtained for phenyl- and pyrazole-labelled fluindapyr). No variation in the enantiomeric ratio of the unchanged fluindapyr was observed for any treatment group, the ratio remaining at around 50 : 50 for R : S enantiomers in representative faeces samples.

Fluindapyr was mainly metabolized through *N*-demethylation, hydroxylation and carboxylation of methyl groups. Other metabolic processes including double hydroxylation, dehydrogenation and conjugation with glucuronic acid were noted to occur to a lesser extent. All metabolites accounting for

5% of AD or more were identified by comparative liquid chromatography–mass spectrometry (LC-MS) analyses against reference standards. Metabolites identified included the following:

- unchanged fluindapyr;
- 1-carboxy-N-desmethyl-fluindapyr (two diastereomers);
- 1-hydroxymethyl-N-desmethyl-fluindapyr (two diastereomers);
- trans-1-carboxy-fluindapyr (isomer #1);
- N-desmethyl-fluindapyr;
- N-hydroxy-fluindapyr.

Excretion levels of 1-carboxy-N-desmethyl-fluindapyr isomer #1 and 1-hydroxymethyl-N-desmethyl-fluindapyr isomer #1 were generally higher in male rats, reaching maximums of 15.51% and 14.76% respectively. Metabolites N-desmethyl-fluindapyr and N-hydroxy-fluindapyr were excreted in female faeces in larger amounts than in males, reaching maximums of 7.96% and 8.43% respectively. Metabolites 1-carboxy-N-desmethyl-fluindapyr isomer #2, and 1-hydroxymethyl-N-desmethyl-fluindapyr isomer #2 were found at similar levels in males and females. The distribution of the main faecal metabolites was very similar regardless of labelling position or dose level.

Other minor metabolites (less than 5% of AD) were identified as:

- 1-hydroxymethyl-fluindapyr (two diastereomers);
- cis-1-carboxy-fluindapyr (isomer #2);
- dihydroxy-N-desmethyl-fluindapyr (isomers);
- dihydroxy-fluindapyr;
- 1-hydroxymethyl-N-desmethyl-fluindapyr glucuronide (isomers);
- 1-hydroxymethyl-dehydro-N-desmethyl-fluindapyr, and its glucuronide;
- 1-carboxy-dehydro-N-desmethyl-fluindapyr, and
- 3-OH-fluindapyr.

Total identified/characterized radioactivity (mean of phenyl- and pyrazole-labelled fluindapyr administrations from various dosage regimes) accounted for 84.66% of AD and 74.73% of AD in excreta of male and female rats respectively. The unextractable radioactivity in all cases represented less than 5% of AD. Chromatographic profiles from plasma samples and excreta were qualitatively very similar. Significantly higher amounts of N-desmethyl-fluindapyr and unchanged fluindapyr were detected in the plasma of female rats than in that of male rats. (Mainolfi & Garau, 2017)

The metabolite profile of ^{14}C-labelled fluindapyr was also evaluated in the bile, urine and faeces of male and female bile duct-cannulated rats following repeated oral administration of [*pyrazole*-^{14}C]fluindapyr (Ghiglieri, 2017). Overall, the elimination of radioactivity was rapid with about 90% and 78% of AD recovered in bile, urine and faeces within 72 hours in bile duct-cannulated males and females, respectively. The unchanged fluindapyr was found to be almost exclusively excreted via the faeces. Chromatographic analyses established that fluindapyr was extensively metabolized. Metabolite profiles in bile and excreta were qualitatively similar in male and female rats, although differences in relative amounts of metabolites were seen between the two sexes. Fluindapyr was metabolized through *N*-demethylation and oxidation to hydroxy and carboxy derivatives. Dehydrogenation also occurred, but to a lesser extent. Extensive phase II metabolism was found in bile samples of male and female rats. Conjugation with glucuronic acid occurred with 1-OH-me-N-desmethyl-fluindapyr (two diastereomers), 1-OH-methyl-deH-N-desmethyl-fluindapyr, 1-COOH-N-desmethyl-fluindapyr (two diastereomers), 1-OH-methyl-fluindapyr (two diastereomers), 1-COOH-fluindapyr (*cis* and *trans* isomers), N-OH-fluindapyr, and to a lesser extent for N-OH-deH-fluindapyr.

Chemical hydrolysis simplified the complex biliary radiochromatographic profiles, allowing the quantification of the deconjugated compounds. In both sexes, the major biliary metabolites were:

- 1-OH-N-desmethyl-fluindapyr (26.48% and 10.11% of AD in males and females respectively).
- 1-COOH-N-desmethyl-fluindapyr (15.12% and 6.05% of AD in male and female rats),
- 1-COOH-fluindapyr (5.23% and 9.41% of AD in male and female rats),
- 1-OH-methyl-fluindapyr (6.41% and 13.45% of AD in male and female rats),
- N-OH-fluindapyr (5.90% and 17.57% of AD in male and female rats),
- 1-OH-Methyl-deH-N-desmethyl-fluindapyr,
- 1-COOH-deH-N-desmethyl-fluindapyr.

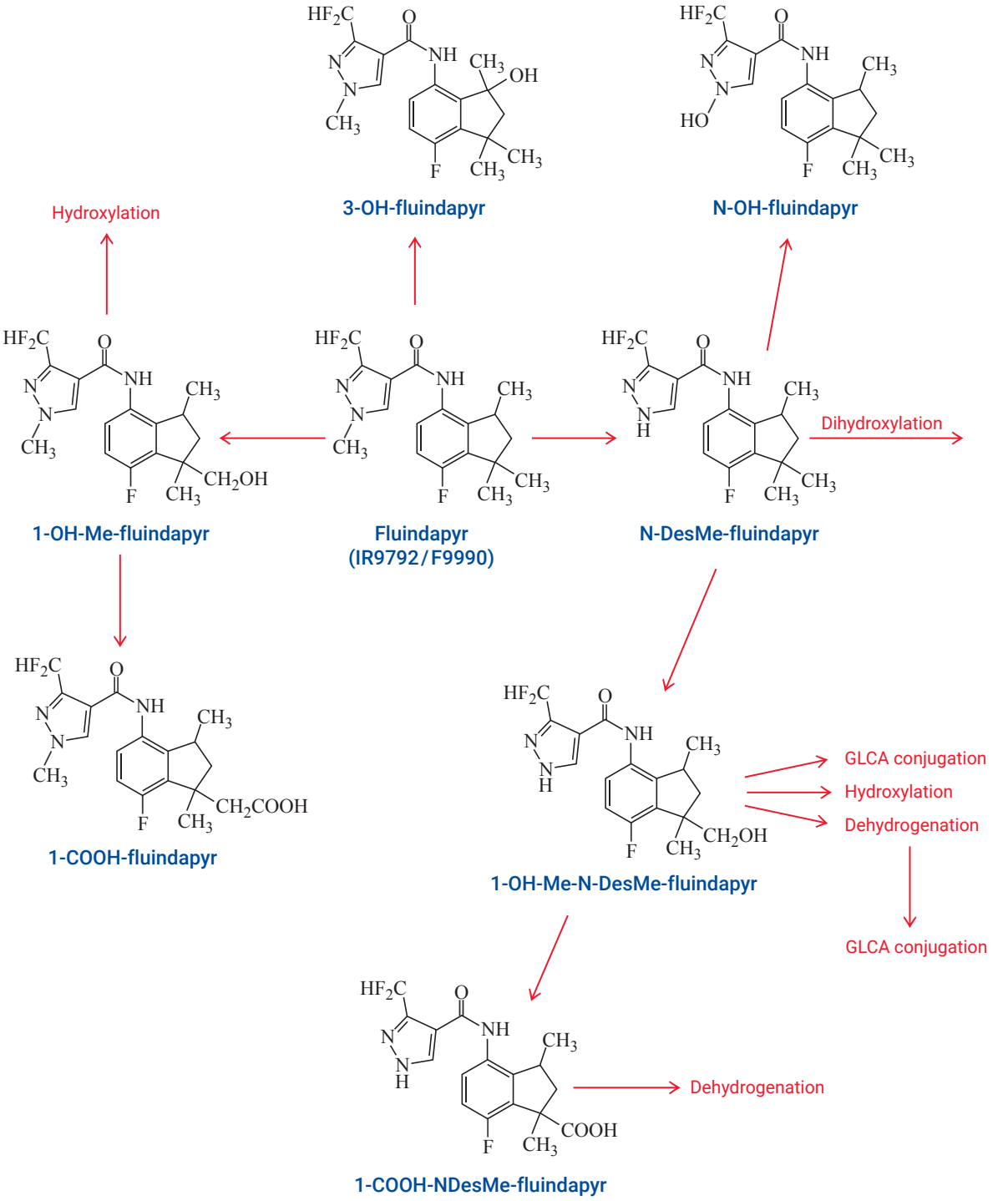
The last two of which were found in hydrolysed bile samples at 2.29% and 1.49% of AD, respectively, but only in the samples from males.

In conclusion, after repeated oral administration of 50 mg/kg bw per day of fluindapyr to bile duct-cannulated male and female rats, the percentage oral absorption of total radioactivity, as the sum of percentage excretion of radioactivity in bile and urine, was estimated to be about 84% for male rats and about 70% for females. The major metabolites (greater than 10% of AD in bile and urine) included 1-hydroxymethyl-fluindapyr, 1-hyroxymethyl-N-desmethyl-fluindapyr, 1-carboxy-fluindapyr, 1-carboxy-N-desmethyl-fluindapyr and N-hydroxy-fluindapyr. There was no evidence of significant cleavage of the core structure.

Overall, the ADME characteristics of fluindapyr in the Sprague Dawley rat, are very well understood. The administered dose is rapidly absorbed (T_{max} 3 hours) with most of the radioactivity eliminated within 48 hours. Biliary excretion constitutes the major elimination route, and up to 84% of total radioactivity is absorbed after oral administration. The systemic exposure to unchanged parent fluindapyr is up to four-fold higher in female rats than males. Fluindapyr is extensively metabolized with only 5% of the parent excreted unchanged in the faeces. No significant qualitative differences in metabolite profile are evident between the labels or genders. Fluindapyr is primarily metabolized via hydroxylation, oxidation and *N*-demethylation, and by formation of the corresponding conjugates. In addition, the ratio of enantiomers of fluindapyr remains unchanged, indicating no evidence of enantiospecific ADME in rats (Mainolfi & Garau, 2017)

The proposed metabolic pathway of fluindapyr in the rat is shown below in Figure 3 .

Figure 3. Proposed metabolic pathway of fluindapyr in the rat



(Redrawn from Mainolfi & Garau, 2017)

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

The results of acute toxicity studies with fluindapyr administered orally, dermally or by inhalation are summarized in Table 8. Fluindapyr was of low toxicity via all routes when tested at the limit doses or limit concentration.

Table 8. Summary of acute toxicity studies with fluindapyr

Species	Strain	Sex	Route	Purity (%)	LD_{50} (mg/kg bw) or LC_{50}(mg/L)	Reference
Rat	Wistar	F	Oral	96.309	> 2000	Bradshaw, 2018a
Rat	Wistar RccHan:WIST	M + F	Dermal	96.309	> 2000	Bradshaw, 2018b
Rat	Wistar RccHan:WIST	M + F	Inhalation	96.309	> 5.19 MMAD 2.89 µm	Griffiths, 2013

bw Body weight; F females; M males; GLP good laboratory practice; LC_{50} median lethal dose; LD_{50} median lethal concentration; MMAD: Mass median aerodynamic diameter; w/w weight per weight

(b) Dermal, ocular and phototoxic effects

The results of dermal and eye irritation and skin sensitization studies with fluindapyr are summarized in Table 9. Fluindapyr was not irritating to skin or eyes but was a skin sensitiser in mice (LLNA).

Table 9. Summary of dermal and eye irritation and skin sensitization studies with fluindapyr

Species	Strain	Sex	End-points	Purity (%)	Result	Reference
Reconstructed Human Epidermis Model	EPISKIN-SM™	Not applicable	Skin irritation, human skin model	97.6	Non-irritant	Warren, 2018
Isolated cow eye	Not applicable	Not applicable	Eye irritation (in vitro)	97.6	Inconclusive	Henzell, 2018
Rabbit	New Zealand White	Female	Eye irritation	96.309	Non irritating	Ben Abdeljelil, 2013
Mouse	CBA/ CaOlaHsd	Female	Skin sensitization (Local Lymph node Assay)	96.309	Sensitizing,	Dony, 2014a
3T3 fibroblasts)	Balb/c 3T3 fibroblasts (clone 31)	Not applicable	Phototoxicity	97.46	Non-phototoxic	Westerink, 2015

2.2 Short-term studies of toxicity

(a) Oral administration

Mouse

Study 1

Fluindapyr (purity 96.3%; Lot no. PL13-015) was administered for 28 consecutive days to four groups of approximately 37-day-old Crl:CD-1 mice, males weighing ca 25–35 g and females ca 20–30 g at randomization. Groups consisting of five mice of each sex were fed at dietary concentrations of 0, 300,

1000, 3000 or 5000 ppm (equal to 0, 61,192, 528 and 1093 mg/kg bw per day for males, 0, 71, 275, 675, and 1339 mg/kg bw per day for females). The concurrent control group was administered the basal diet on a comparable regimen. Following 28 days of exposure, all mice were humanely euthanized.

All animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily, and detailed physical examinations were performed weekly. Individual body weights and food consumption were recorded weekly. Clinical pathology parameters (haematology and serum chemistry) were analyzed for all animals at the scheduled necropsy (day 28). Complete necropsies were conducted on all animals, and selected organs were weighed at scheduled necropsy. Selected tissues from all animals were examined microscopically.

There were no test substance-related effects on survival, body weight, food consumption, haematology parameters, or serum chemistry parameters. In addition, there were no test substance-related clinical observations or macroscopic findings. Test substance-related findings were limited to the liver. These liver effects included:

- higher liver weight (absolute and relative to body and brain weight) at 300 ppm and above; this was statistically significant compared to controls, with a 17% and 21% increase in males when expressed relative to final body weight in the 3000 and 5000 ppm group respectively, and a 21% increase in the 5000 ppm group females;
- the microscopic observation of hepatocellular hypertrophy at 1000, 3000 and 5000 ppm in males and females.

In the absence of any biochemical alteration these liver findings were considered to be adaptive responses and nonadverse, as there were no correlating test substance-related changes in clinical pathology parameters related to the liver.

Based on the results of this study, the no-observed-adverse-effect level (NOAEL) was 5000 ppm (equal to 1093 mg/kg bw per day). (Randazzo, 2016a)

Study 2

In a 90 day dietary study, male and female Crl:CD1(ICR) mice approximately 37 days old, males weighing ca 25–35 g, females ca 20–30 g at randomization, were administered fluindapyr (purity 96.3%; Lot no. PL13-0150) in the diet for at least 90 consecutive days. Groups consisting of 10 mice of each sex were fed fluindapyr at concentrations of 0, 300, 1000 or 3000 ppm (equal to 0, 51, 160 and 529 mg/kg bw per day for males, 0, 79, 254 and 799 mg/kg bw per day for females).

Animals were observed for mortality and moribundity twice daily; clinical signs were evaluated beginning on study day 0 and until the day prior to the scheduled necropsy. Body weights were recorded weekly during the study period and on the day of scheduled necropsy. Food consumption was recorded weekly throughout the study. Blood samples for haematology and clinical chemistry parameters were collected from the surviving animals at termination. A complete necropsy was conducted on all animals. Organ weights were recorded from all animals at necropsy. Histopathological examination of an extensive range of selected tissues was performed.

There was no treatment-related mortality, clinical observations or effects on food consumption, haematology or clinical chemistry parameters, nor any noteworthy gross necropsy observations. The 3000 ppm group males, showed reduced body weight gain (−5.8 g; −22.7%; not statistically significant) when compared to the control group (+7.5 g) for the entire cumulative interval (weeks 0–13), higher liver weight (by 17.3% relative to final body weight; statistically significant) and minimal hepatocellular hypertrophy were observed. The 3000 ppm group females showed higher body weight gain (7.4 g; by 21.3%; not statistically significant) when compared to the control group (6.1 g) for the entire cumulative interval (weeks 0–13). Higher mean liver weights compared to controls (by 13.9% and 17.8%, relative to final body weights; statistically significant) were seen in the 1000 and 3000 ppm group females, respectively, and minimal to mild hepatocellular hypertrophy at 300 ppm and greater was also observed. Higher mean weights for the adrenal gland (by 30.6%, 38.5%, 33.6%; absolute, relative to final body weight, and relative to brain weight, respectively) and pituitary gland (by 24%, 28.6% absolute and relative to final body weight, respectively) were noted in the 3000 ppm group males. These changes were minimal, with only the values of three individual animals' absolute adrenal weights, and two individuals' absolute pituitary weights above the concurrent control group range. There were no

histological correlates, therefore weight changes were not considered to be test substance-related. The microscopic observation of minimal to mild hepatocellular hypertrophy was not considered adverse because increased liver weights did not correlate with observations on serum chemistry parameters.

Table 10. Key findings of the 13-week dietary study in mice

	Sex, dietary dose (ppm)							
	Males				Females			
Finding	**0**	**300**	**1000**	**3000**	**0**	**300**	**1000**	**3000**
Body weights (g)								**0**
Week 13 weight	38.0	38.0	38.2	35.8	29.1	30	30.6	30.5
% difference	-	0	0.5	−6	-	3	5	5
Cumulative body weight change (g)								
Week 1–13 weight	7.5	7.8	8.0	5.8	6.1	6.8	7.2	7.4
% difference	-	4	7	−23	-	11	18	21
Organ weights								
Liver weight								
Absolute (g)	1.98	2.04	2.10	2.17	1.51	1.70	1.82**	1.89
% difference	-	3	6	10	-	12	20	25
Relative to bw[a] (g/100g)	5.2	5.4	5.5	6.1**	5.2	5.6	5.9**	6.1**
% difference	-	4	6	17	-	8	14	18
Relative to brain (g/100g)	394.5	415.0	416.2	441.2	315.4	349.0	367.1*	384.5**
% difference	-	5	6	12	-	11	16	22
Adrenal weight								
Absolute (g)	0.0049	0.0051	0.0053	0.0064**	0.0109	0.0115	0.0105	0.0113
% difference	–	4.1	8.2	30.6	-	5.5	−3.7	3.7
standard deviation	0.00111	0.00114	0.00107	0.00114	0.00217	0.00271	0.00128	0.00243
Relative to bw[a] (g/100g)	0.013	0.014	0.014	0.018**	0.038	0.038	0.035	0.037
% difference	-	7.7	7.7	38.5	-	0.0	−7.9	−2.6
Relative to brain (g/100g)	0.982	1.048	1.054	1.312*	2.269	2.360	2.134	2.285
% difference	-	6.7	7.3	33.6	–	4.0	−5.9	0.7
Pituitary weight								
Absolute (g)	0.0025	0.0022	0.0022	0.0031	0.0031	0.0030	0.0030	0.0028
% difference	-	−12.0	−12.0	24.0	-	−3.2	−3.2	−9.7
Relative to bw[a] (g/100g)	0.007	0.006	0.006	0.009*	0.011	0.010	0.010	0.009
% difference	-	−14.3	−14.3	28.6	-	−9.1	−9.1	−18.2
Histopathology								
Hepatocellular hypertrophy (centrilobular)								
Number examined	10	10	9	10	10	10	10	10
Minimal	0	0	0	7	0	3	5	3
Mild	0	0	0	0	0	0	4	6

[a] Relative to final body weight: Source: Kappeler, 2016a

Statistically significant at: * $p \leq 0.05$, ** $p \leq 0.01$

Based on the results of this study, the NOAEL was 3000 ppm (equal to 529 mg/kg bw per day) the highest dose tested. (Kappeler, 2016a)

Rat

Study 1

Fluindapyr (purity 96.3%; Lot no. PL13-0150) was administered in the diet ad libitum to male and female Crl:CD(SD) rats, arranged in two sets of treatment groups: four toxicology groups (Groups 2–5) for 28 consecutive days and four toxicokinetic groups (Groups 2A–5A) for 26 consecutive days. The

animals were approximately seven weeks old at the initiation of dose administration. Individual body weights ranged from 205 g to 264 g for males and from 154 g to 206 g for females in the toxicology groups, and from 201 g to 255 g for males and 160 g to 210 g for females in the toxicokinetic groups at randomization. Concurrent control groups for each set (Groups 1 and 1A) were offered the basal diet on a comparable regimen. Groups 1–5 each consisted of five animals per sex. Group 1A consisted of three animals of each sex, and Groups 2A–5A each consisted of six animals per sex. The design of the experiment is shown below in Table 11.

Table 11. Design of the experiments; 28-day rat study

Group number	Treatment	Dietary concentration (ppm)	Active ingredient intake (mg/kg bw per day)		Number of animals[a]	
			Males	Females	Males	Females
Toxicology group						
1	Basal diet	0	0	0	5	5
2	+ fluindapyr	300	24	27	5	5
3	+ fluindapyr	1000	81	83	5	5
4	+ fluindapyr	2000	157	183	5	5
5	+ fluindapyr	4000	322	320	5	5
Toxicokinetic group						
1A	Basal diet	0	0	0	3	3
2A	+ fluindapyr	300	24	27	6	6
3A	+ fluindapyr	1000	81	83	6	6
4A	+ fluindapyr	2000	157	183	6	6
5A	+ fluindapyr	4000	322	320	6	6

Source: Randazzo, 2016b

[a] All animals in the toxicology group were euthanized following 28 consecutive days of control or test diet being given; all animals in toxicokinetic group were euthanized following 26 consecutive days of control or test diet being given

All animals were observed twice daily for mortality and morbidity. Clinical examinations were performed daily, whereas body weights and food consumption were measured weekly for all groups. Blood and urine samples for clinical pathology evaluations (haematology, coagulation, serum chemistry and urinalysis) were taken from the toxicology group animals at scheduled necropsy on day 28. Animals in the toxicokinetic groups were monitored/evaluated specifically for measuring fluindapyr. Blood samples for toxicokinetic evaluation were collected from three animals per sex from Groups 2A–5A at approximately 06.00, 10.00, 14.00, 18.00 and 22.00 hours on days 2 and 24, and at approximately 02.00 hours on days 3 and 25. Blood samples were collected from three animals per sex in Group 1A) at approximately 06.00 hours on days 2 and 24. Plasma concentrations of fluindapyr were measured using a validated method. No other analytes were measured. A complete necropsy was performed on all animals and organ weights (adrenals, brain, epididymides, heart, kidneys, liver, ovaries with oviducts, pituitary gland, prostate with seminal vesicles and coagulating glands, spleen, testes, thymus, thyroid with parathyroid, uterus) were recorded. An extensive range of organs and tissues were evaluated histopathologically.

There were no treatment-related clinical signs, effects on mortality, haematology, serum chemistry or urinalysis parameters, or noteworthy gross necropsy or histopathological observations. Lower body weight gains and food consumption were noted in the 4000 ppm group females primarily during the first two weeks of the test diet being offered. This initial suppression of body weight gain in 4000 ppm females resulted in a 16 g lower cumulative body weight gain compared to the control group over days 0–27. Test substance-related findings at scheduled necropsy (day 28) were limited to a higher relative liver to final body weight in females at 300 ppm and above (statistically significant), and slightly higher relative liver to final body weight in males at 1000 ppm and above. These findings were considered nonadverse since no correlating findings in serum chemistry or histopathology were observed. In analysis of metabolizing liver enzymes, a small but statistically significant increase in testosterone 16β-hydroxylation (a marker

for CYP2B1/2) in male rats at 4000 ppm, and a statistically significant and dose-dependent increase in testosterone 6β-hydroxylation (a marker for CYP3A1/2) in male rats up to 4000 ppm were observed. These findings were considered nonadverse since no correlating findings in serum chemistry or histopathology were observed and the same changes were not observed in females.

Dietary administration of fluindapyr to rats resulted in systemic exposure to fluindapyr. Exposure (measured as AUC_{last} and C_{max}) increased with increasing dose over the dose range 300 to 4000 ppm on both evaluation days, except for males on day 2, when C_{max} was lower at 4000 ppm than at 2000 ppm. Using AUC_{last} and C_{max}, normalized for calculated test substance consumption in the main study phase (as mg/kg bw per day), exposure increased nearly proportionally over the dose range 300 to 4000 ppm on both evaluation days in both sexes. However, the relationship was generally greater than proportional between 300 ppm and 1000 ppm or 2000 ppm. Females, assessed in terms of C_{max} on day 24, were an exception to this trend; here the relationship over the dosing range was less than proportional. Exposure to fluindapyr for females (based on dose-normalized AUC_{last} and C_{max}) was two- to four-fold higher than for males on day 2, and two- to six-fold higher on day 24. For males, exposure to fluindapyr appeared to be lower on day 24 than on day 2 at all dosage levels, which, it was thought, was likely to be a consequence of hepatic enzyme induction (CYP2B and CYP3A) leading to increased metabolism and clearance. The details of the above relationships is shown below in Table 12.

Table 12. Toxicokinetic Parameter for Fluindapyr in Rats

	Sex, dietary concentration (ppm)							
	Males				**Females**			
	300	**1000**	**2000**	**4000**	**300**	**1000**	**2000**	**4000**
Parameters	**Study day 2**							
AUC_{last} (ng × hours/mL)	286	2020	2940	3730	947	4690	8660	10900
Dose-normalized AUC_{last}	10.2	21.0	15.4	10.5	31.6	51.0	47.3	32.8
C_{max} (ng/mL)	26.0	223	366	250	88.3	458	797	963
Dose-normalized C_{max}	0.929	2.32	1.92	0.704	2.94	4.98	4.36	2.90
T_{max} of the day (24 hour clock)	02.00	06.00	06.00	02.00	06.00	06.00	06.00	06.00
Parameters	**Study day 24**							
AUC_{last} (ng × hours/mL)	263	1330	1610	2720	1100	4200	7800	10600
Dose-normalized AUC_{last}	12.5	18.0	11.1	9.44	45.8	53.2	46.2	34.8
C_{max} (ng/mL)	18.6	107	149	341	126	310	568	865
Dose-normalized C_{max}	0.886	1.45	1.03	1.18	5.25	3.92	3.36	2.84
T_{max} of the day (24 hour clock)	10.00	06.00	06.00	02.00	06.00	06.00	10.00	02.00
Accumulation Ratio	0.92	0.66	0.55	0.73	11.2	0.90	0.90	0.97

Source: Randazzo, 2016b

The NOAEL in this study was 4000 ppm (equal to 320 mg/kg bw/day) the highest dose tested (Randazzo, 2016b).

Study 2

A 90-day dietary toxicity study in Sprague Dawley rats was undertaken with dual objectives: to evaluate the potential toxicity of fluindapyr when administered orally via the diet for at least 90 consecutive days, and to evaluate the recovery, persistence or progression of any effects following a minimum of a 28-day recovery period following a 90-day exposure to the test article. Fluindapyr (purity 96.3%; Lot no. PL13-0150) was administered to three groups of male and female Crl:CD(SD) rats, approximately seven weeks old, male body weights ranging from 186–246 g, females from 152–198 g at the initiation of diet exposure. Groups consisted of 15 rats of each sex per group for control and high concentration groups, 10 rats of each sex per group for the low and medium concentration groups. Initial dietary concentrations were 0, 100, 450 or 2000 ppm. Considering the available toxicokinetic data from the 28-day dietary toxicity study in rats (Randazzo, 2016b) and the lack of any clinical signs of toxicity or

effects on body weight gains in the 2000 ppm males in the first period, the dietary concentration for these males was increased from 2000 to 6000 ppm beginning on day 28. Mean consumption for males was equal to 0, 8, 36 or 160 mg/kg bw per day from study weeks 0 to 4 and 0, 6, 24 or 330 mg/kg bw per day from weeks 5–13. In females, mean consumption was 0, 11, 41 or 194 mg/kg bw per day from study weeks 0–4, and 0, 7, 30 or 139 mg/kg bw per day from weeks 5–13).

Animals were observed twice daily for mortality and moribundity and once daily for clinical signs. Body weight and food consumption were recorded once weekly except for weeks 12 and 16, when they were recorded twice weekly. Blood and urine samples for clinical pathology evaluations (haematology, coagulation, serum chemistry and urinalysis) were collected from all animals after overnight fasting at scheduled necropsy (week 13 for animals scheduled for necropsy, and week 17 for the recovery groups). Necropsies were performed on 10 animals/sex per group during week 13 and on the remaining five (or fewer) animals per sex following the 28/29-day recovery period. Ophthalmic examinations were performed during week 12. Organ weights (adrenals, brain, epididymides, heart, kidneys, liver, ovaries with oviducts, pituitary gland, prostate with seminal vesicles and coagulating glands, spleen, testes, thymus, thyroid with parathyroid, uterus) were recorded for all animals. An extensive range of organs and tissues was evaluated histopathologically.

One incidental death in the control group occurred during the study. There were no treatment-related clinical signs of toxicity and there were no treatment-related effects observed upon ophthalmoscopic examination or macroscopic examination at necropsy; this in both the primary and recovery groups. Lower body weights (by 4.6% compared with controls) were noted in 2000/6000 ppm group males between weeks 4 and 13. Cumulative body weight gains in the 2000/6000 ppm group males from week 4 to 13 were 15.3% lower than those of the control group. However, these lower body weights did not persist during the recovery period for the 2000/6000 ppm group males. Lower body weights were noted in the 2000 ppm group females from week 0 to 4 and week 4 to 13. Cumulative body weight gains in these 2000 ppm group females from week 4 to13 were 16.4% lower than for the control group. Lower body weights then partially recovered (to just 5.9% lower when compared to the control group at week 17) with higher body weight gains seen during the recovery period. The changes in body weight and body weight gain were not considered to be adverse as the differences were not statistically significant and did not persist during the recovery period.

Haematology and clinical chemistry changes were noted in the 2000 ppm group females (lower white blood cell and lymphocyte counts) at the primary necropsy. These findings did not appear to persist as they were not observed at necropsy of the recovery group 2000 ppm females. Also the original findings were not associated with bone marrow or other microscopic changes and fell within the laboratory's historical control database range of study means. White blood cell and lymphocyte counts in the 2000 ppm group females were similar to control group means at the recovery necropsy. Similar changes were not noted in males. Significantly lower absolute neutrophil counts in the 100, 450 and 2000 ppm group females (by 35.8%, 34.7%, and 36.8%, respectively) recorded only at the primary necropsy were probably due to high individual and mean concurrent control values, which were occasionally at the high end of the laboratory's historical control database range (individual values). There was no dose–response relationship, therefore the change was not considered treatment-related. A higher triglyceride value was noted in the 2000/6000 ppm group males at the primary necropsy. Group means were within the laboratory's historical control range of study means and only one individual value was above the laboratory's historical control reference range. A lower γ-glutamyl transpeptidase (GGTP) value in the 450 ppm group females (0.0 U/L) at primary necropsy was not considered test substance-related given the lack of a dose–response relationship.

Higher liver weights were noted in the 2000/6000 ppm group males and 2000 ppm group females at the primary necropsy, and in the 2000/6000 ppm group males at the recovery necropsy. The absence of related clinical chemistry or pathology findings in the liver indicates that these weight increases were nonadverse. Higher thyroid/parathyroid weights were noted in the 450 and 2000/6000 ppm group males and 2000 ppm group females at primary necropsy, and in the 2000 ppm group females and 2000/6000) ppm group males at the recovery necropsy. There were no directly treatment-related macroscopic or microscopic findings at the primary or recovery necropsies and no test article-related haematology or clinical chemistry changes at the recovery necropsy, that might suggest thyroid toxicity, therefore the weight changes were considered nonadverse.

Table 13. Summary of findings from the 90-day toxicity study with fluindapyr in rats

	Sex, dietary concentration (ppm)							
	Males				Females			
	0	100	450	2000/ 6000	0	100	450	2000
Body weights (g)								
Week 1	298	295	301	298	212	206	213	207
Week 4	421	413	435	421	266	257	271	253
Week 13	592	578	604	565	333	318	352	308
Week 17 (recovery groups)	590	NA	NA	615	375	NA	NA	353
Cumulative body weight change (g)								
Weeks 0–1	56	55	59	55	28	23	29	22
Weeks 0–4	179	173	194	178	82	74	87	68
Weeks 4–13	170	165	169	144	67	61	81	56
Weeks 13–17 (recovery groups)	42	NA	NA	42	21	NA	NA	34*
Clinical chemistry and haematology								
WBC; week 13 (1000/µL)	11.01	11.77	10.76	9.99	7.03	6.91	6.48	5.51
% difference from controls	-	7%	−2%	−9%	-	−2%	−8%	−21.6%
Lymphocytes; week 13 (1000/µL)	8.95	9.12	8.99	7.13	5.74	5.99	5.55	4.67
% difference from controls	-	2%	0.4%	−20%	-	4%	−3%	−19%
Neutrophils; week 13 (1000/µL)	1.49	2.03	1.22	2.28	0.95	0.61*	0.62*	0.60*
% difference from controls	-	36.2%	−18.1%	53.0%	-	−35.8%	−34.7%	−36.8%
Triglycerides; week 13 (mg/dL)	64	70	86	102*	38	38	45	32
% difference from controls	-	9%	34%	59%	-	0%	18%	−16%
Organ weights	**Week 13: primary necropsy**							
Liver (absolute, g)	14.35	13.66	14.40	15.09	7.52	7.63	8.5	8.1
% difference from controls	–	−5%	0.3%	5%	–	2%	13%	7%
Liver (g/100g final bw)	2.462	2.476	2.480	2.820**	2.474	2.527	2.533	2.833**
% difference from controls	–	0.6%	0.7%	15%	–	2%	2%	15%
Liver (g/100 g brain)	672.7	650.1	683.5	747.7	400.8	404.0	441.4	426.5
% difference from controls	–	−3%	2%	11%	–	0.8%	10%	6%
Thyroid/parathyroid (absolute, g)	0.0195	0.021	0.025*	0.025	0.015	0.014	0.018	0.019
% difference from controls	–	8%	28%	26%	–	−6%	14%	26%
SD	0.0027	0.004	0.005	0.006	0.003	0.003	0.004	0.005
Thyroid/parathyroid (g/100g final bw)	0.003	0.004	0.004**	0.005**	0.005	0.005	0.005	0.007*
% difference from controls	–	33%	33%	67%	–	0%	0%	40%
Thyroid/parathyroid (g/100 g brain)	0.912	1.001	1.183*	1.214**	0.815	0.764	0.914	1.013
% difference from controls	–	10%	30%	33%	–	−6%	12%	24%
Organ weights	**Week 17: recovery necropsy**							
Liver (absolute, g)	13.62	NA	NA	14.40	9.44	NA	NA	8.24
% difference from controls	–			6%	–			−13%
Liver (g/100g final bw)	2.416	NA	NA	2.450	2.691	NA	NA	2.509
% difference from controls	–			1.4%	–			−7%
Liver (g/100 g brain)	665.1	NA	NA	687.9	471.0	NA	NA	426.7
% difference from controls	–			3%	–			−9%
Thyroid/parathyroid (absolute, g)	0.0189	NA	NA	0.0206	0.0171	NA	NA	0.0182
% difference from controls	–			9%	–			6%
Thyroid/parathyroid (g/100g final bw)	0.004	NA	NA	0.003	0.005	NA	NA	0.006
% difference from controls	–			−25%	–			20%
Thyroid/parathyroid (g/100 g brain)	0.927	NA	NA	0.985	0.850	NA	NA	0.939
% difference from controls	–			6%	–			11%

WBC: Total leukocyte count; Statistically significant at: * $p \leq 0.05$, ** $p \leq 0.01$ Source: Kapeller, 2016b

Based on the results of this study, which shows no relevant toxicological effect up to the highest dose tested, the NOAEL was 2000/6000 ppm for males (2000 ppm for females), (equal to 139 mg/kg bw per day), the highest dose tested. (Kappeler, 2016b)

Dog

Study 1

In a dose range-finding study, fluindapyr (purity 96.3%; Lot no. PL13-0150) was orally administered daily for 28 consecutive days, undiluted via gelatin capsule, to four groups of beagle dogs, 5–6 months old, weighing 6–9 kg at first test substance administration. Groups consisted of between two and five dogs of each sex per treatment group. The test substance was initially offered in the diet ad libitum for seven consecutive days to four groups of dogs at dietary concentrations of 800, 2800, 8000 and 20 000 ppm (groups 2, 3, 4 and 5, respectively). A concurrent control group was offered the basal diet ad libitum. Due to decreased palatability of the test diet at these dosage levels, the study was restarted following an eight-day pause in dosing, and the route of administration was changed to oral via gelatine capsule. Dosage levels were 20, 70, 200 and 500 mg/kg bw per day (groups 2, 3, 4 and 5, respectively). A concurrent control group (group 1) received empty capsules on a comparable regimen. All animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily at the time of dosing, and approximately 1–2 hours after dose administration. Individual body weights were recorded on day 0 (prior to dosing), biweekly during the study period, and on the day prior to the scheduled necropsy (nonfasted) and final body weights (fasted) prior to the scheduled necropsy. Food consumption was recorded daily. Blood samples for clinical pathology (haematology, coagulation, serum chemistry) were collected from all animals during the acclimation prior to randomization, and again on day 28. Ophthalmic examinations were conducted on all animals during acclimation prior to randomization and on day 25. All animals were euthanized on day 28 and a complete necropsy was conducted. Organ weights were recorded for the following: adrenals, brain, epididymides, heart, kidneys, liver with gallbladder, ovaries, pituitary gland, prostate gland, spleen, testes, thymus, thyroid with parathyroids, uterus with cervix). Pathological evaluation was undertaken on selected organs and histopathology on selected tissues.

All animals survived until the scheduled necropsy. There were no test substance-related clinical or ophthalmic findings, effects on haematology or coagulation parameters. Reduced body weights with correspondingly lower food consumption values were noted in males at 500 mg/kg bw per day, predominantly due to one male that consumed less diet during the dosing period. Microscopic observations revealed dose-dependent hepatocellular hypertrophy in all treated dogs and these findings correlated with higher liver weights and higher serum levels of alkaline phosphatase (ALP). The increased ALP in low-dose males was considered unrelated to treatment as the pretest (day 22) value was more than double the control value. At 70 mg/kg bw per day and above in male dogs and 200 mg/kg bw per day and above in female dogs, panlobular hepatocellular hypertrophy was associated with the increased serum levels of ALP. At 500 mg/kg bw per day, there was observed an elevation of other serum enzymes indicative of liver cell injury; GGTP, alanine transaminase (ALT), and succinate dehydrogenase (SDH). Collectively, pathology findings were consistent with oral exposure to a test substance that induced liver microsomal enzymes (P450). Liver changes were considered to be adverse in males and females at 500 mg/kg bw per day because the increases (greater than 30% compared to controls, relative to body weight) were associated with increases in ALT, ALP, GGTP and SDH activity in serum, and with liver hypertrophy. Liver effects at lower doses were reported not to be adverse according to published criteria (Hall et al., 2012; HED, 2002; Yokoyama et al., 2019).

Table 14. Findings from the 28-day study in dogs treated orally (capsule) with fluindapyr

	Sex, dose (mg/kg bw per day)									
	Males					Females				
	0	20	70	200	500	0	20	70	200	500
Body weight gain (*n* = two dogs)										
Day 0–6 (kg/dog)	0.2	0.1	0.4	0.4	−0.1	0.2	0.3	0.2	0.2	0.1
Day 0–16 (kg/dog)	0.4	0.3	0.6	0.6	−0.3	0.5	0.5	0.5	−0.1	0.3
Day 0–27 (kg/dog)	0.7	0.8	1.0	1.0	−0.5	0.5	0.7	0.7	0.4	0.5

	Sex, dose (mg/kg bw per day)									
	Males					Females				
	0	20	70	200	500	0	20	70	200	500
Food consumption										
Week 0–1 (g/dog)	271	314	339	364	267	301	270	265	250	250
Week 2–3 (g/dog)	265	264	309	344	201	279	275	265	228	231
Week 3–4 (g/dog)	240	243	274	307	228	253	243	230	217	215
Clinical chemistry*										
ALP; day 22 (U/L)	110	229	115	108	133	97	118	142	118	145
ALP; day 28 (U/L)	98	285	201	171	271	90	166	163	218	345
Percentage difference[a]	-	191%	105%	75%	177%	-	84%	81%	142%	283%
Chol.; day 28 (mg/dL)	144	142	126	111	64	138	135	99	75	95
Percentage difference[a]	-	−1%	−13%	−23%	−56%	-	−2%	−28%	−46%	−31%
Albumin; day 28 (g/dL)	3.2	2.9	2.5	2.8	2.4	3.2	2.8	2.7	2.8	2.8
Percentage difference[a]	-	−9%	−22%	−13%	−25%	-	−12.5%	−15.6%	−12.5%	−12.5%
TP; day 28 (g/dL)	5.6	5.5	5.0	5.3	4.6	5.8	5.0	4.9	5.0	4.9
Percentage difference[a]	-	−2%	−11%	−5%	−18%	-	−14%	−16%	−14%	−15%
ALT; day 28 (U/L)	41	30	30	41	92	42	35	34	45	35
Percentage difference[a]	-	−27%	−27%	0%	124%	-	−17%	−19%	7%	−17%
GGTP; day 28 (U/L)	0.0	0.0	0.5	0.0	7.0	0.0	1.0	0.0	1.5	1.0
Percentage difference[a]	-	NA	NA	NA	NA	-	NA	NA	NA	NA
SORD; day 28 (U/L)	4	4	3	4	10	5	5	4	7	4
Percentage difference[a]	-	0%	−25%	0%	150%	-	0%	−20%	40%	−20%
Organ weights										
Liver (absolute, g)	192	232	311	318	304	180	212	256	240	244
Percentage difference[a]	-	21%	62%	66%	58%	-	18%	42%	34%	36%
Liver: relative to bw[b], (g/100g)	2.44	2.89	3.74	3.74	4.56	2.72	3.05	3.52	3.49	3.69
Percentage difference[a]	-	19%	53%	53%	87%	-	12%	29%	28%	36%
Liver: relative to brain, (g/100g)	266	319	451	432	422	265	317	358	356	341
Percentage difference[a]	-	20%	69%	62%	58%	-	20%	35%	35%	29%
Thymus (absolute, g)	7.94	9.32	7.37	7.90	4.03	6.04	10.29	10.25	8.81	7.79
Percentage difference[a]	-	17%	−7.2%	−0.5%	−49.2%	-	70%	70%	45.9%	29%
Spleen (absolute, g)	105.72	100.94	84.46	118.93	65.99	69.96	74.09	80.50	111.50	87.17
Percentage difference[a]	–	−5%	−20.1%	−13%	−38%	-	5.9%	15%	59.4%	24.6%
Heart (absolute, g)	64.03	64.60	66.98	62.57	50.14	53.24	52.05	57.69	53.02	58.66
Percentage difference[a]	-	1%	5%	−2%	−21.7%	-	−2.2%	8.4%	−0.4%	10.2%
Liver histopathology (two tissues sampled in each case)										
Hepatocellular hypertrophy; panlobular	0	0	2	2	2	0	0	1	2	2
Minimal	-	-	1	0	0	-	-	1	1	0
Mild	-	-	1	2	0	-	-	0	1	1
Moderate	-	-	0	0	2	-	-	0	0	1
Hepatocellular hypertrophy; periportal	0	2	0	0	0	0	2	1	0	0
Minimal	-	2	-	-	-	-	2	1	-	-

[a] Percentage difference from concurrent controls; NA Not applicable; Source: Kappeler, 2016c

[b] bw refers to final body weight; * Bilirubin levels were essentially zero in all groups male and females.

ALP: Alkaline phosphatase; Chol.: Total cholesterol; TP: Total protein; ALT: Alanine transaminase; GGTP: γ-glutamyl transpeptidase; SORD: Sorbitol dehydrogenase

The NOAEL for this study was 200 mg/kg bw per day, based on severely reduced body weights with corresponding lower food consumption in males and adverse liver effects (increased liver weights, increased GGTP and ALP) and microscopic observations (moderate panlobular hepatocellular hypertrophy) at the LOAEL of 500 mg/kg bw per day for males and females (Kappeler, 2016c).

Study 2

In a 90-day oral toxicity study, fluindapyr (purity 97.46%; Lot no. P/13/013) was orally administered for 13 weeks to four groups of beagle dogs via gelatin capsules at doses of 0, 10, 40 or 200 mg/kg bw per day. The animals were approximately six months old and body weights ranged from 5.8–7.5 kg for males and 6.1–7.5 kg for females at the first dose administration. The concurrent control group (group 1) received empty capsules on a comparable regimen. Each group consisted of four males and four females.

Following a minimum of 90 consecutive days of dose administration, all animals were euthanized. The animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily, and detailed physical examinations were performed around weekly (within a two day window). Individual body weights were recorded around weekly (within a two day window). Food weights were recorded daily, and food consumption recorded weekly. Samples were taken for clinical pathology parameters (haematology, coagulation, serum chemistry, and urinalysis) during acclimation prior to randomization (week −1), during week 6, and on the day of the scheduled necropsy (week 12/13). Blood samples for measuring fluindapyr in plasma were collected from all animals approximately three hours after dose administration on study days 0, 45 and 89/90. Ophthalmic examinations were performed during study weeks −1 and 12. Complete necropsies were performed on all animals, and selected organs were weighed at scheduled necropsy. Selected tissues were examined microscopically from all animals.

All animals survived to the scheduled necropsy. There were no test substance-related clinical observations or effects on body weight, food consumption, ophthalmology, haematology, blood coagulation or urinalysis parameters. In addition, there were no test substance-related macroscopic findings at necropsy. Test substance-related serum chemistry alterations included higher ALP values in males at 200 mg/kg bw per day and females at 40 and 200 mg/kg bw per day, lower albumin, albumin to globulin (A : G) ratios, and calcium values in the 200 mg/kg bw per day males, and a lower cholesterol value in 200 mg/kg bw per day females. Additionally, individual females in the 40 and 200 mg/kg bw per day groups exhibited higher ALT, aspartate transaminase (AST), GGTP and/or sorbitol dehydrogenase (SORD) values; lower albumin, total protein and cholesterol values were also noted in a single female at 200 mg/kg bw per day. Test substance-related higher liver weights were noted in all fluindapyr-treated male and female groups. Test substance-related microscopic findings of minimal to mild hepatocellular panlobular hypertrophy in the liver were noted in all fluindapyr-treated male and female groups. In addition, minimal to mild bile duct hyperplasia in the liver was noted in one animal each from the 40 and 200 mg/kg bw per day group females. Given the extensive biliary excretion in rats and an absence of this latter findings in any other groups, it was considered treatment-related and potentially adverse.

Table 15. Findings from the 90-day study in dogs treated orally (capsule) with fluindapyr

	Sex, dose (mg/kg bw per day)							
	Males				**Females**			
	0	**10**	**40**	**200**	**0**	**10**	**40**	**200**
Clinical chemistry								
ALP; week 12/13 (U/L)	72	115	136	438**	91	121	212	402
Percentage difference[a]	-	60%	89%	508%	-	33%	133%	342%
GGTP; week 12/13 (U/L)	0	0	0.3	1.0	0	0	0.5	5.5
Albumin; week 12/13 (g/dL)	3.2	3.2	3.1	2.7**	3.4	3.0	3.1	2.8
Percentage difference[a]	-	0%	−3%	16%	-	−12%	−9%	−18%
Chol.; week 12/13 (mg/dL)	146	142	118	88	169	151	129	87**
Percentage difference[a]	-	−3%	−19%	−40%	-	−11%	−24%	−49%
Body weight (kg)								
at week 13	9.2	8.1	8.7	8.3	7.7	8.7	8.4	7.4
Percentage difference[a]	-	−12.0	−5.4	−9.8	-	13	9.1	−3.9

	Sex, dose (mg/kg bw per day)							
	Males				Females			
	0	10	40	200	0	10	40	200
Organ weights								
Liver (absolute, g)	214.2	251.0	280.6*	332.0**	202.8	291.8**	317.4**	265.5*
Percentage difference[a]	-	17%	31%	55%	-	44%	57%	31%
Liver: relative to bw[b], (g/100g)	2.41	3.22**	3.41**	4.19**	2.7	3.5**	3.9**	3.7**
Percentage difference[a]	-	34%	42%	74%	-	30%	45%	36%
Liver: relative to brain, (g/100g)	289.4	345.1	389.5**	445.7**	292.1	393.1**	423.2**	403.2**
Percentage difference[a]	-	19%	35%	54%	-	35%	45%	38%
Liver histopathology (four tissues sampled in each case)								
Hypertrophy; panlobular	0	2	2	4	0	2	4	4
minimal	-	2	2	1	-	2	3	0
mild	-	0	0	3	-	0	1	4
Hyperplasia; bile duct	0	0	0	0	0	0	1	1
minimal	-	-	-	-	-	-	1	0
mild	-	-	-	-	-	-	0	1
Toxicokinetics; serum fluindapyr (ng/mL)								
Day 0 Mean	-	113	566	1021	-	335	382	871
SD		± 64	± 636	± 1566		± 386	± 196	± 624
Day 45 Mean	-	362	251	638	-	323	323	464
SD		± 328	± 47	± 502		± 328	± 228	± 188
Day 89/90 Mean	-	340	1428	2458	-	322	1326	1537
SD		± 388	± 1864	± 3679		± 335	± 608	± 2013

[a] Percentage difference from concurrent controls; SD: Standard deviation Source: Kappeler, 2016d

ALP: Alkaline phosphatase; Chol.: Total cholesterol; GGTP: γ-glutamyl transpeptidase;

Statistically significant at: * $p \leq 0.05$, ** $p \leq 0.01$

The NOAEL in this study was therefore 10 mg/kg bw per day based on evidence of bile duct hyperplasia in females at the LOAEL of 40 mg/kg bw per day. (Kappeler, 2016d)

Study 3

A 12-month dog study employed beagle dogs, 5–6 months old, with body weights ranging from 5.5–9.0 kg for males and 5.4–7.8 kg for females at the initiation of dose administration. Four dogs of each sex per group were administered undiluted fluindapyr (purity 97.46%; Lot no. P/13/013) orally via gelatin capsule at dose levels of 0, 4, 8, 40 and 100 mg/kg bw per day for males, and 0, 2, 4, 8 and 40 mg/kg bw per day for females. All animals received capsules daily for 12 consecutive months except the 100 mg/kg bw per day males, which received the test substance for only 10 consecutive months. Two of the 100 mg/kg bw per day males were euthanized during week 44 following 10 consecutive months of dose administration; the remaining two high-dose males were euthanized during week 52 following an eight-week, undosed recovery period.

Animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily and detailed physical examinations weekly. Individual body weights were recorded weekly. Individual food weights were recorded daily. Samples were taken for determination of clinical pathology parameters (haematology, coagulation, serum chemistry, urinalysis) prior to the initiation of dose administration, during weeks 26, 30 (serum chemistry only) and 44 (males in group 1 for serum chemistry only, and group 5 for all parameters), and at scheduled necropsy at week 52. Ophthalmic examinations were performed during weeks −1 and 51. Complete necropsies were performed on all animals, and selected organs were weighed (adrenals, brain, epididymides, heart, kidneys, liver with gallbladder, ovaries, pituitary, prostate, spleen, testes, thymus, thyroid with parathyroids, uterus with cervix) at the two early terminations and scheduled necropsies. Selected tissues from all animals were examined microscopically.

All animals survived to the scheduled necropsies during weeks 44 or 52. There were no test treatment-related effects on survival, ophthalmic findings or urinalysis parameters. Clinical findings included thin body condition (in 40 and 100 mg/kg bw per day males and 40 mg/kg bw per day females), clear material around the mouth and/or salivation (in 8 and 40 mg/kg bw per day females and 100 mg/kg bw per day males) and reddened gums (in 100 mg/kg bw per day males and 40 mg/kg bw per day females). These effects were noted as early as day 0 (clear material around the mouth/salivation), day 21 (thin body condition) and day 217 (reddened gums). Lower mean cumulative body weight gains (or even losses) were noted in the 40 and 100 mg/kg bw per day males and the 4, 8 and 40 mg/kg bw per day females. These lower weight gains resulted in mean body weights at week 52 that were 21.7% (40 mg/kg bw per day males), 17.4% (100 mg/kg bw per day males), 9.4% (4 mg/kg bw per day females), 15.1% (8 mg/kg bw per day females) and 25.5% (40mg/kg bw per day females) lower, than for the control group. Lower mean cumulative body weight gains compared to controls were noted in the three female groups beginning as early as study week 1 (40 mg/kg group), week 2 (8 mg/kg group) and week 3 (4 mg/kg group) and this continued until the scheduled necropsy. Corresponding lower mean food consumption was noted for the 100 mg/kg bw per day males and 40 mg/kg bw per day females during the first 16 to 20 weeks of dosing. During the eight-week recovery period from week 44 to 52, mean body weight gains for the 100 mg/kg bw per day males were generally higher than for control group males (although mean body weights still remained slightly lower), indicating the reversibility of the effects seen during the dosing period.

At 40 and 100 mg/kg bw per day for males and 40 mg/kg bw per day for females, effects were reported that included higher mean liver enzyme activity (ALP, ALT, AST, GGTP, SORD), longer mean prothrombin times, lower mean cholesterol, urea nitrogen, albumin, total protein, A : G ratios and calcium values. Also observed were gross observations of a mottled (males only) or pale liver (100 mg/kg bw per day males only) and histopathologic findings in the liver, including hepatocellular hypertrophy, bile duct hyperplasia and hepatocellular necrosis. Increased intracellular accumulation of brown pigment (presumed to be bile) was noted in a single male animal administered the test substance at 100 mg/kg bw per day for 44 weeks. The lower mean cholesterol, urea nitrogen and albumin values and longer mean prothrombin times that had been observed were consistent with decreased liver function.

Higher mean liver weights in males and females of the 4 and 8 mg/kg bw per day groups were accompanied by higher mean ALP values, while histopathological findings of minimal hepatocellular hypertrophy and minimal bile duct hyperplasia were only seen at 8 mg/kg bw per day. No correlating gross observations were noted at 4 or 8 mg/kg bw per day and there were no histopathological findings in the liver at 4 mg/kg bw per day. Therefore the hepatocellular hypertrophy and alterations in liver weights and mean ALP values were considered to be an adaptive response to test substance administration. Based on a weight-of-evidence approach, test substance-related alterations in the liver in 4 and 8 mg/kg bw per day males and females were not considered adverse, but were considered adverse in 40 mg/kg bw per day males and females and in 100 mg/kg bw per day males.

Test substance-related lower prostate weights were noted in the 8 and 40 mg/kg bw per day males at primary necropsy, but these were without correlating histopathologic findings and all individual values were within the laboratory's historical control database mean range (within two standard deviations of the mean). Additionally, there were no statistically significant alterations in the weights of testes and epididymides (with exception of the testes weight relative to final body weight at 40 mg/kg bw per day) or test substance-related histopathologic findings in the testes or epididymides consistent with an endocrine-related effect. Therefore, the alterations in prostate weights in the 8 and 40 mg/kg bw per day groups were not considered adverse.

In male dogs that had received fluindapyr at 100 mg/kg bw per day for 44 weeks, partial recovery from test substance-related effects was achieved in the eight weeks following cessation of administration. Most test substance-related clinical pathology alterations returned to levels seen during the acclimation period or were within the laboratory's historical control database mean range (within two standard deviations of the mean) with the exception of individual ALP and urea nitrogen values.

Individual liver weights, while still above the concurrent control range, were within the laboratory's historical control database range as described, with the exception of the liver to final body weight from one animal. Individual prostate weights remained within two standard deviations of the laboratory's historical control database mean. With the exception of bile duct hyperplasia, all test substance-related histopathologic findings observed at 100 mg/kg bw per day at week 44 and at 40 mg/kg bw per day at week 52 were not present at the necropsy carried out at the end of the recovery period. The observed bile duct hyperplasia was of minimal severity, indicating no progression of this finding after cessation of test substance administration. Selected data from the 12 month study in dogs are shown below in Table 16.

Table 16. Findings from the 12-month study in dogs treated orally (capsule) with fluindapyr [a]

	Sex, dose (mg/kg bw per day)										
	Males					**Females**					
	0	**4**	**8**	**40**	**HCD**	**0**	**2**	**4**	**8**	**40**	**HCD**
Mean body weight (kg)											
Week 52	11.5	10.3	11.3	9.0		10.6	10.6	9.6	9.0	7.9	
% difference	-	−10	−2	−22		-	0	−9	−15	−26	
Standard deviation	1.70	1.48	1.39	1.17		1.21	1.59	1.53	0.81	1.22	
Cumulative body weight											
Weeks 0–2	0.5	0.6	0.6	0.2		0.7	0.5	0.6	0.4	0.0**	
Weeks 0–52	4.7	3.7	4.2	2.4**		4.4	4.6	3.5	2.7	1.8**	
Haematology											
Prothrombin time (seconds)											
Week 26	7.7	8.1	8.1	8.7**	6.7–	7.5	7.5	7.9	7.6	8.7**	6.8–
% difference	-	5	5	13	7.9	-	0	5	1	16	8.2
Week 52	7.9	8.3	8.2	8.8	7.0–	7.6	7.6	7.9	8.0	9.0**	7.2–
% difference	-	5	4	11	7.7		0	4	5	18	7.6
Clinical chemistry											
Alkaline phosphatase, ALP (U/L)											
Week −1	130	139	131	127		131	103	150	152	147	
Week 52	31	118	122	858**	37–67	58	64	115	204	753**	27–98
% difference	-	280	294	2668		-	10	98	252	1198	
Standard deviation	6.4	59.9	48.1	516.8		17.9	26.8	73.8	90.2	546.2	
ALT (U/L); Week 52	38	38	43	242*		30	32	29	37	190**	
% difference	-	0	13	537	27–89	-	7	−3	23	533	16–37
Standard deviation	4.3	10.4	11.0	164.0		3.8	4.3	3.9	10.3	101.5	
AST (U/L); Week 52	39	34	36	48		35	37	37	37	64**	
% difference	-	−13	−8	23	18–38	-	6	6	6	83	22–36
Standard deviation	6.4	2.6	7.7	13.6		1.3	13.1	4.6	4.2	20.9	
γ-glutamyl transpeptidase, GGTP (U/L)											
Week −1	3	3	3	3		3	3	3	3	3	
Week 52	0	0	0.8	26.5	0.0–	0.0	0.0	1.0	1.8	17.5**	0.0–
% difference	-	NA	NA	NA	0.0	-	NA	NA	NA	NA	0.0
Standard deviation	0.00	0.00	0.96	29.26		0.00	0.00	0.82	1.26	7.05	
SORD (U/L); Week 52	3	3	1	8		4	3	5	2	4	
% difference	-	0	−67	167	1.0–	-	−25	25	−50	0	2.0–
Standard deviation	0.6	1.5	1.5	4.9	8.0	1.7	2.2	2.4	2.4	4.0	12.0
Chol. (mg/dL); Week 52	128	136	140	89		199	183	200	151	66**	
% difference	-	6	9	−31	111–	-	−8	1	−24	−67	109–
Standard deviation	5.4	14.8	33.6	56.4	164	63.1	64.0	55.1	14.0	30.9	226
Albumin (g/dL) , Week 52	3.3	3	3	2.5**	3.2–	3.4	3.3	3.1*	3.0**	2.6**	3.0–
% difference	-	−9	−9	−24	3.7	-	−3	−9	−12	−24	3.4 [b]

	Sex, dose (mg/kg bw per day)										
	Males					Females					
	0	4	8	40	HCD	0	2	4	8	40	HCD
Organ weights (at week 52)											
Liver weights											
Absolute (g)	266	300.3	370.0*	392**		273	305.3	315.8	297.0	322.5	
% difference	-	13	39	47		-	12	16	9	18	
Standard deviation	32.88	39.42	42.21	51.74		14.05	70.56	42.3	28.52	5.53	
Relative to bw[c], (g/100g)	2.326	2.91**	3.29**	4.37**	2.060–	2.57	2.84	3.34**	3.29**	4.10**	2.125–
% difference	-	25	41	88	3.013	-	10	30	28	59	3.567
Relative to brain (g/100 g)	334.9	397.7	494**	514**		384.1	430.7	437.1	424.2	456.7	
% difference	-	19	47	53		-	12	14	10	19	
Prostate; absolute (g)	11.1	8.5	7.32*	6.87*		-	-	-	-	-	
% difference	–	−23.3	−34.1	−38.2							
Testes											
Absolute (g)	12.78	13.80	12.74	14.32		-	-	-	-	-	
% difference	–	8.0	−0.3	12.1							
Relative to bw[c], (g/100g)	0.11	0.134	0.113	0.160**		-	-	-	-	-	
% difference	–	19.6	0.9	42.9							
Thyroid; absolute (g)	0.93	0.98	1.0	1.0		0.77	0.82	0.81	0.87	0.22	
Histopathology at week 52 (four tissues sampled from each group)											
Liver; hypertrophy, hepatocellular	0	0	1	4		0	0	0	1	4	
minimal	-	-	1	4		-	-	-	1	4	
Hyperplasia, bile duct	1	0	2	3		0	0	0	2	4	
minimal	1	-	2	2		-	-	-	2	4	
mild	0	-	0	1		-	-	-	0	0	
Necrosis, hepatocellular	0	0	0	1		0	0	0	0	0	
minimal	-	-	-	1		-	-	-	-	-	
Pigment, brown											
Increased	0	0	0	0		0	0	0	0	0	

HCD: Historical control data; ALT: Alanine transaminase; AST: Aspartate transaminase; Source: Randazzo, 2017a

[a] A high dose of 100mg/kg bw per day was administered to a group of males only for 44 weeks, whereas the figures in the rest of Table 16 are at the end of 52 weeks, therefore since they were not comparable these data were not included;

[b] At week 44; SORD: Sorbitol dehydrogenase; Chol: Total cholesterol;

[c] Relative to final body weight; Statistically significant at: * $p \leq 0.05$, ** $p \leq 0.01$

In consideration of the above findings, the NOAEL was 4 mg/kg bw per day, based on hyperplasia of the bile duct in males and females at the LOAEL of 8 mg/kg bw per day (Randazzo, 2017a).

(b) Dermal application

Rat

Fluindapyr (purity: 97.46%; Lot No. P/13/013) was applied dermally to Wistar RccHanTM: WIST(SPF) rats. Groups consisted of 10 rats of each sex, about seven weeks old; males weighed in the range 225.8–253.8 g (mean 238.4 g), and females 146.2–191.4 g (mean 161.7 g). Dose levels were 0, 100, 300 or 1000 mg/kg bw per day, administered five days per week for 21 days. There were no deaths, and no clinical signs of systemic toxicity were seen during daily/weekly detailed or functional observational battery (including mean grip strength and locomotor activity), no effects on mean food consumption or body weight gain, no ophthalmoscopic changes, no toxicologically relevant differences in the mean haematology or urinalysis parameters, and no test item-related effects of mean absolute or relative organ weights. Macroscopical evaluation at necropsy was unremarkable. Test item-related local findings were generally restricted to a marginally higher incidence of skin crusts and localized desquamation. The severity was marginally higher in the test item-treated rats and signs occurred sooner than in controls. The very limited degree of these findings and the lack of any indication of discomfort were suggestive of typical nonadverse changes seen in repeat dose dermal toxicity studies. Because the mean potassium levels of control and treated animals exceeded the upper limit of the historical control data, these differences were considered to be an artefact. The administration of fluindapyr to Wistar rats by dermal application five days per week for three weeks did not induce any histological effects at the application site or on internal organs at dose levels up to 1000 mg/kg bw per day.

Based on the results of this study, minor local findings were observed in treated rats but were not considered adverse, therefore the NOAEL for dermal application of fluindapyr was 1000 mg/kg bw per day (Braun, 2015).

(c) Exposure by inhalation

Rat

A four-week study employed male and female Sprague Dawley Crl:CD® (SD)BR rats, aged around nine weeks, the males weighing 250–322 g, females: 194–236 at the start of treatment. Four groups consisting of five rats of each sex were exposed, nose-only, to fluindapyr (purity 97.46%; Lot no. P/13/013) as a powder aerosol via inhalation at concentrations of 0 (air control), 0.06, 0.33 or 0.98 mg/L of for five consecutive days per week for four weeks. Target exposure levels were 0, 0.06, 0.30 and 1.15 mg/L with corresponding target doses of 0, 15, 75 and 300 mg/kg bw per day. In practice the average mass median aerodynamic (MMAD) was 2.2–2.6 µm (geometric standard deviation 1.5–3.0). At week 2 the particle size sample value for the nominal 0.33 mg/L groups was outside the protocol target MMAD range of 1.5–3.0 µm but was included in mean. Animals were observed in their cages twice daily for mortality, moribundity and signs of severe toxic or pharmacological effects, and weekly for clinical signs. Ophthalmoscopic examinations were performed pretest and during week 4. Animals were weighed twice pretest, at weekly intervals, twice each week during treatment, and again immediately prior to termination, after fasting. Food consumption was measured weekly. Blood for haematology, coagulation and clinical chemistry parameters was obtained from fasted animals at study termination. All animals were necropsied. Organs weighed included the following: adrenals, brain, epididymides, heart, kidneys, liver, lungs and bronchi, ovaries, pituitary, prostate and seminal vesicles (weighed together), spleen, testes, thymus, thyroid with parathyroid, uterus and cervix. Microscopic examination was performed on a range of tissues from the control and high-dose groups and on all gross lesions.

All animals survived until study termination. Exposure was associated with clinical signs including decreased activity, hunched appearance and abnormal gait in both genders at 0.98 mg/L, most notably during the first week of exposure. There were no treatment-related organ weight findings. A number of statistically significant changes in absolute and/or relative organ weights were seen, but these were small, independent of the level of exposure and/or lacked a microscopic correlate. Inhalation exposure to fluindapyr for four weeks was associated with nasal pharyngeal mucous cell hyperplasia at 0.33 mg/L and above. Nasal pharyngeal mucous cell hyperplasia in this case was not considered adverse due its minimal or slight grade and the absence of functional deficits. Incidences of mucous cell hyperplasia were not increased in the nasal turbinate, a frequent area first and most severely affected by inhalation. Mucous cell hyperplasia is frequently observed in the anterior nasal cavity of rodents in

response to repeated inhalation of irritants. Additionally, there is no evidence that mucous cell hyperplasia is preneoplastic (Renne et al., 2009). Nasal pharyngeal mucous cell hyperplasia could be due to the physical effect of particles rather than any specifically chemical effect.

Table 17. Test substance-related findings in the nasal pharynx and turbinate of rats exposed to fluindapyr (five tissues examined from each treatment group)

		Sex, exposure level (mg/L)							
		Males				Females			
		0	0.6	0.33	0.98	0	0.6	0.33	0.98
Nasal pharynx									
Mucous cell hyperplasia	minimal	0	0	2	3	0	0	1	3
	slight	0	0	0	2	0	0	0	2
	Total	0	0	2	5	0	0	1	5
Nasal turbinate									
Mucous cell hyperplasia	Minimal	3	1	2	0	0	1	2	0
	Slight	0	1	0	2	0	0	1	0
	Total	3	2	2	2	0	1	3	0

Source: Hoffman, 2016

The no observed-adverse-effect concentration (NOAEC) was 0.33 mg/L based on the adverse effects on clinical signs at the highest exposure level of 0.98 mg/L. Indications of local effects on the nasopharyngeal mucosa were seen at 0.33 mg/L and above (Hoffman, 2016).

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

An 18-month carcinogenicity study in mice employed main groups of 50 male and 50 female CD-1 mice. At the initiation of the test diet these were around six weeks old, with male body weights in the range 22.1–32.0 g, females 18.2–25.7 g in the carcinogenicity groups, and in the satellite groups 25.3–29.9 g for males and 19.5 g–25.4 g for females. Fluindapyr (purity 98.65%; Lot no. P/13/013) was provided in the diet at concentrations of 0, 100, 500 or 3000 ppm (equal to 0, 13, 67 and 410 mg/kg bw per day for males, 0, 18, 81 and 527 mg/kg bw per day for females). Main group animals (50/sex per group) were treated for 78 consecutive weeks. Satellite groups (six/sex per group) were treated for 28 consecutive days and were used for a toxicokinetics assessment.

For carcinogenicity assessment, all animals were observed twice daily for mortality or moribundity. Clinical examinations were performed daily, and detailed physical examinations and palpable mass examinations were performed weekly. Individual body weights and food weights were recorded weekly until week 14 and every other week thereafter. Blood smears were prepared for possible examination from all animals euthanized in extremis and from all surviving animals at scheduled necropsy. Complete necropsies were conducted on all animals, and selected organs were weighed at the scheduled necropsy. Selected tissues from all animals were examined microscopically. For toxicokinetic evaluation, blood samples were collected from three mice of each sex in the satellite group on days 2 and 27. All satellite animals were euthanized following the final blood collection, their livers weighed and saved for potential analysis, and other remains discarded.

Exposure to fluindapyr did not affect survival, and there were no treatment-related clinical observations or effects on body weight, body weight gain, cumulative body weight gain or food consumption. On days 2 and 27, fluindapyr was observed in the plasma of all test substance-treated male and female animals and was seen to increase in a dose-dependent manner.

In the 3000 ppm males and females, hepatocellular hypertrophy and liver weight increases compared to controls (by 41.4% in males and 26.9% in females, relative to body weight), were observed. Given that a higher incidence of pigmented macrophages, often accompanied by hepatocellular

degeneration, was observed in the 3000 ppm males, the changes in liver histopathology and weight were considered adverse. The hepatocellular hypertrophy in 3000 ppm females, in the absence of any adverse findings, was considered an adaptive response and therefore not adverse.

There was no evidence that fluindapyr is carcinogenic in mice. Among the 3000 ppm group males there was a slightly higher incidence of hepatocellular adenomas and carcinomas compared to concurrent controls as shown in Table 18. Incidences of adenoma and carcinoma, singly or combined, were not statistically significant in a pairwise comparison using Fisher's exact test. A Cochran–Armitage test, analysis using linear, probit, and logistic regression techniques as well as non-parametric tests further supported the absence of a trend. The actual numerical difference in hepatocellular carcinoma incidence between concurrent controls and 3000 ppm males was only by one animal (n = 2/50 in controls compared to 3/49 for the 3000 ppm males) indicating that this difference is highly likely to be spontaneous in nature. It is therefore concluded that fluindapyr is not carcinogenic in the mouse.

Table 18. Summary of carcinogenicity in mice treated orally with fluindapyr

	Sex, dietary dose (ppm)								
	Males					Females			
	0	**100**	**500**	**3000**	**HCD**	**0**	**100**	**500**	**3000**
Average intake (mg/kg bw per day)	0	13	67	410		0	18	81	527
Organ weights									
Liver; number examined	10	10	10	10		10	10	10	10
Weight; absolute (g)	2.09	2.25	2.28	3.16**		1.84	2.12	1.72	2.19
standard deviation	0.26	0.45	0.32	0.54		0.37	1.21	0.22	0.42
Weight; relative to bw (g/100g)	5.13	5.14	5.48	7.26**		4.76	5.49	4.92	6.04
standard deviation	0.58	0.34	0.81	1.43		0.69	2.13	0.58	0.85
Microscopic pathology									
Neoplastic findings									
Number of mice	50	50	50	49		50	50	50	50
Survival to 18 months (number of mice)	44	41	44	43		33	41	39	39
Liver [a]									
Hepatocellular adenoma; single or multiple	3	3	4	6	3.33%–	1	0	0	0
percentage	6%	6%	8%	12%	20% [b]				
Hepatocellular carcinoma; single or multiple	2	1	0	3	0–4.0% [b]	0	0	0	0
percentage	4%	2%	0%	6%	0–15% [c]				
Hepatocellular adenoma and/or carcinoma	5	4	4	9	3.33–	1	0	0	0
percentage	10%	8%	8%	18%	20.0% [b]				
Uterus									
Uterine endometrial Polyp(B)	-	-	-	-	-	0	0	2	2
Uterine endometrial Sarcoma(M)	-	-	-	-	-	3	0	0	1
Non-neoplastic findings									
Liver [a]	50	50	50	49		50	50	50	50
Focus of cellular alteration; all types	0	2	2	7**		0	0	1	0
Focus of cellular alteration; basophilic	-	2	1	1		-	-	1	-
Focus of cellular alteration; eosinophilic	-	-	1	4		-	-	-	-
Focus of cellular alteration; mixed	-	-	-	1		-	-	-	-
Focus of cellular alteration; vacuolated	-	-	-	1		-	-	-	-
Hypertrophy, hepatocellular; all types	12	21	25	38		0	1	3	13
minimal	11	16	15	13		-	1	2	11
mild	1	5	7	18		-	-	1	2
moderate	-	-	3	7		-	-	-	-

	Sex, dietary dose (ppm)								
	Males					Females			
	0	100	500	3000	HCD	0	100	500	3000
Pigmented macrophages	2	3	4	12		11	12	7	7
percentage	4%	6%	8%	24%	4.6%[c]				
minimal	1	3	1	7		7	10	6	4
mild	0	-	3	5		4	2	0	3
moderate	1	-	-	-		-	-	1	-
Necrosis; hepatocellular, single cell	2	2	1	6		1	1	0	1
Minimal	1	2	1	3		1	0	-	1
Mild	0	-	-	3		-	1	-	-
Marked	1	-	-	-		-	-	-	-
Necrosis; hepatocellular centrilobular	0	1	1	1		0	0	0	0
Mild	-	0	0	1		-	-	-	-
Moderate	-	1	1	-		-	-	-	-
Necrosis; hepatocellular	3	4	4	8		5	1	1	6
minimal	0	3	2	5		1	1	1	2
mild	0	1	2	3		3	-	-	2
moderate	2	-	-	0		1	-	-	1
marked	1	-	-	-		-	-	-	1

[a] Week 78 – scheduled necropsy, early deaths and scheduled sacrifice; Source: Randazzo, 2018

[b] Historical control data from the test facility from seven 78-week studies (394 animals) conducted during 2001–2016;

[c] Giknis & Clifford, 2010; Significantly different from the control group using Dunnett's test: * $p = 0.05$, ** $p = 0.01$

In view of the above findings, the NOAEL for systemic toxicity was 500 ppm (equal to 67 mg/kg bw per day) based on higher incidence of pigmented macrophages often accompanied by hepatocellular degeneration at 3000 ppm (equal to 410 mg/kg bw per day. The NOAEL for carcinogenicity was 3000ppm (equal to 410mg/kg bw per day) the highest dose tested. Fluindapyr was not carcinogenic in mice. (Randazzo, 2018)

Rat

Fluindapyr (purity 98.65%; Lot no. P/13/013) was offered on a continuous basis in the diet to groups of Crl:CD(SD) rats, around six weeks old at the start of dose administration, males weighing 129–192 g, females 96–140 g for females in the chronic toxicity/carcinogenicity groups, while males in the toxicokinetic groups weighed 134–183 g and females 111–132 g. Rats received the diet for 52 or 104 consecutive weeks (chronic toxicity and carcinogenicity groups, respectively), or for 89 consecutive days (toxicokinetic groups). Dosage concentrations were 0, 100, 400 or 1600 ppm (equal to 0, 4, 16 and 64 mg/kg bw per day for males, 0, 5, 20 and 82 mg/kg bw per day for females), and 4800 ppm for males only (equal to 195 mg/kg bw per day). Concurrent control groups received just the basal diet on a comparable regimen. Each chronic toxicity/carcinogenicity group consisted of 60 rats of each sex, whilst toxicokinetic groups consisted of three animals of each sex. Ten animals/sex per group were assigned to the interim necropsy (chronic group, necropsy at 12 months) and the remaining up to 50 animals/sex per group (carcinogenicity group) were assigned to the primary necropsy at 24 months).

All animals were observed twice daily for mortality or moribundity. Clinical examinations were performed daily, and detailed physical examinations and palpable mass examinations were performed weekly. Individual body and cage food weights were recorded weekly until week 14, and every two weeks thereafter. Ophthalmic examinations were performed predose and during week 51. Samples for clinical pathology parameters (haematology, coagulation, serum chemistry and urinalysis) were taken from 10 rats/sex per group during week 25 (excluding coagulation) and for all chronic toxicity animals at the scheduled interim necropsy during week 52. Blood smears were prepared for possible examination from all animals euthanized in extremis. Blood was collected from three animals/sex per group from the

chronic toxicity groups for toxicokinetic evaluation at 12 months (prior to scheduled interim necropsy). A complete necropsy was conducted on all animals, and selected organs were weighed at the scheduled interim necropsy. Selected tissues were examined microscopically from all animals found dead or euthanized in extremis, from all control and high-dose chronic toxicity animals at the primary necropsy, and from all animals at the terminal schedule necropsy. Gross lesions were examined from all chronic toxicity animals in the low-, mid-, and high-dose groups, and males in the 4800 ppm dose groups, at interim necropsy. In addition, as the liver was identified as a potential target tissue, livers were examined from all animals at the interim necropsy. For the toxicokinetic group, blood samples were collected on days 2, 14 and 90. All surviving animals were euthanized following the final blood collection on day 90.

There were no treatment-related clinical signs of toxicity, alterations in haematology, coagulation, or serum chemistry (biochemical) parameters, nor ophthalmology or macroscopic findings. Incidence of palpable masses was unaffected by test substance administration. Mortality was not affected by treatment up to the 12-month necropsy. At 24 months mortality was higher in 100 and 400 ppm group males than in other groups; whilst this attained statistical significance it was not considered to be treatment-related as there was no dose–response relationship (see Table 19).

Lower mean body weights were noted in 1600 and 4800 ppm males and 1600 ppm females throughout the study. By week 52 (interim necropsy), respective mean body weights for the 1600 and 4800 ppm males and 1600 ppm females were lower by 3.9%, 9.5% and 12.1% respectively than for concurrent controls. By the week 104 terminal schedule necropsy, respective mean body weights for the 1600 and 4800 ppm males and 1600 ppm females were lower by 8.7%, 15.6% and 13.1% than for concurrent controls. Marked reductions in body weight gain were also observed; by week 104 body weight gains in the 4800 ppm males and 1600 ppm females were suppressed by 20%–21% compared to those of the control groups. The impact on body weight and body weight gain indicated that a maximum tolerated dose (MTD) had been reached. Decreases in body weight and body weight gain were accompanied by slight reductions in food consumption by the high-dose groups, and to a lesser extent also by male rats in the 1600 ppm group. Differences from control values were generally statistically significant from the first interval throughout the first month, but less significant thereafter.

Increased liver weight was noted at the interim and primary necropsies in 4800 ppm males and 1600 ppm females, with associated centrilobular hepatocellular hypertrophy in all treated groups of males and the 1600 ppm females at the terminal schedule necropsy. Karyomegaly was also noted in the treated groups of females at the interim or primary necropsy.

No test substance-related neoplasms were noted at either the interim or main necropsies. There were no statistically significant changes in neoplasm incidence, alterations in the time to tumour onset, and no induction of rare tumours. At the main necropsy an increase in the incidence and/or severity of uterine endometrial hyperplasia was noted in the 400 and 1600 ppm females, and an increased incidence of uterine luminal dilation was observed in the females on 1600 ppm. Uterine dilation incidences were generally within the testing facility historical control ranges, although the incidence of severe dilation was slightly above. Incidences of endometrial hyperplasia, including those in control animals, were above the testing facility historical control ranges, but were graded minimal to mild and not associated with neoplastic findings. There was also an increase in mammary gland adenocarcinoma in females at 400 and 1600 ppm.

On days 2, 14 and 90, fluindapyr was detected in all plasma samples collected from treated males and females. Mean plasma concentrations followed a dose-related pattern in both sexes. Comparing plasma levels on day 2 to those on days 14 and 90, for males and females at 100, 400 and 1600 ppm the values across time were very similar, whilst for 4800 ppm males plasma levels decreased over time. By day 364 only the high-dose males and females had plasma concentrations of fluindapyr above the analytical limit of quantification, with the exception of one sample each in the 1600 ppm males and 400 ppm females. A summary of results for this study is shown below in Table 19.

Table 19. Summary of results from the two-year chronic toxicity/carcinogenicity study in rats treated orally with fluindapyr

	Sex, dietary dose (ppm)								
	Males					Females			
	0	100	400	1600	4800	0	100	400	1600
Average intake (mg/kg/day)	0	4	16	64	195	0	5	20	82
Survival [a]									
at week 52 (interim termination)	98	92	85	93	95	97	95	97	100
at week 80	82	75	58	75	81	89	87	83	88
at week 104 (main termination)	54	29	25	41	49	52	55	44	64
p-value	-	0.0223*	0.0003*	0.1897	0.9370	-	NT	NT	NT
Clinical signs	No treatment-related effects								
Body weight									
Week 13, (*N*)	60	58	57	59	60	60	60	59	60
mean (g/rat)	551	545	539	523**	504**	285	284	285	271**
standard deviation	43.0	46.9	51.3	48.5	48.6	25.8	24.1	22.2	20.1
Week 52, (*N*)	59	55	51	56	57	58	57	58	60
mean (g/rat)	769	753	755	739	696**	387	381	376	340**
standard deviation	82.4	80.8	79.5	82.1	84.1	58	57	58	60
Week 104, (*N*)	27	14	12	20	24	26	27	22	32
mean (g/rat)	812	774	756	741	685**	459	458	474	399*
standard deviation	28.3	30.8	49.6	30.7	74.9	26	27	22	32
Body weight gain									
Week 13, (*N*)	60	58	57	59	60	60	60	59	60
mean (g/rat)	337	333	327	310**	291**	136	134	136	123**
standard deviation	41.9	41.2	42.8	40.1	40.5	23.1	19.6	18.0	15.0
Week 52, (*N*)	59	55	51	56	57	58	57	58	60
mean (g/rat)	555	540	544	527	483**	238	231	227	192**
standard deviation	82.5	76.2	72.7	75.8	75.0	45.3	47.9	45.2	37.8
Week 104, (*N*)	27	14	12	20	24	26	27	22	32
mean (g/rat)	598	558	539	524	470**	313	308	326	251*
standard deviation	148.7	102.0	168.4	132.3	68.6	94.0	91.0	66.0	60.3
Food consumption (g/rat per day)									
Weeks 1–2	24	22**	23*	22**	22**	15	15	15	13**
Weeks 51–52	25	27	27*	27	25	19	18*	18*	16**
Weeks 103–104	24	25	22	23	21	18	16	18	16
Ophthalmoscopy	No treatment-related effects								
Haematology	No treatment-related effects								
Biochemistry	No treatment-related effects								
Urinalysis									
pH; week 25	7.0	7.3	7.1	6.8	6.9	6.1	6.2	6.3	6.5**
pH; week 52	6.8	6.9	6.8	6.6	6.6	6.1	6.1	6.1	6.3
Organ weights; mean weight (g)									
Liver; week 52									
Number examined	10	10	10	10	10	9	10	10	10
Weight; absolute:	20.91	19.31	17.52*	18.19	20.92	9.56	9.49	9.42	9.83
standard deviation	2.518	3.415	3.610	2.639	2.426	1.271	1.367	0.978	0.641
Weight; relative to bw	2.669	2.652	2.578	2.646	3.042**	2.714	2.761	2.761	3.207**
standard deviation	0.1517	0.2627	0.2426	0.1519	0.1446	0.2007	0.2935	0.2281	0.3438

	Sex, dietary dose (ppm)								
	Males					Females			
	0	100	400	1600	4800	0	100	400	1600
Liver; week 104									
Number examined	27	14	12	20	24	26	27	22	32
Weight; absolute:	20.12	19.56	20.13	20.22	20.19	12.42	12.65	12.95	12.43
standard deviation	4.259	3.572	5.181	4.404	2.476	2.497	2.413	2.474	1.645
Weight; relative to bw	2.657	2.643	2.837	2.985	3.096	2.915	3.022	2.923	3.373**
standard deviation	0.6203	0.3212	0.6689	0.2626	0.0533	0.5191	0.7184	0.4815	0.4889
Microscopic pathology – at interim sacrifice									
Neoplastic findings	No treatment-related effects								
Non-neoplastic findings									
Liver									
Number examined	10	10	10	10	10	9	10	10	10
Centrilobular hepatocyte hypertrophy									
minimal	0	0	0	0	2	0	0	1	5
Karyomegaly	0	0	0	0	0	1	2	2	3
minimal	-	-	-	-	-	1	2	2	2
mild	-	-	-	-	-	0	0	0	1
Microscopic pathology – all animals from terminal sacrifice, interim kill and unscheduled deaths									
Neoplastic findings									
Thyroid follicular cell adenoma	1	0	1	2	5	0	0	1	0
Thyroid follicular cell carcinoma	3	0	0	3	2	0	1	1	0
Thyroid C-cell adenoma	5	3	5	4	5	7	5	3	4
Thyroid C-cell carcinoma	3	1	0	1	2	0	0	0	0
Non-neoplastic findings									
Liver									
Number examined	60	60	60	60	60	60	60	60	60
Centrilobular hepatocyte hypertrophy	0	0	5	7	13	0	0	1	20
minimal	−0	−0	5	7	11	0	0	−1	17
mild	−0	−0	0	0	2	0	0	0	3
Karyomegaly	0	1	0	1	0	1	10	8	25
minimal	-	-	-	-	-	−1	10	4	11
mild	-	-	-	-	-	−0	0	4	13
moderate	-	-	-	-	-	−0	00		1

	Sex, dietary dose (ppm)								
	Males					Females			
	0	100	400	1600	4800	0	100	400	1600
Uterus									
Number examined	NA	NA	NA	NA	NA	60	60	60	60
Uterine luminal dilation [b]		NA	NA	NA	NA	2	8	7	13
minimal	-	-	-	-	-	0	6	1	5
mild	-	-	-	-	-	0	0	1	3
moderate	-	-	-	-	-	2	1	4	2
severe	-	-	-	-	-	0	1	1	3
Uterine endometrial hyperplasia, diffuse [c]		NA	NA	NA	NA	4	4	7	14
minimal	-	-	-	-	-	2	3	3	11
mild	-	-	-	-	-	2	1	4	2
moderate	-	-	-	-	-	0	0	0	1
Cervix; hyperplasia of squamous epithelium		NA	NA	NA	NA	14	6	15	31
minimal	-	-	-	-	-	5	1	5	11
mild	-	-	-	-	-	9	5	9	19
moderate	-	-	-	-	-	0	0	1	1
Mammary gland									
Total number examined						59	48	48	56
Malignant adenocarcinoma arising in fibro-adenoma [#]						1	1	1	3
p-value							0.7200	0.7211	0.3500
Malignant adenocarcinoma [#]						2	5	9	9
p-value							0.1890	0.0216	0.0357
Benign fibroma [#]						0	1	1	0
p-value							0.5098	0.4884	1.0000
Benign fibro-adenoma [#]						17	19	16	16
p-value						0.3253	0.4879	0.6966	0.7001
Benign adenoma [#]						4	3	3	3
p-value							0.7245	0.7394	0.7392
Adenocarcinoma/adenoma						5	8	12	12
p-value							0.1974	0.0346	0.0294
Adenocarcinoma/adenocarcinoma arising from fibro-adenoma						3	5	10	11
p-value							0.3155	0.0302	0.0318

NT Not tested (in accordance with protocol statistical methodology); NA Not applicable; Source: Randazzo, 2017b

\# Neoplasm;

(a) Kaplan–Meier estimates (%) of survival, *p*-value based on comparisons using vehicle group

(b) Historical control ranges:
Females: 1.54%–13.85% per study, minimal incidence in 22 studies with overall 3.29% incidence;
1.43%–10.34% per study mild incidence in 24 studies with overall 3.18% incidence;
1.54–6.90% per study moderate incidence in 14 studies with overall 1.28% incidence;
1.43–3.57% per study severe incidence in 7 studies with overall 0.36% incidence;

(c) Historical control ranges
Females: 1.43–2.17% per study minimal incidence in 2 studies with overall 0.10% incidence;
1.54% per study mild incidence in 1 study with overall 0.05% incidence;

Significantly different from the control group using Dunnett's test: * $p = 0.05$, ** $p = 0.01$

The NOAEL for toxicity and carcinogenicity was 100 ppm (equal to 5 mg/kg bw per day) based on increases in mammary gland adenocarcinomas in females at 400 ppm (equal to 21 mg/kg bw per day). Fluindapyr is carcinogenic in female rats and not in male rats (Randazzo, 2017b).

2.4. Genotoxicity

Fluindapyr was tested for genotoxicity in ten studies, three of which were in vivo (see Table 20). Fluindapyr showed no evidence of genotoxicity either from in vivo or in vitro studies. On the basis of these studies, the Meeting concluded that fluindapyr is not genotoxic.

Table 20. Summary of genotoxicity studies with fluindapyr

Type of study	Organisms/cells	Concentration/ dose range tested	Purity (%)	Result	Reference
		In vitro			
Bacterial mutagenicity (Ames)	*Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537	Both ± S9 mix: Experiment I: 3, 10, 33, 100, 333, 1000, 2500, 5000 µg/plate Experiment II: 10, 33, 100, 333, 1000, 2500, 5000 µg/plate	96.309	Negative ±S9	Sokolowski, 2013
Bacterial mutagenicity (Ames)	*Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 *E. coli* WP2 uvrA	Both ± S9 mix: Experiment I and II: 10, 31.6, 100, 316, 1000, 2500, 5000 µg/plate	97.60	Negative ±S9	Schreib, 2017
Bacterial mutagenicity (Ames)	*Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 *E. coli* WP2 uvrA	Both ± S9 mix: Experiment I and II: 10, 31.6, 100, 316, 1000, 2500, 5000 µg/plate	96.84	Negative ±S9	Schreib, 2018a
Mouse lymphoma gene mutation test	Mouse lymphoma cells	Experiment I: 3.5, 7.0, 14.0, 28.0, 56.0, 84.0, 112.0 µg/mL (–S9 mix) 3.9, 7.9, 15.8, 31.5, 63.0, 84.0 µg/mL (+S9 mix) Experiment II 0.2, 0.4, 0.8, 1.5, 3.0, 4.5, 6.0 µg/mL (–S9 mix) 3.9, 7.9, 15.8, 31.5, 280, 63.0, 84.0 µg/mL (+S9 mix)	96.309	Negative ±S9	Wollny, 2014
Mouse lymphoma gene mutation test	Mouse lymphoma cells	Experiment I 5, 10, 25, 50, 60 µg/mL (–S9 mix) 10, 50, 60, 80, 82, 84 µg/mL (+S9 mix)	97.60	Negative ±S9	Schreib, 2018b
Chromosome aberration (clastogenicity)	Human lymphocytes	Experiment I 41.3, 72.3, 126.6, 221.5 µg/mL (–S9 mix) 72.3, 126.6, 221.5 µg/mL (+S9 mix) Experiment II 72.3, 126.6, 221.5 µg/mL (–S9 mix) 50.0, 75.0, 200.0, 225.0, 250.0, 300.0, 400.0 µg/mL (+S9 mix)	96.309	Negative ±S9	Bohnenberger, 2014
Chromosome aberration (clastogenicity)	Human lymphocytes	Experiment I 10, 20, 40 µg/mL (–S9 mix) 10, 20, 50, 100 µg/mL (+S9 mix) Experiment II 20, 40, 60, 80 µg/mL (–S9 mix)	97.60	Negative ±S9	Tiessen, 2018

Type of study	Organisms/cells	Concentration/ dose range tested	Purity (%)	Result	Reference
		In vivo			
Bone marrow micronucleus	Male (Crl:NMRI) mouse bone marrow cells	2000 mg/kg bw	96.309	Negative	Dony, 2014b
Peripheral blood micronucleus	Male and female (Crl:NMRI) mouse peripheral blood erythrocytes	Male: 500, 1000, 2000 mg/kg bw Female: 250, 500, 1000 mg/kg bw	97.60	Negative	Donath, 2018a
Peripheral blood micronucleus	Male and female (Crl:NMRI) mouse peripheral blood erythrocytes	Male: 500, 1000, 2000 mg/kg bw Female: 125, 250, 500 mg/kg bw	96.84	Negative	Donath, 2018b

There were seven in vitro studies and three in vivo studies on the genotoxicity of fluindapyr; all were negative. Three in vivo studies submitted were in mice, one bone marrow micronucleus test and two micronucleus tests in peripheral blood. To ascertain the proof of target tissue exposure in mice, in the absence of any ADME study in mice, the kinetic study along with the study of carcinogenesis in mice was considered, and there it was observed that fluindapyr was absorbed and sufficiently distributed in mice through oral route.

2.5 Reproductive toxicity

(a) Multigeneration studies

Preliminary study

A preliminary reproduction study was conducted to determine suitable dose levels of fluindapyr for a subsequent main two-generation reproduction study in the Han Wistar rat. Fluindapyr (purity 96.309%; Lot no.: 30414/64) was administered via the diet at concentrations of 0, 400, 1200, 4000 or 12000 ppm to males for 14 days prior to pairing, during pairing and after pairing, for a total of 61 days and to females for 14 days prior to pairing, during the pairing, gestation and lactation periods until postpartum day (PPD) 21, for a total of at least 56 days. Each group consisted of five male and five female rats aged around 11 weeks, males weighing 318–355 g, females 178–224 g at the start of treatment. Dams were allowed to rear their pups until PPD 21. After delivery was complete, offspring were examined for litter size, number of live and still births and any gross anomalies. Viability/mortality and clinical observations were made daily. Sex determination was carried out on PPDs 0 (if possible), 1, 4 and 21. Body weights were measured on PPDs 0 (if possible), 1, 4, 7, 14 and 21.

The mean achieved dose levels of fluindapyr are given below in the Table 21.

Table 21. Mean achieved dose levels of fluindapyr in F0 parental rats throughout a preliminary multigenerational study in rats

	Sex, dietary dose (ppm)									
	Male					**Female**				
	0	**400**	**1200**	**4000**	**12000**	**0**	**400**	**1200**	**4000**	**12000**
F0 generation – achieved chemical intake (mg/kg bw per day)										
Pre-pairing period	0	25	76	232	645	0	30	90	260	639
After pairing period	0	19	63	204	625	-	-	-	-	-
Gestation period	-	-	-	-	-	0	29	91	288	862
Lactation period	-	-	-	-	-	0	59	170	535	1371

Source: Dettwiler, 2014

All animals surviving to the end of the observation period were euthanized. All parental animals and pups were examined macroscopically for any structural changes. For the parent animals, special attention was directed at the organs of the reproductive system. The uteruses of all dams were placed in a solution of ammonium sulfide to visualize possible haemorrhagic areas of implantation sites, and the number of implantation sites was noted.

At the scheduled sacrifice liver weights were measured for all F0 animals. Organs (epididymides, liver, ovaries, prostate gland, seminal vesicles with coagulating glands, testes, uterus (including oviducts, cervix and vagina) from all F0 generation animals which failed to produce offspring were preserved for possible further examination. Organs were trimmed from any adherent tissue and preserved in neutral phosphate buffered 4% formaldehyde solution. Pups found dead, except those excessively cannibalized, were preserved in phosphate buffered 4% formaldehyde solution for possible further examination.

All animals survived until the scheduled necropsy. No test item-related clinical signs were noted in males or females at any dose level. A dose dependent reduction in food consumption and relative food consumption was observed at 4000 and 12 000 ppm in both sexes. Food consumption and relative food consumption at 400 and 1200 ppm were considered not to be affected by the treatment. In males, a reduction in food consumption was observed at 4000 and 12 000 ppm during pre-pairing. Thereafter it recovered and was similar to the controls during the remainder of the study. Reduction in food consumption was accompanied by a slight reduction in relative food consumption at 4000 and 12 000 ppm at the beginning of the study.

In females, reduction in food consumption was recorded at 4000 and 12 000 ppm during pre-pairing. During gestation it recovered slightly, although remained lower than control values at both 4000 and 12 000 ppm. Significant decreases in food consumption were observed once again during lactation at these doses. Relative food consumption showed similar dynamics; it declined at both doses during pre-pairing, recovered during gestation and declined again during lactation. In both sexes, effects on food consumption occurring at the beginning of the treatment period were attributed to a lack of palatability of the test item and were reversible after animals became accustomed to the taste. Effects on food consumption that occurred over a longer period as treatment continued were considered to be due to the toxic potential of the test item.

A dose-dependent reduction in body weights and body weight gain was recorded at 4000 and 12 000 ppm in both sexes. At 400 and 1200 ppm, body weight and body weight gain were considered not to be affected by the treatment. In males, treatment with the test item caused slight body weight loss followed by a reduced body weight gain and lower body weight at 4000 and 12 000 ppm during pre-pairing. A recovery in body weight gain and body weight was recorded at both 4000 and 12 000 ppm during pairing. Thereafter a decrease in body weight gain resulting in reduced body weights was observed again at 12 000 ppm in the period after pairing. A similar but less pronounced reduction in body weight and body weight gain was also noted at 4000 ppm in the period after pairing. In females, body weight loss at the beginning of treatment followed by a reduced body weight gain and accompanied by reduced body weights were recorded at 4000 and 12 000 ppm during pairing. Body weight gain recovered slightly but body weights remained reduced at both 4000 and 12 000 ppm during gestation. During lactation, body weight gain reduced again, to the extent of causing body weight loss, at both doses at the end of this period. Body weights were reduced at both doses during lactation. The initial reduction in body weight gain and body weights was considered to be a result of reduced food consumption due to poor palatability of the test item. Effects on body weight and body weight gain, becoming more pronounced during treatment over a longer period, were considered to be due to the toxic potential of the test item.

Mating performance, fertility and duration of gestation were not affected by treatment with the test item. A slightly reduced number of implantation sites was noted at 4000 and 12 000 ppm resulting in slightly lower number of living pups at first litter check. At 12 000 ppm four pups from one litter were missing during the first four days after birth. The increase in postnatal loss at this dose was statistically significant.

Treatment with the test item caused significant increases in liver weights at 1200, 4000 and 12 000 ppm, and a slight increase in liver weight at 400 ppm in both sexes. Mean absolute liver weights at 400, 1200, 4000 and 12 000 ppm were higher than the controls by 1.5%, 11.3%, 10.2% and 21.1% respectively in males, and 15.9%, 27.1%, 45.2% and 45.8% respectively in females. Mean relative liver weight to body weight ratios at 400, 1200, 4000 and 12 000 ppm were higher than the controls by 4.8%, 15.3%, 16.6% and 28.1% respectively in males, and 16.4%, 33.4%, 64.5% and 88.4% respectively in females. No other organ weights were measured. No macroscopic findings were noted in males or females at any dose level.

Body weights of pups were similar at all doses on PPD 1. During lactation, reduced body weight gain was recorded at 4000 and 12 000 ppm, resulting in significantly reduced body weights of pups on PPD 21 at these doses. Mean pup weights at 4000 and 12 000 ppm were lower than those of controls by 23.8% and 47.3% respectively. Pup body weights and bodyweight gains at 400 and 1200 ppm were considered not to be affected by the treatment. The sex ratios were unaffected by treatment with the test item at any dose level. Body cavities of reduced size were noted in several pups from two different litters at 12 000 ppm. No further test item-related effects were noted at any dose level. No microscopic examinations were undertaken in this study.

Based on the results of this range-finding study, a high-dose level corresponding to fluindapyr concentration in feed of higher than 1200 ppm but lower than 4000 ppm was considered to be appropriate for the subsequent two generation reproduction toxicity study in Han Wistar rats. At 4000 ppm there was a significant decrease in food consumption and body weight gain of dams, an increase in liver weight and decrease in pup body weight at weaning (Dettwiler, 2014).

Main two-generation study

In the main, two-generation reproduction study, fluindapyr (purity: 97.46%; Lot no. P/13/013) was administered continuously in the diet at concentrations of 0, 100, 400 and 1600 ppm to male and female Wistar Hannover RccHan:WIST(SPF) rats, aged seven weeks, males weighing 219–260 g, females 139–186 g at the start of treatment. Treatment groups consisted of 24 rats of each sex per group. Dose levels were set on the basis of a preliminary study (Dettwiler, 2014), however, after treatment for four weeks no significant effects on food consumption, body weight, or body weight gain had been observed in males or females of any group, therefore the concentration of fluindapyr for the high-dose level was increased from 1600 ppm to 3200 ppm on day 36 of the pre-pairing period of the F0 generation; this group is referred to as "3200 ppm" in this report. During the lactation period the fluindapyr concentrations in the diet were reduced by half for the F0 and F1 females to maintain the dams at the desired target dose of fluindapyr during this period of significantly increased food consumption, but all of these dose levels are referred to by their initially applied concentrations in ppm.

The F0 generation was administered test diets for 10 weeks prior to mating and throughout mating, gestation and lactation. From the F1 litters, 24 male and 24 female offspring were selected to form the F1 generation and received control or treated diets for a minimum of 90 days prior to mating and throughout mating, gestation and lactation. The F2 offspring were sacrificed at weaning.

The mean intakes of fluindapyr at various dietary doses and at various stages are shown below in Tables 22a and b.

Table 22a. Test item intake for the multigenerational study in rats – males

		Mean achieved dose level (mg/kg bw per day)			
Male group number	**Dietary dose (ppm)**	**F0 generation**		**F1 generation**	
		Pre-pairing	**After pairing**	**Pre-pairing**	**After pairing**
1	0	0	0	0	0
2	100	6.3	4.8	7.8	4.8
3	400	25	19.8	31.5	19.6
4	1600 [a]	112.1 [a]	-	-	-
	3200 [b]	174.1 [b]	160.9	266.2 [b]	164.1

[a] From day 1 to 36 of the pre-pairing period in F0 generation; Source: Dettwiler, 2015a

[b] From day 36 of the pre-pairing period in F0 generation onwards and during the entire pre-pairing period in F1

Table 22b. Test item intake for the multigenerational study in rats – females

Female group number	Dietary dose (ppm)	Mean achieved dose level (mg/kg bw per day)					
		F0 generation			F1 generation		
		Pre-pairing	Gestation	Lactation	Pre-pairing	Gestation	Lactation
1	0	0	0	0	0	0	0
2	100	7.7	6.6	6.7	8.6	6.7	6.6
3	400	29.7	26.0	28.1	35.3	26.9	28.4
4	1600 [a]	137.9 [a]	-	-	-	-	-
	3200 [b]	209.0 [b]	201.1	213.8	293.7[b]	219.4	214.6

[a] From day 1 to 36 of the pre-pairing period in F0 generation; Source: Dettwiler, 2015a

[b] From day 36 of the pre-pairing period in F0 generation onwards and during the entire pre-pairing period in F1

All parental animals (F0 and F1 generation) were observed twice daily for mortality or moribundity. Clinical observations were made daily and females were observed for signs of difficult or prolonged parturition and behavioural abnormalities in nesting or nursing. Detailed clinical examinations of males were made once prior to the first administration (F0 generation only) and weekly thereafter (from start of pre-pairing for F1 generation). Females also underwent such examinations once prior to the first administration and weekly during the pre-pairing and pairing periods, then on postcoitum days 0, 6, 13 and 20 and PPDs 1, 4, 7 and 14. Individual body weights were recorded once during acclimatization, weekly during pre-pairing (for males and females), pairing and after pairing periods (for males) and, for females only on postcoitum days 0, 7, 14 and 21 and PPDs 1, 7, 14 and 21. Food consumption was recorded at the same time as body weights, except during the pairing period and during the lactation period, after PPD 14, since pups begin to consume maternal feed. Vaginal smears were prepared starting 21 days prior to pairing and throughout pairing, until the smear was sperm-positive or a copulation plug was observed. Vaginal smears were taken daily and stage of estrus determined. Females of the F0 and F1 generations were sacrificed after weaning of the offspring on PPD 21. Males of the F0 and F1 generation were sacrificed on the day after completion of the treatment when they were no longer necessary for the assessment of reproductive performance. Females that lost their litter were sacrificed and necropsied together with other dams after weaning of the pups. If birth did not occur on the expected date (postcoitum day 21), the female was sacrificed and examined on day postcoitum day 25. The sperm of F0 and F1 parental males was analyzed at necropsy. At the scheduled sacrifice, selected organs from all F0 and F1 parental animals were weighed and preserved. This was also done for animals that died spontaneously or had to be terminated in extremis.

On PPD 4 the F1 litters were culled to yield as nearly as possible four males and four females per litter. All pups were observed twice daily (F1 pups) or once daily (F2 pups) for mortality or moribundity. Offspring were examined as soon as possible after completion of delivery for litter size, sex of pups, number of live and still births and any gross abnormalities. Sex was determined on PPD 0 (if possible), and on PPDs 1, 4 and 21. Clinical observations were made daily for any physical or behavioural abnormalities. Body weights were recorded on PPDs 0 (if possible), 1, 4, 7, 14, 21 and 28, and weekly thereafter for F1 pups. for F2 pups the same was done but on PPDs 0 (if possible), 1, 4, 7, 14 and 21. For all F1 pups only the day of onset of ear and eye opening, lower incisor eruption and hair growth were recorded. Age and body weight at which vaginal opening or preputial separation occurred were recorded. For F2 pups only the anogenital distance was measured on PPD 1 following observation of a delay in F1 female pup sexual maturation. F1 pups selected for organ weights and preservation as well as all F2 pups were sacrificed after weaning on PPD 21. After weaning, 24 male and 24 female pups were randomly selected with at least one pup per sex per litter, to create the F1 generation. For both males and females, the start of the pre-pairing period was taken to be after the youngest animal had reached PPD 28. The remaining F1 offspring (those not selected for F1 generation) were sacrificed after the youngest litter reached PPD 28. At the scheduled sacrifice selected organs (brain, liver, spleen, thymus and all gross lesions) from 24 male and 24 female pups per group, at least one pup per sex per litter, if possible, were weighed and preserved.

No deaths related to the treatment occurred among parent animals. No clinical signs were noted during the daily or weekly detailed observations. At 3200 ppm food consumption was reduced in F0 and F1 generation females for most of the study period. A slight and transient reduction in food consumption was also noted in F0 males at 3200 ppm, and F0 females receiving 400 ppm during the pre-pairing period. At 3200 ppm, body weight gain and mean body weights were reduced in both F0 males and females during most of the study period. At 3200 ppm F1 males and females had reduced mean body weights already at the start of the pre-pairing period (as a result of reduced body weight gain in F1 pups) and although body weight gain increased in the F1 parental animals, the absolute body weights remained lower until termination when compared to their respective control values.

In F0 females, reproduction and breeding parameters (i.e. estrous cycle, mating performance, fertility, duration of gestation, implantation rate and post-implantation loss, litter size at first litter check, viability of pups until weaning on PPD 21) were not affected in any group. In the F1 generation, treatment at 3200 ppm caused a decrease in the number of females with regular cycles and an increase in the number of females with irregular cycles. Furthermore, the mean length of the cycles was prolonged in these females, however without attaining statistical significance. Remaining reproductive and breeding parameters (mating performance, fertility, duration of gestation, implantation rate and post-implantation loss, litter size at first litter check, viability of pups until weaning on PPD 21) were not affected in any group.

At a dietary dose of 3200 ppm increased liver weights, together with minimal or slight diffuse midzonal/centrilobular hypertrophy were observed in F0 and F1 generation males and females. Liver enlargement was also observed for some F0 and F1 females at necropsy, as was midzonal/centrilobular hypertrophy in F0 females at 400 ppm. An increased incidence of minimal thyroid follicular hypertrophy/hyperplasia was also noted in males and females at 3200 ppm , in both the F0 and F1 generations. Reduced thymus weight was recorded in females of the F0 and F1 generations at 3200 ppm, and in F1 females at 400 ppm, with moderate lymphoid atrophy/involution of the thymus seen in F1 females at 3200 ppm. This occurred in the presence of general toxicity and therefore may be a secondary effect, a explanation that is supported by the lack of similar findings in males, which were less severely affected in terms of general toxicity than females. An increase in seminal vesicle weight was recorded in F0 generation males at 3200 ppm, without any supporting findings either from histopathological examination, seminology, or reproductive performance. Sperm analyses gave no indication of any treatment-related effect in any generation at any dose level. In males at 3200 ppm of both generations, cortical tubular yellow-brown (lipofuscin-like) pigment was observed in the kidney.

Follicle and corpora lutea count revealed no significant differences between the control and the 3200 ppm group animals in the F0 generation. Changes in the reproductive organs were observed in females at 3200 ppm in both the F0 and F1 generations. Reduced ovarian and uterine weights were recorded in F0 females, together with an increase in the number of females with severe vacuolated corpora lutea and/or moderate attenuated endometrium and/or moderate vaginal epithelial mucification observed at histopathology. Females of the F1 generation revealed reduced pituitary and uterus weights, and a macroscopically smaller uterus was found in some females at necropsy. An increased number of F1 females had moderate attenuated endometrium and/or anestrus epithelium in the vagina, and the antral follicle number was reduced in their ovaries, although the mean number of antral follicles remained within the range of historical control data. No histopathological correlate was noted in the pituitary, and the reduced organ weight occurred in the presence of a significant delay in body weight development and reduced brain weight, so that it could be secondary to the overall delay in growth rather than a specific effect on the organ. The combination of vacuolated corpora lutea (F0 generation), attenuated endometrium, increased mucification (F0 generation) and anestrus epithelium (F1 generation) of the vagina may be regarded as a delayed return to normal estrous cycles during lactation in the high-dose females. The marginally significant reduction in antral follicles in 3200 ppm F1 females may also reflect disturbed estrus in these animals. A slight increase in attenuated endometrium in the 400 ppm F0 females was not observed in the 400 ppm F1 females, and the slight increase in vaginal epithelium mucification at 100 and 400 ppm in the F0 females was less than the incidence of this finding in F1 control females (15/24). Therefore, the findings in the F0 females at 100 and 400 ppm were not considered adverse or related to treatment.

At 3200 ppm, retarded pup growth was observed in both generations. Body weights at birth were

not affected by the treatment in F1 or F2 pups, however during lactation a reduction in body weight gain was noted in both generations, resulting in significantly reduced pup body weights during this period. Furthermore, in female pups, the time of vaginal patency (assessed in F1 pups) was delayed. No further findings were recorded in any group or generation. In particular, no abnormal findings were recorded in pups during lactation. The pinna unfolding, incisor eruption, onset of coat development and opening of eyes were similar in all groups. Also the pup sex ratio gave no indication of any effect in any generation.

Table 23. Summary of results from two-generation toxicity study in rats treated orally with fluindapyr

	Sex and dietary dose (ppm)							
	Males				Females			
	0	100	400	3200[a]	0	100	400	3200[a]
	F0 generation							
Mortality	No treatment-related deaths							
Clinical signs	No treatment-related effects							
Body weight (g)								
Before pairing; day 1	236	236	234	234	158	159	160	157
Before pairing; day 70	480	465	466	459	252	255	256	241
Pairing; day 10	485	470	473	463*	-	-	-	-
After pairing; day 40	526	512	513	501*	-	-	-	-
During gestation; day 0	-	-	-	-	252	253	252	240*
During gestation; day 7	-	-	-	-	277	279	277	261**
During gestation; day 21	-	-	-	-	368	374	369	348*
During lactation; day 7	-	-	-	-	292	304	298	278*
During lactation; day 21	-	-	-	-	288	297	296	275*
Estrus (24 females in each group)								
Irregular cycle, shortened	-				1	2	0	5
Irregular cycle, prolonged					3	4	3	4
Historical control data								
Irregular cycle, shortened	-				11/23 [44%], 5/23 [20.8%]			
Irregular cycle, prolonged					7/23 [28.0%], 4/23 [16.7%]			
Adults: organ weights (g)								
Liver	15.40	15.20	15.62	17.62**	12.42	12.68	13.41	17.47**
Thymus	0.401	0.374	0.370	0.371	0.240	0.239	0.225	0.175**
Seminal vesicle	1.67	1.75	1.79	2.00**	-	-	-	-
Ovary (left)	-	-	-	-	0.061	0.059	0.057	0.046**
Ovary (right)	-	-	-	-	0.054	0.054	0.049	0.045
Uterus	-	-	-	-	0.86	0.83	0.84	0.54**
Microscopic findings (*N*)	24	24	24	24	24	24	24	24
Thyroid Follicular hypertrophy/ hyperplasia, diffuse[b]	5	4	8	13	2	3	3	18
Liver Midzonal/centrilobular hypertrophy, diffuse[g]	0	0	4	22	1	1	11	22
Ovaries Vacuolated corpora lutea	-	-	-	-	3	0	1	12[c]
Uterus Attenuated endometrium[d]	-	-	-	-	1	0	5	20
Vagina Epithelial mucification[e]	-	-	-	-	5	9	9	17[f]

	Sex and dietary dose (ppm)							
	Males				Females			
	0	100	400	3200[a]	0	100	400	3200[a]
F1 Offspring: body weight (g)								
Day 1	6.3	6.2	6.2	6.6	6.1	5.9	5.8	6.3
Day 14	30.5	33.0	30.3	28.8	30.9	32.3	29.0	28.1*
Day 28	82.1	87.1	81.9	68.7**	77.8	81.6	75.8	65.8**
F1 Offspring: organ weight								
Thymus								
Absolute (g)	0.21	0.22	0.20	0.18*	0.22	0.22	0.20	0.18**
Relative to brain (%)	14.56	15.08	14.05	12.37*	14.56	15.4	14.2	12.1
Spleen								
Absolute (g)	0.25	0.29**	0.23	0.19**	0.25	0.27	0.25	0.21**
Relative to brain (%)	16.96	20.30**	16.40	13.50**	16.96	18.3	18.4	15.
	F1 generation							
Mortality	No treatment-related deaths							
Clinical signs	No treatment-related effects							
Body weight (g)								
Before pairing (day 1)	79	84*	80	67**	76	79	75	64**
Before pairing (day 78)	418	427	425	400	245	261**	249	229*
Pairing (day 1)	436	444	441	417	-	-	-	-
Pairing (day 14)	443	452	448	426	-	-	-	-
After pairing (day 42)	445	454	451	428	-	-	-	-
After pairing (day 42)	479	494	493	461	-	-	-	-
During gestation (day 0)	-	-	-	-	251	263	251	231**
During gestation (day 21)	-	-	-	-	359	381*	362	327**
During lactation (day 1)	-	-	-	-	257	283**	262	247
During lactation (day 21)	-	-	-	-	283	289	282	262**
Estrus (*N*)					24	24	24	24
Regular cycles (four days)					16	12	16	4**
Irregular cycle (shortened)					1	0	0	3
Irregular cycle (prolonged)					1	2	0	3
Acyclic					3	7	8	13**
HCD								
Irregular cycle, shortened	-				0/23 [0%], 0/23 [0%]			
Irregular cycle, prolonged					0/23 [0%], 3/23 [12.5%]			
Balanopreputial separation (days)	26.8	26.2	25.9*	27.1	-	-	-	-
bw (g) at day of attainment	75.73	76.78	71.12	64.76	-	-	-	-
Vaginal patency (days)	-	-	-	-	32.9	32.8	32.0	35.5*
bw (g) at day of attainment	-	-	-	-	103.00	106.64	97.48	101.20
Adults: organ weights (g)								
Pituitary	0.010	0.011	0.011	0.010	0.014	0.014	0.013*	0.012**
Liver	14.48	14.86	15.10	16.77**	11.99	12.20	13.00	18.55**
Thymus	0.355	0.364	0.374	0.343	0.278	0.253	0.224**	0.191**
Ovary (L)	-	-	-	-	0.061	0.055	0.055	0.055
Ovary (R)	-	-	-	-	0.058	0.060	0.058	0.049*
Uterus	-	-	-	-	0.087	0.80	0.85	0.52**

	Sex and dietary dose (ppm)							
	Males				Females			
	0	100	400	3200[a]	0	100	400	3200[a]
Microscopic findings								
Thyroid (*N*)	24	24	24	24	24	24	24	24
Follicular hypertrophy/ hyperplasia, diffuse [b]	7	3	6	10	5	4	6	15
Liver (*N*)	24	24	24	24	24	24	24	24
Midzonal/centrilobular hypertrophy, diffuse [h]	0	0	3	18	0	0	1	24
Kidney (*N*)	24	24	24	24	24	1	-	24
Cortical tubular pigment yellow-brown [i]	4	8	8	20	5	0	-	8
Ovaries (*N*)	-	-	-	-	24	24	24	24
Vacuolated corpora lutea antral follicle (count)					1 78.70	4 90.90	2 89.20	4 53.40*
Uterus (*N*)	-	-	-	-	24	24	24	24
Attenuated endometrium [d]					0	0	1	18
Thymus (*N*)	24	-	-	24	24	24	24	24
Lymphoid atrophy,involution [j]	4	-	-	4	5	4	5	12
Vagina (*N*)					24	24	24	24
Epithelial mucification	-	-	-	-	15	7	4	10
F2 Offspring: body weight (g)								
Day 1	6.1	6.3	6.1	6.3	5.8	5.8	5.9	6.0
Day 14	31.0	33.0*	31.3	29.4	30.4	31.2	30.6	28.2*
Day 21	50.8	53.2	50.7	45.1**	49.6	49.9	49.4	43.3**
F2 Offspring: organ weights (g)								
Thymus								
Absolute (g)	0.235	0.252	0.240	0.194**	0.254	0.250	0.246	0.215*
Relative to brain (%)	16.1	17.3	16.3	13.5**	17.7	17.8	17.5	15.4
Spleen								
Absolute (g)	0.26	0.28	0.27	0.20**	0.27	0.28	0.25	0.21**
Relative to brain (%)	17.5.	19.2	18.3	14.1**	18.7	20.0	17.5	14.8**
Liver								
Absolute (g)	2.02	2.05	2.01	1.81	2.01	1.96	1.94	1.77*
Relative to brain (%)	138.6	141.5	136.8	126.0	139.9	139.3	138.6	127.3

N: Number examined; HCD: Historical control data; Source: Dettwiler, 2015a

[a] 1600 ppm from day 1 to 36, increased to 3200 ppm from day 36 to 70;

[b] Follicular hypertrophy/hyperplasia, diffuse (minimal and slight);

[c] Grades: 7 minimal, 2 slight, 2 moderate and 1 severe;

[d] Attenuated endometrium (minimal, slight and moderate);

[e] Epithelial mucification (minimal, slight, moderate);

[f] Grades: 7 minimal, 6 slight, 4 moderate;

[g] Midzonal/centrilobular hypertrophy, diffuse (minimal and slight);

[h] Midzonal/centrilobular hypertrophy, diffuse (minimal, slight and moderate);

[i] Cortical tubular pigment yellow-brown (lipofuscin-like) (minimal, slight and moderate);

[j] Lymphoid atrophy, involution (minimal, slight and moderate);

* Dunnett-test based on pooled variance significant at 5% level or Fisher's exact test, significant at 5%, or Steel-test significant at 5% level;

** Dunnett-test based on pooled variance significant at 1% level, or Fisher's exact test, significant at 1%

Table 24. Sperm parameters of male rats in two-generation rat study with fluindapyr

	Parental generation, dose level (mg/kg bw per day)							
	F0 generation				F1 generation			
	0	100	400	3200[a]	0	100	400	3200[a]
Sperm count (10^6/g)								
Testis	125.15	-	-	124.57	130.94	-	-	129.45
Cauda epididymis	682.69	-	-	731.47	736.36	-	-	732.53
Sperm motility (%)	69	69	67	60**	69	68	67	64
Abnormal sperm (%)	6.1	5.9	7.3	6.2	4.0	-	-	4.2

[a] Dose of 1600 ppm from day 1 to 36, increased to 3200 ppm from day 36 to 70; Source: Dettwiler, 2015a

Statistically significant using Fisher's exact test at: * $p = 0.05$, ** $p = 0.01$

Table 25. Reproduction parameters of female rats in two-generation rat study with fluindapyr

	Parental generation, dose level (mg/kg bw per day)							
	F0 generation				F1 generation			
	0	100	400	3200[a]	0	100	400	3200[a]
Number of animals per group	24	24	24	24	23	22	22	23
Female mating index (%)	100	100	100	100	100	100	100	100
Female fertility index (%)	95.8	91.7	95.8	95.7	100	95.8	91.7	95.8
Precoital interval, median (days)	3	3	3	3	2	3	2	3
Precoital interval, mean (days)	2.8	2.7	3.2	3.2	2.4	2.7	2.7	2.8
Implantations, total	259	240	274	280	304	285	285	270
Post-implantation loss (mean %)	8.1	9.6	11.3	12.1	10.9	10.5	8.8	15.2
Gestation index (%)	95.7	90.9	91.3	95.7	95.8	95.7	100.0	100.0
Birth index (%)	96.5	95.8	90.1**	87.9**	89.1	90.9	91.2	84.8
Live born pups per litter	22/22	18/20	18/21	21/22	22/23	19/22	19/22	22/23

[a] Dose of 1600 ppm from day 1 to 36, increased to 3200 ppm from day 36 to 70; Source: Dettwiler, 2015a

Statistically significant at: * $p = 0.05$, ** $p = 0.01$

Table 26. Pup survival and sex ratio in two-generation rat study with fluindapyr

	Parental generation, dose level (mg/kg bw per day)							
	F0 generation				F1 generation			
	0	100	400	3200[a]	0	100	400	3200[a]
Number of litters	22	20	21	22	23	22	22	23
with live-born pups	22	18	18	21	22	19	19	22
with still-born pups	0	2	3	1	1	3	3	1
Viability index [%]	98.4	98.3	90.1**	87.9**	95.9	93.4	98.8*	97.4
Weaning index [%]	100	100	100	98.8	99.4	100.0	100.0	100.0
Live pups at day 4 pre-cull	165	144	163	172	176	165	176	166
litters at day 4 pre-cull	22	20	21	22	23	22	22	23
Live pups at day 21	165	144	163	170	175	165	176	166
litters at day 21	22	20	21	22	23	22	22	22

[a] Dose of 1600 ppm from day 1 to 36, increased to 3200 ppm from day 36 to 70; Source: Dettwiler, 2015a

Statistically significant at: * $p = 0.05$, ** $p = 0.01$

The NOAEL for parental toxicity was 400 ppm (equal to 19.6 mg/kg bw per day) based on the thyroid follicular cell hypertrophy at 3200 ppm (equal to 161 mg/kg bw per day in males and 213 mg/kg bw per day in females). The NOAEL for reproductive toxicity was also 400 ppm (equal to 26 mg/kg bw per day) based on the effects on the female reproductive organs associated with changes in estrous cycle observed at 3200 ppm (equal to 201.1 mg/kg bw per day). The offspring NOAEL was 400 ppm (equal to 28.1 mg/kg bw per day), based on reduced body weight observed at 3200ppm (equal to 161 mg/kg bw per day) (Dettwiler, 2015a).

(b) Developmental toxicity

Rat

A developmental toxicity study was conducted to detect effects on the pregnant rat and development of the embryo and fetus following daily exposure of the dams to fluindapyr (purity 96.309%; Lot No. 30414/64) from postcoitum day 6 (implantation) to postcoitum day 20 (the day prior to caesarean section). Four groups of 22 mated female rats were used, 11 weeks old and weighing 193–248 g at postcoitum day 0. These were treated by gavage once daily at 0, 60, 300 or 1000 mg/kg bw per day, using 0.5% low viscosity CMC in double-distilled water containing 0.1% Tween 80 as the vehicle and at a dosing volume of 10 mL/kg bw. The animals were observed twice daily throughout the study for viability/mortality. Clinical signs and body weights were recorded daily from postcoitum day 0 to 21. Food consumption was recorded for the periods postcoitum day 0–3, 3–6, 6–9, 9–12, 12–15, 15–18 and 18–21. All surviving females were sacrificed on postcoitum day 21 and the fetuses were removed by caesarean section. The following parameters and end-points were also evaluated in this study: necropsy observations, organ weights, uterine contents (including uterine weights) and fetal external, visceral, and skeletal alterations.

No maternal deaths related to treatment with fluindapyr occurred. No treatment-related clinical signs were noted at the daily observations.

At 300 and 1000 mg/kg bw per day, transient but statistically significant and dose-dependent reductions in body weight, body weight gain, corrected body weight gain (corrected for the gravid uterus weight) and food consumption were recorded. By the end of the study, food consumption values were similar to those of the control group, and therefore effects on food consumption were not considered adverse. Although body weight and body weight gain tended to recover by the end of the study, the corrected body weight gain recorded at termination was reduced to a statistically significant extent compared with controls, and therefore the effects on body weight gain were considered to be adverse at 300 and 1000 mg/kg bw per day.

A statistically significant increase (greater than 10%) in absolute liver weights was noted at 300 and 1000 mg/kg bw per day and a statistically significant increase in liver to body weight ratio was also noted, in this case in all dose groups. This effect was considered an adaptive change due to the increased metabolic burden caused by exposure to a xenobiotic.

Post-implantation loss and the number of fetuses per dam were not affected at any dose level. During visceral examination of fetuses, an increased incidence of variations at 300 and 1000 mg/kg bw per day were noted. At 1000 mg/kg bw per day the incidence of dilated renal pelvis (including a severely dilated renal pelvis and ureter in one of the nine affected fetuses) exceeded the control value and was slightly higher than the historical control range. This observation was considered to possibly indicate a disturbance in fetal development related to the treatment. However, because the finding is a variation, which normally occurs in this strain of rat and reverses after birth, it was considered not to be adverse and most likely secondary to the toxic effects on the dams.

No further test item-related findings were noted in fetuses at any dose level.

Table 27. Findings in the rat developmental toxicity study after exposure to fluindapyr

	Dose level (mg/kg bw per day)			
Parameter	**0**	**60**	**300**	**1000**
Dam data				
Number of mated females	22	22	22	22
Number of pregnant females	21	21	17	20
Number surviving to day 21 with live litters	21	21	17	20
Number killed in extremis	0	0	0	0
Number found dead	0	0	0	0
Clinical signs	NTRE	NTRE	NTRE	NTRE
Food consumption; mean (g/animal per day)				
Days 6–9	20.9	20.5	16.8**	12.0**
Days 9–12	22.4	21.8	21.4	20.0**
Days 18–21	24.7	24.5	23.3	23.2
Body weight; absolute value (g)				
Day 12	269	266 -	265 -	256 *
Day 18	316	312 -	309 -	305 -
Day 20	342	334 -	334 -	330 -
Body weight gain; mean (percentage)				
Day 7	2	1	–1**	–3**
Day 14	15	13	12*	10**
Day 21	45	43	42	40
Liver weight; mean (g)				
Day 21	13.01	13.78	15.09**	16.72**
Reproductive parameters				
Number of dams	21	21	17	20
Corpora lutea	268	272	217	261
Mean (percentage)	12.8%	13%	12.8%	13.1%
Standard deviation	2.7	2.7	1.9	2.5
Pre-implantation loss	16	22	8	11
Percentage of corpora lutea	6%	8.1%	3.7%	4.2%
Mean	0.8	1	0.5	0.6
Standard deviation	1.4	2.4	1.1	1.6
Number of dams affected	8	8	4	4
Post-implantation loss	2	8	4	6
Percentage of corpora lutea	0.8%	3.2%	1.9%	2.4%
Mean	0.1	0.4	0.2	0.3
Standard deviation	0.3	0.8	0.4	1.1
Number of dams affected	2	5	4	2
Embryonic/fetal deaths total	2	8	4	6
Fetuses; total number	250	242	205	244
Percentage of implantation sites	99.2%	96.8%	98.1%	97.6%
Mean number	11.9	11.5	12.1	12.2
Standard deviation	3.2	3.3	2.3	3.3
Males; total number	128	107	105	111
Females; total number	122	135	100	133

Parameter	Dose level (mg/kg bw per day)			
	0	60	300	1000
Weights of live fetuses[a] (g)				
Fetuses, total number	250	242	205	244
Mean weight	4.9	4.8**	4.9	4.7**
Standard deviation	0.4	0.4	0.4	0.4
Male fetuses, number	128	107	105	111
Mean	5	4.9	5	4.9**
Standard deviation	0.4	0.4	0.4	0.4
Female fetuses, number	122	135	100	133
Mean	4.8	4.7*	4.7	4.6*
Standard deviation	0.3	0.4	0.4	0.4
Litter responses				
Renal pelvis dilated				
Fetuses, number affected [percentage]	2 [2%]	0	2 [2%]	8 [6%]
Litters, number affected [percentage]	2 [10%]	0	2 [12%]	6 [30%]
Ureter dilated				
Fetuses, number affected [percentage]	1 [1%]	0	0	0
Litters, number affected [percentage]	1 [5%]	0	0	0

[a] On an individual basis; NTRE No treatment related effects; Source: Dettwiler, 2015b

Statistically significant at: * $p = 0.05$, ** $p = 0.01$

In view of these results, the NOAEL for maternal toxicity was 300 mg/kg bw per day based on reductions in body weight, and depression in body weight gain at 1000 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Dettwiler, 2015b).

Rabbit

A study was conducted to detect effects on the pregnant rabbit and development of the embryo and fetus following daily exposure of dams to fluindapyr (purity 96.309%; Lot no. 30414/64) by gavage, from postcoitum day (PCD) 6 (implantation) to PCD 27 (the day prior to caesarean section). Fluindapyr was administered daily at 0, 50, 250 or 750 mg/kg bw per day to 24, 22, 25 and 25 mated female New Zealand White rabbits respectively. Rabbits were 19–23 weeks old and weighed 2.8–4.4 kg on PCD 0. The animals were observed twice daily throughout the study for viability/mortality. Clinical signs were recorded daily from PCD 0 to 28 and body weights were recorded PCD 0 and from PCDs 6 to 28. Food consumption was recorded for the periods PCD 0–3, 3–6, 6–9, 9–12, 12–15, 15–18, 18–21, 21–24 and 24–28. All surviving females were sacrificed on PCD 28 and all fetuses were removed by caesarean section. The following parameters and end-points were also evaluated in this study: necropsy observations, organ weights, uterine contents (including uterine weights) and fetal external, visceral, and skeletal alterations.

No maternal deaths related to treatment with fluindapyr occurred. No treatment-related clinical signs were noted during the daily observations. At 750 mg/kg bw per day, body weight gain and absolute and relative food consumption were reduced to a statistically significant extent. Increased relative liver weights were recorded but macroscopic pathology and histopathology did not reveal any lesions in the liver that could be attributed to the treatment. All findings recorded were considered to be within the range of normal lesions that might be seen in rabbits of this strain and age and under the experimental conditions used in this study, and were comparable with the controls.

At 750 mg/kg bw per day pre-implantation loss was higher than for controls, showing statistical significance. This finding was not considered treatment-related, because administration started on day 6 of pregnancy, which was recognised as the day of implantation. No statistically significant alterations in the other reproduction parameter (corpora lutea or post-implantation loss) were recorded. No noteworthy differences were recorded at hysterectomy on embryo/fetal development. Fetal weight and sex ratio were unaffected by treatment.

The macroscopic external examination of fetuses revealed a range of abnormalities and variations in all groups, however there was no indication of a treatment-related trend in the type or incidence of these anomalies. A total of five fetuses from two litters in the control group, two fetuses from two litters at 50 mg/kg bw per day, two fetuses from one litter at 250 mg/kg bw per day and five fetuses from four litters at 750 mg/kg bw per day were affected by malformations and/or variations, with one fetus in each group presenting one or more malformations. At skeletal examination no noteworthy alterations were recorded. A slightly higher percentage of litters with incompletely ossified phalanx of the forelimbs (left and right) was observed at 750 mg/kg bw per day, and a higher percentage of flying ribs was recorded among fetuses at 250 and 750 mg/kg bw per day compared to the controls. However, these findings were devoid of any toxicological significance and were attributed to the normal biological variability recorded in the process of ossification in the rabbit. Skeletal malformations already recorded at external examination were also confirmed.

A visceral serial head examination confirmed anencephaly, and other abnormalities already recorded at the external examination for the malformed fetus at 750 mg/kg bw per day. A total of six fetuses showed visceral alterations, classified as simple variations; a slightly dilated brain lateral ventricle was recorded in a total of five fetuses (two from the control group, and one each from the treated groups). Considering the low incidence of these abnormalities, the lack of a apparent dose–effect relationship or any evidence that the treatment could significantly increase the occurrence of these abnormalities, these finding were considered to be due to spontaneous variation.

Table 28. Findings in the rabbit developmental toxicity study after exposure to fluindapyr

	Dose level (mg/kg bw per day)			
Parameter	**0**	**50**	**250**	**750**
Dam data				
Number of mated females	24	22	25	25
Number of pregnant females	20	19	22	21
Number surviving to day 21 with live litters	20	19	20	20
Number killed in extremis	0	0	0	0
Number found dead	1	0	2	1
Clinical signs	NTRE			
Food consumption; mean (g/animal per day)				
Days 6–9	140.4	141.1	129.5	88.3**
Days 12–15	127.5	127.4	129.6	108.2*
Days 24–28	103.6	93.8	108.9	108.6
Body weight; absolute value (kg)				
Day 15	3.73	3.67	3.68	3.55
Day 21	3.73	3.66	3.68	3.58
Day 28	3.88	3.81	3.84	3.72
Body weight gain; mean (percentage)				
Day 9	0.0	0.1	−0.8	−2.2**
Day 13	1.6	2.1	1.4	0.1*
Day 28	7.6	8.3	8.5	8.0
Liver weight; mean (g)				
Day 28	89.2	88.3	99.2	107.1**
Reproductive parameters				
Corpora lutea	193	176	208	193
Pre-implantation loss	36	21	51	53
Percentage of corpora lutea	18.7%	11.9%*	24.5%	27.5%*
Post-implantation loss	12	12	11	9

Parameter	Dose level (mg/kg bw per day)			
	0	50	250	750
Litter responses – fetal data				
External examination; number of fetuses examined	145	143	146	131
Head: acrania, anencephaly, absent eye & small ear (unilateral), Palate, lip & mandible cleft, protruding tongue	0	0	0	1
Abdominal cavity: oedema	0	0	1	0
Forelimb: hyperflexion/short (unilateral)	0	1	0	0
Forepaw				
Hyperflexion (unilateral)	1	0	0	1
Malrotated (unilateral)	0	0	0	1
One digit absent (bilateral)	1	0	0	0
Few digits absent (unilateral)	0	1	0	0
Small (unilateral)	0	1	0	0
Hindpaw: malrotated (unilateral)	0	0	0	1
Tail: Hooked	4	1	1	1
Amniotic fluid: discoloured, reddish	0	0	0	2
Visceral fresh; number of fetuses examined	145	143	146	131
Brain: perimeningeal space, large	0	0	0	1
Abdominal cavity: fluid-filled (transparent)	0	0	1	0
Heart and great vessels				
Large heart and right atrium with dilated aorta and pulmonary artery; ventricles and great vessels in normal proportion	0	0	1	0
Pulmonary artery apparently absent; aorta dilated and entry between both ventricles	0	0	0	1
Skeletal examination; number of fetuses examined	136	143	143	123
Forelimbs (left and right)				
Incompletely ossified				
Metacarpal 1, digits 1–5, proximal & medial phalanx, total – left and right (sum)	20	24	29	21
Non-ossified				
Metacarpal 1 and 4, digits 1–5 (proximal and medial phalanx), total – left and right (sum)	32	39	25	37
Ribs				
Flying rib 13, left	8	6	19	13
Flying rib 13, right	5	7	6	10
Visceral head examination; number of fetuses examined	71	72	74	64
Dilated brain lateral ventricles	2	1	1	1
Dilated olfactory lobe	1	0	0	0

NTRE: No treatment related effects observed; Source: Canut, 2015

Statistically significant at: * $p = 0.05$, ** $p = 0.01$

In view of the above findings, the maternal NOAEL) was 250 mg/kg bw per day based on significantly decreased food consumption and depressed body weight gain recorded at 750 mg/kg bw per day. The embryo/fetal NOAEL was 750 mg/kg bw per day, the highest dose tested (Canut, 2015).

2.6 Special studies

(a) Neurotoxicity

Acute neurotoxicity

To evaluate the acute neurotoxic potential of fluindapyr technical (purity 97.46%; Lot no. P/13/013) it was administered as a single oral dose to Crl:CD(SD) rats by gavage. The neurotoxic potential of the test substance was evaluated using a neurotoxic screening battery consisting of a functional observational battery (FOB), motor activity, and neuropathological assessments.

Fluindapyr was made up in a vehicle consisting of 0.5% (w/v) CMC and 0.1% Tween 80 in deionized water. This was administered orally by gavage as a single dose to Groups 2, 3 and 4, each consisting of 10 male and 10 female rats. Initial dose levels were 125, 500 and 2000 mg/kg bw. A similar concurrent control group (Group 1) received the vehicle on a comparable regimen. Due to significant reductions in motor activity noted at all dose levels during the initial phase, an additional phase (Phase II) was added to determine a NOAEL for neurotoxicity.

In Phase II, the test substance or vehicle was administered orally by gavage as a single dose to four groups of 10 male and 10 females Crl:CD(SD) rats at dose levels of 0, 15, 30 or 60 mg/kg. Animals in both phases were approximately six weeks old at the initiation of dose administration. Phase I male rats weighed 206–272 g, females134–168 g. Phase II male rats weighed 179–281 g and females146–190 g. In all cases the dose volume was 10 mL/kg bw. All animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily, on FOB assessment days. Individual body weights were recorded weekly (days −7, 0, 7 and 14). Motor activity and FOB data were recorded for all animals prior to the initiation of dose administration, at the time of peak effect (around five hours following dose administration) on day 0, and on days 7 and 14. All animals were deeply anaesthetized on day 15 and perfused in situ; brain weights and brain dimensions (excluding olfactory bulbs) were recorded. In addition, for Phase I animals, a neuropathological evaluation of selected tissues from the central and peripheral nervous systems was performed on five animals per sex in the control and 2000 mg/kg bw groups. Neuropathological evaluations were not performed on the Phase II animals because there were no treatment-related microscopic findings noted at 2000 mg/kg bw during Phase I of the study.

All Phase I animals survived to the scheduled euthanasia (day 15). No test substance-related clinical signs were noted during the daily examinations. Mean body weight and body weight gains were unaffected by fluindapyr administration at 125, 500 and 2000 mg/kg bw. In Phase I test substance-related FOB findings were noted at the time of peak effect on day 0 for males in the 500 and 2000 mg/kg bw groups, and females in the 125, 500 and 2000 mg/kg bw groups. The FOB parameters affected included open field (impaired mobility, altered gait, low arousal, bizarre behaviour, and/or decreased rearing) for males at 2000 mg/kg bw and females at 125, 500 and/or 2000 mg/kg bw, sensorimotor (abnormal air righting reflex) for males at 2000 mg/kg bw and females at 500 and 2000 mg/kg bw, and physiological (lower body temperature) for males and females at 500 and 2000 mg/kg bw. Lower mean total and ambulatory motor activity counts were noted for males and females in the 125, 500, and 2000 mg/kg bw groups in Phase I, primarily during the first and second subintervals (0–10 minutes and 11–20 minutes, respectively) at the time of peak effect on day 0. As a result of the initial decreases in motor activity counts, lower mean cumulative total and ambulatory counts compared to the control group were observed for males and females at 125, 500 and 2000 mg/kg bw when the entire testing session (0–60 minutes) was evaluated. The effects on FOB and motor activity for males and females at all dose levels did not persist to the day 7 or 14 evaluations. No remarkable shifts in the pattern of habituation occurred in any of the treated groups when the animals were evaluated on days 0, 7 and 14. There were no test substance-related macroscopic or microscopic findings or effects on brain weight or brain dimensions for males or females at any dose level in Phase I.

All Phase II animals survived to scheduled euthanasia on day 15. No test substance-related clinical signs were noted during the daily examinations. Mean body weight and body weight gains were unaffected by fluindapyr technical administered at 15, 30 or 60 mg/kg bw. No test substance-related effects were apparent when FOB evaluations were performed at the time of peak effect on day 0 or on days 7 and 14 in Phase II. The FOB evaluations collectively involved home cage, handling, open field, sensorimotor, neuromuscular, and physiological parameters. In Phase II, no test substance-related

effects were apparent in mean ambulatory or total motor activity counts at the time of peak effect on day 0 or on days 7 and 14. No remarkable shifts in the pattern of habituation occurred in any of the test substance-treated groups when the animals were evaluated on days 0, 7 and 14. There were no macroscopic findings or test substance-related effects on brain weight or brain dimensions in males or females at 15, 30 or 60 mg/kg bw.

Table 29. Findings in the acute neurotoxicity study in rats after exposure to fluindapyr

	Males; dose levels (mg/kg bw)					
Parameter	**0**	**60**	**0**	**125**	**500**	**2000**
Mortality	0	0	0	0	0	0
Body Weight (g)						
Day 0	223	221	233	235	232	236
Day 7	281	276	283	287	280	280
Day 14	337	329	331	335	328	330
Functional observation battery (FOB)						
FOB: home cage and handling parameters	No treatment-related effects					
FOB: sensory and neuromuscular observations	No treatment-related effects					
FOB: open field parameters (individuals affected/examined)[a]						
Mobility[b]	0/10	0/10	0/10	0/10	0/10	1/10
Gait[c]	0/10	0/10	0/10	0/10	0/10	1/10
Gait score[d]	0/10	0/10	0/10	0/10	0/10	1/10
Arousal[e]	0/10	0/10	0/10	0/10	0/10	1/10
FOB: Physiological observations						
Mean body temperature (°C)	37.6	37.2	38.1	37.6	37.2**	35.4**
Motor activity						
Total (Day 0, 0–10 minutes)						
Mean	1030	1106	1060	901	703	237
% difference from control	-	7.4	-	−15.0%	−33.7%	−77.6%
Standard deviation	33	250	380.3	308	261.9	163
Number examined	10	10	10	10	10	10
Linear trend *p*-value	-	-	-	0.199	0.006*	< 0.001*
Ambulatory (Day 0, 0–10 minutes)						
Mean	325	330	312	266	203	56
% difference from control	-	1.5	-	−14.7%	−34.9%	−82.1%
Standard deviation	116	91	121.8	92.3	95.3	48.2
Number examined	10	10	10	10	10	10
Linear trend *p*-value	-	-	-	0.269	0.011*	< 0.001*
Neurohistopathology	No treatment-related microscopic lesions were observed in any of the central or peripheral nervous system tissues examined from five animals in the 2000 mg/kg bw group.					
	Females; dose levels (mg/kg bw)					
Parameter	**0**	**60**	**0**	**125**	**500**	**2000**
Mortality	-	-	0	0	0	2
Body Weight						
Day 0	167	168	151	150	150	147
Day 7	192	194	176	176	175	172
Day 14	218	224	202	204	204	198

	Females; dose levels (mg/kg bw)					
Parameter	**0**	**60**	**0**	**125**	**500**	**2000**
Functional observation battery (FOB)						
FOB: home cage and handling parameters	No treatment-related effects					
FOB: sensory and neuromuscular observations	No treatment-related effects					
FOB: open field parameters (individuals affected/examined)[a]						
Mobility[b]	0/10	0/10	0/10	0/10	0/10	2/10
Gait[c]	0/10	0/10	0/10	0/10	0/10	2/10
Gait score[d]	0/10	0/10	0/10	0/10	0/10	3/10
Arousal[e]	0/10	0/10	0/10	0/10	0/10	0/10
Bizarre/stereotypical behaviour[f]	0/10	0/10	0/10	0/10	0/10	1/10
Rearing (counts)	7.7(6.9)	5.8(2.4))	6.4	3.7*	1.8**	1.1**
FOB: physiological observations						
Mean body temperature (°C)	38.2	38.1	37.9	37.5	35.4**	34.6**
Motor activity						
Total (Day 0, 0–10 minutes)						
Mean	1171	1105	1401	884	355	261
% difference from control	-	5.6	-	−36.9%	−74.7%	−81.4%
Standard deviation	197	301	392.1	347	93	169.8
Number examined	10	10	10	10	10	10
Linear trend *p*-value	-	-	-	< 0.001*	< 0.001*	< 0.001*
Ambulatory (Day 0, 0–10 minutes)						
Mean	397	375	506	315	97	74
% difference from control	-	5.5		−37.7%	−80.8%	−85.4%
Standard deviation	97	130	175.7	126.5	46.7	67.9
Number examined	10	10	10	10	10	10
Linear trend *p*-value	-	-	-	< 0.001*	< 0.001*	< 0.001*
Neurohistopathology	No treatment-related microscopic lesions seen in any central or peripheral nervous system tissues examined from five animals in the 2000 mg/kg bw group.					

Statistically significant at: * $p = 0.05$, ** $p = 0.01$; Source: Herberth 21016a

[a] Where there are multiple definitions the highest number of animals affected (for any one of them) is presented;

[b] Slightly impaired.

[c] In males: body drags, abdomen contacts surface, body sways and/or hunched body;
In females: body drags, abdomen contacts surface, body sways and/or hindlimbs splayed or dragging, unable to support weight and/or hunched body and/or ataxia, excessive sway, rocks or lurches as proceeds forward;

[d] Slight impairment, but definite; [e] Low – somewhat stuporous;

[f] Head flick (head shaking or backward flip of head) and/or head search (stereotyped, repetitive turning of head from side to side) and/or circling (tendency to move in circles around objects, or in an open environment)

Because adverse effects were noted for all groups receiving the test article (125, 500 and 2000 mg/kg bw) tested in Phase I (Moser,1991), a NOAEL could not be determined. Phase 2 was conducted with males and females at dose levels of 0 15, 30, and 60 mg/kg bw to determine a NOAEL. Based on the results of the FOB and motor activity evaluations in Phase II, none of the functional domains described by Moser (1991) were affected by a single dose of fluindapyr technical at 15, 30, or 60 mg/kg bw. In addition, there was no evidence of neuropathological changes noted in males and females at 2000 mg/kg bw during Phase I of the study. Therefore, the NOAEL for systemic toxicity following a single oral (gavage) dose of fluindapyr technical to male and female Crl:CD(SD) rats was considered to be 60 mg/kg bw, based on reduced motor and locomotor activity in females at 125 mg/kg bw. There were neuropathological changes at the highest dose tested (Herberth, 2016a).

Subchronic neurotoxicity

The objective of this study was to evaluate the potential of fluindapyr to cause neurotoxic effects in Crl:CD(SD) rats when administered continuously in the diet for 13 weeks. Fluindapyr (purity 98.65%; Lot no. P/13/013) was offered to groups of10 animals per sex, approximately six weeks old, males weighing 175–233 g, and females 139–179 g for females at the initiation of test diet administration. Dietary concentrations of fluindapyr were 0, 200, 600 or (males) 5000 ppm (females) 2000 ppm (equal to 0, 12, 34 and 296 mg/kg bw per day for males, 0, 13, 39 and 123 mg/kg bw per day for females). The following parameters were monitored/evaluated: mortality, clinical signs, body weight, food consumption, FOB, motor activity, plasma analysis, neuropathology. Plasma samples were taken for enantiomer analysis to determine the relative proportions of the two enantiomers (F9991 and F9992) of fluindapyr. Motor activity and FOB data were recorded for all animals pretest and then during the second, fourth, eighth and thirteenth week of test substance administration (weeks 1,3,7 and 12 respectively).

All animals survived to the scheduled necropsy (week 13), and no treatment-related clinical observations were noted at the weekly examinations. Treatment-related lower mean body weight gains and food consumption were noted in the 2000 ppm females during days 0–7 and/or 7–14, then sporadically thereafter. As a result, mean body weight gain was lower than controls by 22.8% in these females (days 0–91) and mean body weights lower than controls by 11.0% on day 91. The effects on mean body weight gains and food consumption in the 2000 ppm group females were therefore considered adverse. There were no treatment-related effects on any FOB parameter in any group, motor activity in all groups was unaffected by exposure at all time points evaluated, and no treatment-related macroscopic findings were noted in the brain or spinal cord. No treatment-related microscopic findings were observed at any exposure level.

Table 30. Summary of rat subchronic neurotoxicity study findings with exposure to fluindapyr

	Sex, dietary dose (ppm)							
	Males				Females			
Parameter	**0**	**200**	**600**	**5000**	**0**	**200**	**600**	**2000**
Mean proportion of each isomer in plasma, as percentage of total fluindapyr								
% F9991 (*R*-isomer)	-	45.9	45.5	44.2	-	43.2	35.1	37.3
Standard deviation	-	2.9	4	2.8	-	6.9	0.8	0.7
% F9992 (*S*-isomer)	-	54.1	54.5	55.8	-	56.8	64.9	62.7
Standard deviation	-	2.9	4	2.8	-	6.9	0.8	0.7
Mean body weight (g)								
Day 28	394	419	407	378	243	240	236	234
% difference from control	-	6.3%	3.3%	−4.1%	-	−1.2%	−2.9%	−3.7%
Day 56	496	535	513	474	282	275	273	259
% difference from control	-	7.9%	3.4%	−4.4%	-	−2.5%	−3.2%	−8.2%
Day 91	567	617	590	536	310	300	299	276
% difference from control	-	8.8%	4.1%	−5.5%	-	−3.2%	−3.5%	−11%
Mean body weight change (g)								
Days 0–7	55	57	58	45*	26	25	23	19*
Days 28–35	28	34	29	28	13	14	11	5*
Days 63–70	7	13	12	12	4	0	0	−2
Days 84–91	10	12	16	7	0	3	1	−5
Days 0–91	366	408	386	335	149	139	143	115**
Food consumption (g/animal per day)								
Day 0–7	22	23	21	19*	17	16	15	14*
Day 28–35	24	25	25	22	17	16	18	15
Day 63–70	22	24	23	23	15	15	15	14

	Sex, dietary dose (ppm)							
	Males				Females			
Parameter	**0**	**200**	**600**	**5000**	**0**	**200**	**600**	**2000**
Day 84–91	23	25	25	23	15	15	15	14
FOB assessments	No treatment-related effects observed.							
Motor activity	No treatment-related effects observed.							
Brain weights and dimensions	No treatment-related effects observed.							
Neuropathology macroscopic findings	No treatment-related effects observed.							
Neuropathology neurohistopathology	No treatment-related effects observed.							

Statistically significant at: * $p = 0.05$, ** $p = 0.01$; Source: Herberth 21016b

In view of the above observations, the NOAEL for repeat-dose neurotoxicity of fluindapyr was 2000 ppm (equal to 123 mg/kg bw per day), the highest dose tested. The NOAEL for systemic toxicity of fluindapyr was 600 ppm (equal to 39 mg/kg bw per day) based on reductions in body weight gain and food consumption at 2000 ppm (equal to 123 mg/kg bw per day) (Herberth, 2016b).

(b) Immunotoxicity

No immunotoxicity data had been provided. There was no evidence of immunotoxicity from standard toxicity studies

(c) Toxicity studies on metabolites

A table summarizing the various data on significant plant and livestock metabolites is shown on page 490 in the Comments section of this monograph.

Toxicological data on metabolites

Toxicity studies were conducted on the following metabolites of fluindapyr:

- 3-hydroxy-fluindapyr,
- 1-trans-COOH-fluindapyr,
- 1-cis-COOH-fluindapyr,
- 1-OH-met-fluindapyr.

3-hydroxy-fluindapyr

Metabolite 3-hydroxy-fluindapyr was not acutely toxic via the oral route with an estimated LD_{50} of greater than 2000 mg/kg bw (Slonina, 2017).

The mutagenic potential of 3-hydroxy-fluindapyr was studied in bacteria and mammalian cells in vitro in gene mutation and chromosome aberration assays. The material was negative in the in vitro bacterial and mammalian cell gene mutagenicity assays (Gijsbrechts, 2017, 2018). The cytogenetic study using human lymphocytes indicated that 3-hydroxy-fluindapyr was nonclastogenic both in the presence and absence of S9 activation (Verbaan, 2018).

1-trans-COOH-fluindapyr

Metabolite 1-trans-COOH-fluindapyr was not acutely toxic via the oral route with an estimated LD_{50} of greater than 2000 mg/kg bw (Lowe, 2017a).

The mutagenic potential of 1-trans-COOH-fluindapyr was studied in bacteria and mammalian cells in vitro in gene mutation and chromosome aberration assays. The material was negative in the in vitro bacterial and mammalian cell gene mutagenicity assays (Šoltésová, 2018a; Woods, 2018a). The cytogenetic study using human lymphocytes indicated that 1-trans-COOH-fluindapyr was not clastogenic either in the presence or absence of S9 activation (Woods, 2018b).

In a 28-day dietary toxicity study in rats there were no treatment-related adverse effects. The NOAEL was 8000 ppm (equivalent to 614 mg/kg bw per day), the highest dose level tested (Froud, 2018, 2019).

1-cis-COOH-fluindapyr

Metabolite 1-cis-COOH-fluindapyr was not acutely toxic via the oral route with an estimated LD_{50} of greater than 2000 mg/kg bw (Lowe, 2017b).

Metabolite 1-cis-COOH-fluindapyr was negative in the bacterial reverse mutation assay (Šoltésová, 2018b) and gave positive results in mammalian cell gene mutation (Šoltésová, 2018c) and in vitro cytogenetic assays (Woods, 2018c). In an in vivo erythrocyte micronucleus test conducted in male rats, 1-cis-COOH-fluindapyr gave negative results (Herring, 2018). Adequate systemic exposure was shown in a supporting study which demonstrated the presence of radiolabel in plasma and bone marrow of rats following oral administration of cis-1-carboxy-fluindapyr (Shen, 2018).

In a 28-day dietary toxicity study in rats there were no treatment-related adverse effects. The NOAEL was 8000 ppm (equivalent to 651 mg/kg bw per day), the highest dose level tested (Chase, 2018a, b).

1-OH-met-fluindapyr

Metabolite 1-OH-met-fluindapyr is not found at significant levels in rat urine, however it was detected at greater than 10% in rat bile in the 14-day bile duct cannulation study of Ghiglieri (2017). Results of this study support the systemic nature of 1-OH-met-fluindapyr in rats and on this basis the metabolite may be considered to be covered by toxicity studies conducted with the active parent substance.

Genotoxicity profiling of 1-OH-met-fluindapyr was undertaken using QSAR tools; this process did not give rise to any specific alerts for genotoxic potential. It was concluded that 1-OH-met-fluindapyr is not of toxicological concern.

3. Observations in humans

More than 1400 workers were involved in the research and development of fluindapyr, a new active ingredient. For the synthesis, manufacture and analytical processes relating to fluindapyr and its formulations, more than 250 workers were involved. With regard to the global research and development effort, for regulatory studies, over 40 contract research organizations were involved and an estimated 400 personnel may have been exposed to fluindapyr. With regard to the global in-field development, more than 700 people may have been exposed. The sponsors, FMC Corporation, had not received any reports of disease or adverse health effects attributable to exposure associated with the handling, testing or manufacture of fluindapyr and/or its formulations (FMC, 2020).

No other human health information could be found from literature available in public domain.

4. Microbiological aspects

There was no information available in the public domain and no experimental data were submitted that addressed the possible impact of fluindapyr residues on the human intestinal microbiome

Comments

Biochemical aspects

When labelled fluindapyr is administered orally to rats, radioactivity is rapidly absorbed, with a time to maximum concentration (T_{max}) of three hours. After single and repeated oral administration of 50 mg/kg body weight (bw) per day, the oral absorption was about 70–80% (Mainolfi & Garau, 2017).

After a single oral dose of 1000 mg/kg bw maximum concentration (C_{max}) values for radioactivity were approximately 3–4 times higher than after a single oral 50 mg/kg bw dose and the area under the concentration–time curve (AUC) values was approximately 8–12 times higher, confirming that the extent of systemic exposure increased less than in direct proportion to dose. There was no accumulation of radioactive material in the analyzed tissues. After repeated doses of unlabelled compound at 50 mg/kg bw followed by a single labelled dose, the highest levels of radioactivity at T_{max} were found in the liver.

The elimination of radioactivity was rapid with most of the radioactivity excreted in the first 24 hours after administration. Less than 0.2% of the radioactive dose was recovered from the carcass at 168 hours. The majority of radioactivity was excreted via bile (c 60%), with 10–20% of the administered radioactivity appearing in the urine of bile duct-cannulated animals. Higher levels of urinary excretion (c 30%) occurred in non-cannulated rats, indicating some degree of enterohepatic recirculation. The overall mean radioactivity recovered in excreta accounted for over 90% of the dose. The excretion profile was similar in male and female rats (Ghiglieri, 2016b).

Fluindapyr was extensively metabolized, primarily through *N*-demethylation, oxidation of methyl groups to hydroxymethyl and further to carboxylic acids. Unchanged fluindapyr was not excreted via urine. The main metabolite found in the urine was 1-carboxy-N-desmethyl-fluindapyr at up to 10% of administered dose (AD). Biliary excretion was lower in females than males. The main biliary metabolites were 1-carboxy-N-desmethyl-fluindapyr (11% in males, 3.9% in females) and 1-OH-methyl-fluindapyr (20% in males, 8% in females) (Mainolfi & Garau, 2017, 2018).

Toxicological data

The acute oral median lethal dose (LD_{50}) for fluindapyr was greater than 2000 mg/kg bw (Bradshaw, 2018a) and the dermal LD_{50} was greater than 2000 mg/kg bw (Bradshaw, 2018b). The inhalation median lethal concentration (LC_{50}) for fluindapyr was greater than 5.19 mg/L (Griffiths, 2013). Fluindapyr was not irritating to the skin or eyes of rabbits (Warren, 2018; Ben Abdeljelil, 2013), and was shown to be sensitizing, in a local lymph node assay (LLNA) test in mice (Dony, 2014a). Fluindapyr was not phototoxic (Westerink, 2015).

In repeat-dose toxicity studies on mice, rats and dogs, the main effects were on body weight, and liver (increased weight and hepatocellular hypertrophy), with changes in clinical chemistry parameters.

In a 90-day dietary toxicity study in mice, fluindapyr was administered at dietary concentrations of 0, 300, 1000 or 3000 ppm (equal to 0, 51, 162 and 529 mg/kg bw per day for males, 0, 81, 274 and 799 mg/kg bw per day for females). The NOAEL was 3000 ppm (equal to 529 mg/kg bw per day), the highest dose tested (Kappeler, 2016a).

In a 90-day dietary toxicity study in rats, fluindapyr was administered at dietary concentrations of 0, 100, 450 or 2000/6000 ppm (equal to 0, 6, 24 and 330 mg/kg bw per day for males, 0, 7, 30 and 139 mg/kg bw per day for females). The NOAEL was 2000/(6000) ppm (equal to 139 mg/kg bw per day), the highest dose tested (Kappeler, 2016b).

In a 90-day oral toxicity study in dogs, fluindapyr was administered in gelatine capsules at dose levels of 0, 10, 40 or 200 mg/kg bw per day. The NOAEL was 10 mg/kg bw per day based on bile duct hyperplasia at 40 mg/kg bw per day (Kappeler, 2016d).

In a one-year, oral toxicity study in dogs, fluindapyr was administered by capsule at dose levels of 0, 4, 8, 40 or 100 mg/kg bw per day for males and 0, 2, 4, 8 or 40 mg/kg bw per day for females. The NOAEL was 4 mg/kg bw per day based on hyperplasia of the bile duct at 8 mg/kg bw per day (Randazzo, 2017a).

In an 18-month carcinogenicity study in mice, fluindapyr was administered at dietary concentrations of 0, 100, 500 or 3000 ppm (equal to 0, 14, 67 and 412 mg/kg bw per day for males, 0, 18, 84 and 538 mg/kg bw per day for females). The NOAEL for toxicity was 500 ppm (equal to 67 mg/kg bw per day) based on hepatotoxicity (increased weights, hypertrophy, necrosis and higher incidences of pigmented macrophages) at 3000 ppm (equal to 412 mg/kg bw per day). The NOAEL for carcinogenicity was 3000 ppm (equal to 412 mg/kg bw per day), the highest dose tested (Randazzo, 2018).

In a 104-week combined chronic toxicity and carcinogenicity study in rats, fluindapyr was administered in the diet at concentrations of 0, 100, 400 or 1600 ppm (equal to 0, 4, 17 and 67 mg/kg bw per day for males, 0, 5, 21 and 83 mg/kg bw per day for females), and additionally 4800 ppm (equal to 202 mg/kg bw per day) for males only. The NOAEL for toxicity and carcinogenicity was 100 ppm (equal to 5 mg/kg bw per day) based on increases in mammary gland adenocarcinomas in females at 400 ppm (equal to 21 mg/kg bw per day) (Randazzo, 2017b).

The Meeting concluded that fluindapyr is carcinogenic in female rats but not in mice or male rats.

Fluindapyr was tested for genotoxicity in an adequate range of in vitro and in vivo assays (Bohnenberger, 2014; Donath, 2018a, b Dony, 2014b; Schreib, 2017; Schreib, 2018a, b; Sokolowski, 2013; Tiessen, 2018; Wollny, 2014). No evidence of genotoxicity was found.

The Meeting concluded that fluindapyr is unlikely to be genotoxic.

In view of the lack of genotoxicity and the absence of carcinogenicity in mice and male rats, and the fact that the carcinogenicity in female rats exhibits a threshold, the Meeting concluded that fluindapyr is unlikely to pose a carcinogenic risk to humans at levels occurring due to exposure from the diet.

In a two-generation reproductive toxicity study in rats, fluindapyr was administered at dietary concentrations of 0, 100, 400 or 1600/3200 ppm (equal to of 0, 4.8, 19.6, and 161 mg/kg bw per day for males, 0, 6.7, 26, and 201 mg/kg bw per day for females). The NOAEL for parental toxicity was 400 ppm (equal to 19.6 mg/kg bw per day) based on thyroid follicular cell hypertrophy at 3200 ppm (equal to 161 mg/kg bw per day). The reproductive NOAEL was 400 ppm (equal to 26 mg/kg bw per day) based on the effects on female reproductive organs associated with changes in the estrous cycle observed at 3200 ppm (equal to 201 mg/kg bw per day). The offspring NOAEL was 400 ppm (equal to 28.1 mg/kg bw per day), based on reduced body weights at 3200 ppm, (equal to 161 mg/kg bw per day) (Dettwiler, 2015a).

In a developmental toxicity study in rats, fluindapyr was administered by gavage at dose levels of 0, 60, 300 or 1000 mg/kg bw per day from gestation day (GD) 6–20. The maternal NOAEL was 300 mg/kg bw per day based on reductions in body weight and body weight gain at 1000 mg/kg bw per day. The embryo/fetal NOAEL was 1000 mg/kg bw per day, the highest dose tested), (Dettwiler, 2015b).

In a developmental toxicity study in rabbits, fluindapyr was administered by gavage at dose levels of 0, 50, 250 or 750 mg/kg bw per day from GD 6–27. The maternal NOAEL was 250 mg/kg bw per day based on decreased food consumption and body weight gain at 750 mg/kg bw per day. The embryo/fetal NOAEL was 750 mg/kg bw per day, the highest dose tested (Canut, 2015).

The Meeting concluded that fluindapyr is not teratogenic.

In an acute neurotoxicity study in rats, fluindapyr was administered by gavage at doses of 0, 125, 500 or 2000 mg/kg bw and in a follow-up at 0, 15, 30 or 60 mg/kg bw. The NOAEL for systemic toxicity was 60 mg/kg bw based on reduced motor and locomotor activity in females at 125 mg/kg bw. The NOAEL for neurotoxicity was 2000 mg/kg bw, the highest dose tested (Herberth, 2016a).

In a 90-day neurotoxicity study in rats, fluindapyr was administered at dietary concentrations of 0, 200, 600 or 5000 (males)/2000(females) ppm (equal to 0, 12, 34 and 296 mg/kg bw per day for males, 0, 13, 39 and 123 mg/kg bw per day for females). The systemic NOAEL was 600 ppm (equal to 39 mg/kg bw per day) based on reductions in body weight gain and food consumption at 2000 ppm (equal to 123 mg/kg bw per day). The NOAEL for repeat-dose neurotoxicity was 2000 ppm (equal to 123 mg/kg bw per day), the highest dose tested (Herberth, 2016b).

The Meeting concluded that fluindapyr is not neurotoxic.

No immunotoxicity data had been provided. There was no evidence of immunotoxicity from the standard toxicity studies.

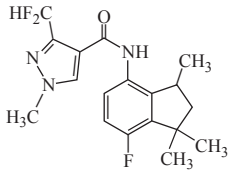
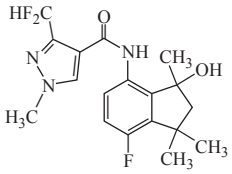
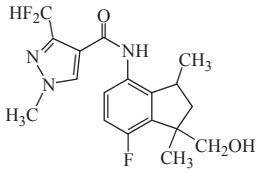
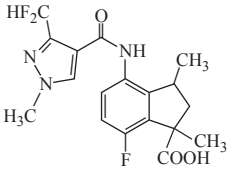
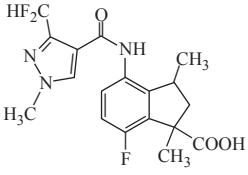
The Meeting concluded that fluindapyr is unlikely to be immunotoxic.

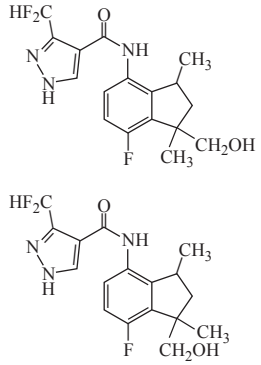
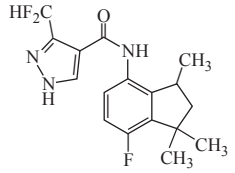
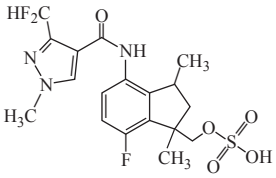
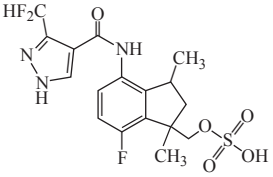
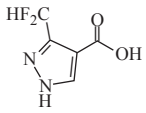
Toxicological data on metabolites and/or degradates

The plant metabolites 3-OH-fluindapyr, 1-OH-met-fluindapyr, 1-cis-COOH-flunidapyr, 1-trans-COOH-flunidapyr, 1-OH-met-N-desmethyl-fluindapyr, N-desmethyl-fluindapyr, 1-SO_4-met-fluindapyr, 1-SO_4-desmethyl-fluindapyr and N-desmethylpyrazole carboxylate were assessed.

A number of the plant and livestock metabolites listed below are important from the point of view of dietary risk assessment. The Meeting evaluated the toxicological information for these metabolites for recommending health based guidance values (HBGVs).

Summary overview of toxicological characterization of plant/livestock metabolites

Compound, codes and structure	Rat ADME (greater than 10% of AD)	Genotoxicity assessment (data, QSAR, read-across)	General toxicity	Conclusion	Refs
Fluindapyr 		Full data set Negative	Full data set		
3-OH-fluindapyr 	Minor metabolite Structure very similar to parent	Unlikely to be genotoxic (Ames, mammalian cell gene mutation and cytogenetic study using human lymphocytes)	LD_{50}: 2000 mg/kg bw	Covered by parent ADI/ARfD due to similarity of structure	
1-OH-met-fluindapyr 	Present at > 10% in bile	QSAR – no alerts for genotoxicity		Covered by parent ADI/ARfD as it is a major rat metabolite	
1-COOH-fluindapyr (cis and trans)  	Structure very similar to parent	Unlikely to be genotoxic based on data. **trans** Gene mutation in vitro (bacterial and mammalian cells); in vitro micronucleus **cis** Gene mutation in vitro (bacterial and mammalian cells); in vitro micronucleus, and in vivo micronucleus	**trans** Acute oral LD_{50}: > 2000 mg/kg bw (rat) 28-day NOAEL: 614 mg/kg bw per day (rat) **cis** Acute oral LD_{50}: > 2000 mg/kg bw (rat) 28-day NOAEL: 651mg/kg bw per day (rat)	Covered by parent ADI/ARfD due to similarity of structure	Chase, 2018a, 2018b

Compound, codes and structure	Rat ADME (greater than 10% of AD)	Genotoxicity assessment (data, QSAR, read-across)	General toxicity	Conclusion	Refs
1-OH-met-N-desmethyl-fluindapyr 	Present at > 10% in bile	No data	No data	Covered by parent ADI/ARfD as it is a major rat metabolite.	
N-desmethyl-fluindapyr 	Structure very similar to parent and 1-OH-met-N-desmethyl-fluindapyr which is a major metabolite in bile	QSAR – no alerts for genotoxicity	No data	Covered by parent ADI/ARfD due to similarity of structure to parent and a major rat metabolite	
1-SO_4-met-fluindapyr 	Not present in rat Conjugate of 1-OH-met-fluindapyr which is a major rat metabolite.	QSAR – no alerts for genotoxicity.	No data	Covered by parent ADI/ARfD as it is a conjugate of a major rat metabolite	
1-SO_4-desmethyl-fluindapyr 	Not present in rat. Conjugate of 1-OH-met-N-desmethyl fluindapyr which is a major rat metabolite	QSAR – no alerts for genotoxicity.	No data	Covered by parent ADI/ARfD as it is a conjugate of a major rat metabolite	
N-desmethyl-pyrazole carboxylate 	Not a rat metabolite	QSAR – no alerts for genotoxicity	No data	TTC – Cramer class III, 1.5 µg/kg bw per day	

Microbiological data

There was no information available in the public domain and no experimental data were submitted which addressed the possible impact of fluindapyr residues on the human intestinal microbiome.

Human data

The sponsor had not received any reports of disease or adverse health effects attributable to exposure associated with the handling, testing or the manufacture of fluindapyr and/or formulations (FMC, 2020).

No information on accidental or intentional poisoning in humans is available.

The Meeting concluded that the existing database on fluindapyr was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

Toxicological evaluation

The Meeting established an ADI of 0–0.04 mg/kg bw, based on the NOAEL of 4 mg/kg bw per day in the one-year oral toxicity study in dogs, supported by the two-year rat combined toxicity and carcinogenicity study and using a safety factor of 100. The margin between the upper bound ADI and the LOAEL for tumours in rats is 525 times.

The Meeting established an ARfD of 0.6 mg/kg bw on the basis of the NOAEL of 60 mg/kg bw, in the acute neurotoxicity study in rats and using a safety factor of 100.

Levels relevant to risk assessment of fluindapyr

Species	Study	Effect	NOAEL	LOAEL
Mouse	78-week study of toxicity and carcinogenicity [a]	Toxicity	500 ppm, equal to 67 mg/kg bw/day	3000 ppm, equal to 412 mg/kg bw/day
		Carcinogenicity	3000 ppm, equal to 412 mg/kg bw/day [c]	-
Rat	Acute neurotoxicity study [b]	Systemic toxicity Neurotoxicity	60 mg/kg bw 2000 mg/kg bw [c]	125 mg/kg bw -
	Two-year study of toxicity and carcinogenicity [a]	Toxicity	100 ppm, equal to 5 mg/kg bw/day	400 ppm, equal to 21 mg/kg bw/day
		Carcinogenicity	100 ppm, equal to 5 mg/kg bw/day	400 ppm, equal to 21 mg/kg bw/day
	Two-generation study of reproductive toxicity [a]	Reproductive toxicity	400 ppm, equal to 26 mg/kg bw/day	3200 ppm, equal to 201 mg/kg bw/day
		Parental toxicity	400 ppm, equal to 19.6 mg/kg bw/day [c]	3200 ppm, equal to 161 mg/kg bw/day
		Offspring toxicity	400 ppm, equal to 19.6 mg/kg bw/day [c]	3200 ppm, equal to 161 mg/kg bw/day [c]
	Developmental toxicity study [b]	Maternal toxicity	300 mg/kg bw/day	1000 mg/kg bw/day
		Embryo/fetal toxicity	1000 mg/kg bw/day [c]	-
Rabbit	Developmental toxicity study [b]	Maternal toxicity	250 mg/kg bw/day	750 mg/kg bw/day [d]
		Embryo/fetal toxicity	750 mg/kg bw/day [c]	-
Dog	One-year study of toxicity [e]	Toxicity	4 mg/kg bw/day	8 mg/kg bw/day

[a] Dietary administration [b] Gavage administration [c] Highest dose tested
[d] Lowest dose tested [e] Capsule administration

*Acceptable daily intake (ADI)**

0–0.04 mg/kg bw

*Acute reference dose (ARfD)**

0.6 mg/kg

**applies to fluindapyr, 3-OH-fluindapyr, 1-OH-met-fluindapyr, cis/trans 1-COOH-fluindapyr, 1-OH-met-N-desmethyl-fluindapyr, N-desmethyl-fluindapyr, 1-SO_4-met-fluindapyr and 1-SO_4-desmethyl-fluindapyr*

Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure.

Critical end-points for setting guidance values for exposure to fluindapyr

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapidly absorbed; (T_{max} 3 h), oral absorption is > 70% (rat)
Dermal absorption	No data
Distribution	Wide; highest concentrations in liver
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	Rapid and nearly complete via urine and faeces; 80% within 24 hours
Metabolism in animals	Extensively metabolised mainly through *N*-demethylation, oxidation of methyl groups to hydroxymethyl and further to carboxylic acid
Toxicologically significant compounds in animals and plants	Fluindapyr (parent); 3-OH-fluindapyr, 1-OH-met-fluindapyr, cis/trans 1-COOH-fluindapyr, N-desmethyl-fluindapyr, 1-OH-met-N-desmethyl-fluindapyr, 1-SO_4-met-fluindapyr, 1-SO_4-desmethyl-fluindapyr, N-desmethyl-pyrazole carboxylate
Acute toxicity	
Rat, LD_{50}, oral	> 2000 mg/kg bw
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.19 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Not irritating
Mouse, dermal sensitization	Sensitizing (LLNA)
Short-term studies of toxicity	
Target/critical effect	Body weight, and liver; increased weight, clinical chemistry, bile duct hypertrophy (dog)
Lowest relevant oral NOAEL	4 mg/kg bw per day (dog)
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Mammary tumours (rat) Liver (mouse)
Lowest relevant NOAEL	5 mg/kg bw per day (rat)
Carcinogenicity	Mammary tumours in female rats [a]
Genotoxicity	Unlikely to be genotoxic
Reproductive toxicity	
Target/critical effect	Lower body weight (pup and parent), thyroid toxicity, altered estrous cycling
Lowest relevant parental NOAEL	19.6 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	19.6 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	26 mg/kg bw per day (rat)
Developmental toxicity	
Target/critical effect	Reduction in body weight and body weight gain
Lowest relevant maternal NOAEL	250 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	750 mg/kg bw per day (rabbit)

Neurotoxicity	
Acute neurotoxicity NOAEL	60 mg/kg bw, (systemic toxicity, rat) 2000 mg/kg bw, the highest dose tested (neurotoxicity, rat)
Subchronic neurotoxicity NOAEL	123 mg/kg bw per day, the highest dose tested (rat)
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity	No evidence from routine studies
Studies on toxicologically relevant metabolites	
3-OH-fluindapyr	Acute oral LD_{50}: > 2000 mg/kg bw (rat) Unlikely to be genotoxic
1-trans-COOH-fluindapyr	Acute oral LD_{50}: > 2000 mg/kg bw (rat) 28-day NOAEL: 614 mg/kg bw per day (rat) Unlikely to be genotoxic
1-cis-COOH-fluindapyr	Acute oral LD_{50}: > 2000 mg/kg bw (rat) 28-day NOAEL: 651 mg/kg bw per day (rat) Unlikely to be genotoxic
Microbiological data	No data available
Human data	No clinical cases or poisoning incidents have been recorded

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

Summary

	Value	Study	Safety factor
ADI	0–0.04 mg/kg bw[a]	One-year dog study supported by a two-year rat study.	100
ARfD	0.6 mg/kg bw	Acute neurotoxicity study	100

[a] Applies to fluindapyr and 3-OH- fluindapyr, 1-OH-met-fluindapyr, cis/trans-1-COOH-fluindapyr, 1-OH-met-N-desmethyl-fluindapyr, N-desmethyl-fluindapyr, 1-SO_4-met-fluindapyr and 1-SO_4-desmethyl-fluindapyr

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Inpyrfluxam

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Explanation

Inpyrfluxam (code S-2399) is the ISO common name for the *R* enantiomer of: 3-(difluoromethyl)-1-methyl-*N*-[(*3R*)-1,1,3-trimethyl-2,3-dihydro-*1H*-inden-4-yl]-*1H*-pyrazole-4-carboxamide (IUPAC) with the Chemical Abstracts Service number 1352994-67-2. Inpyrfluxam belongs to the pyrazolecarboxamide group of fungicides. It acts as a succinate dehydrogenase inhibitor (SDHI) at complex II in the mitochondrial respiratory chain. Inpyrfluxam has not previously been evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and was reviewed by the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR).

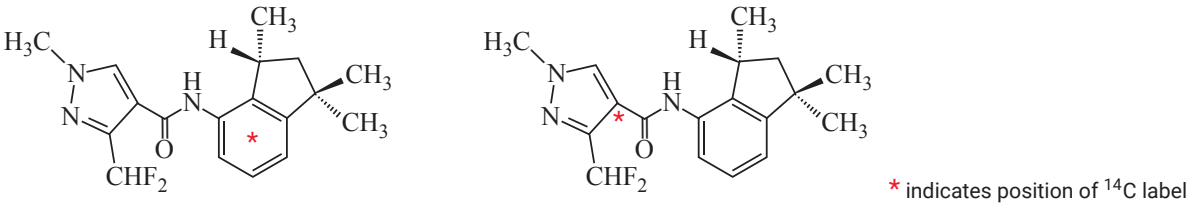
All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with relevant national or international test guidelines, unless otherwise indicated. No additional information from a literature search was identified that complemented the toxicological information submitted for the current evaluation.

Evaluation for acceptable daily intake

1. Biochemical aspects

The positions of the radiolabels used in the absorption, distribution, metabolism and excretion (ADME) studies with inpyrfluxam are shown in Fig.1 .

Figure 1. Inpyrfluxam radiolabelled with ^{14}C in the phenyl and pyrazolyl moieties



1.1 Absorption, distribution and excretion

(a) Oral route

In a study to investigate the ADME of inpyrfluxam, groups of male and female Wistar Hannover rats were gavaged once with 1 or 150 mg/kg bw of inpyrfluxam labelled with ^{14}C at the 4-position in the pyrazole group, [*pyrazolyl-4-*^{14}C]inpyrfluxam, or with 1 mg/kg bw inpyrfluxam labelled with ^{14}C uniformly distributed in the phenyl ring, [*phenyl-U-*^{14}C]inpyrfluxam. To examine excretion of the radiolabel, concentrations were measured in plasma, blood and bile (in the biliary excretion study only), ^{14}C-tissue residues, and the metabolic profiles were determined for up to seven days after administration.

Table 1. Study design for ADME study in rats using ^{14}C-labelled inpyrfluxam

Group	Label position	Study item/s	Dose (mg/kg bw)	Number of rats	
				M	F
A	Pyrazolyl	^{14}C excretion ^{14}C – tissue residue Metabolite profiling	1	4	4
B	Pyrazolyl	^{14}C concentration in blood and plasma	1	4	4
C	Pyrazolyl	^{14}C distribution Metabolite profiling	1	12	12
D (bile duct cannulation)	Pyrazolyl	^{14}C excretion Metabolite profiling	1	4	4
E	Pyrazolyl	^{14}C excretion ^{14}C – tissue residue Metabolite profiling	150	4	4
F	Pyrazolyl	^{14}C- concentration in blood and plasma	150	4	4
G	Pyrazolyl	^{14}C distribution Metabolite profiling	150	12	12
H	Phenyl	^{14}C excretion Metabolite profiling	1	4	4

M: Male; F: Female; Source: Nagahori, 2016a

At the low dose of 1 mg/kg bw with the pyrazolyl-labelled inpyrfluxam, excretion was almost complete within two days via urine and faeces (males 97.3%, females 95.6%), and complete excretion occurred within seven days: 97.2–101.9% of administered dose (AD) in males, and 97.9–101.7% of AD in females. Excretion in urine accounted for 59.7% in males, and 61.0% in females; excretion in faeces accounted for 42.2% in males, and 40.7% in females, all as percentage of AD. There was no excretion via expired air. At the high dose of 150 mg/kg bw with pyrazolyl-labelled inpyrfluxam,

extensive excretion was seen within three days of administration (89–91% AD), and total excretion within seven days of dosing was 98.9% AD (urine 49.5%; faeces 49.3%) in males, and 96.9% AD in females (urine 53.3%; faeces 43.6%). No noteworthy sex-, dose-, or radiolabel-related differences were observed in ^{14}C excretion, although a slightly larger proportion of radioactivity in urine was recorded in female rats.

At the low dose of 1 mg/kg bw, with the phenyl-labelled inpyrfluxam, excretion was almost complete after two days (males 93.6%, females 95.3% of AD) with further excretion having occurred at seven days (males 97.2%, females 97.9% of AD). Excretion in urine accounted for 49.2% AD in males, and 58.5% in females; excretion in faeces accounted for 47.9% AD in males, and 39.4% in females. There was no detectable excretion via expired air.

In a biliary excretion study, bile duct-cannulated rats (four per group) were administered 1 mg/kg bw pyrazolyl-labelled inpyrfluxam. Total excretion of radiolabel within three days was 99.2% AD in males (urine 22.9%; bile 73.6%; faeces 2.6%) and 98.1% AD in females (urine 48.4%; bile 46.9%; faeces 2.8%). Based on ^{14}C excretion into urine and bile, and radiolabel residue in the carcass (0.2% AD in males, 0.4% in females), the oral absorption rate of inpyrfluxam was 95.8% and 95.7% AD in male and female rats respectively.

At the low dose of 1 mg/kg bw, concentration of radiolabel in plasma rapidly increased to a maximum (C_{max}) of 0.161 and 0.144 µg equiv./g (ppm) at one hour after administration in male and female rats, respectively. Concentrations of radiolabel in plasma then declined with half-lives ($t_{½}$) of 13 and 12 hours respectively in males and females. The area under the plasma concentration–time curve ($AUC_{0–\infty}$) was 1.77 and 1.63 µg equiv./g·hour, respectively for males and females. At the high dose of 150 mg/kg bw, concentration of radiolabel in plasma reached a C_{max} of 8.0 and 7.2 µg equiv./g (ppm) at eight and 24 hours in male and female rats respectively. Concentrations in plasma then declined with $t_{½}$ of 14 and 17 hours in male and female rats respectively; $AUC_{0–\infty}$ was 270 and 382 µg equiv./g·hour, respectively. Mean blood : plasma radioactivity concentration ratios in males and females were ranged between 0.5–2 at both dose levels. No noteworthy sex-related difference was observed in the concentrations of radiolabel in blood or plasma, and the ratio of AUC at high dose to AUC at low dose was approximately proportional to the dose administered.

After a low dose of 1 mg/kg bw, radiolabel concentrations in organs and tissues of both male and female rats were measured at 0.25, 1, 8 and 169 hours after dosing. Levels increased rapidly, reaching their peak between 0.25 and 1 hour after dosing, except in the gastrointestinal (GI) tract. Concentrations in liver (1.74–2.02 ppm), kidney (1.04–1.12 ppm), adrenal (1.06 ppm in females), and heart (0.68 ppm in females) were relatively high compared to the plasma concentration. The elimination half-lives of radiolabel residue in organs and tissues were determined as 2–8 hours, except for the GI tract. At 168 hours after administration the total residue radiolabel in the carcass was 0.1–0.2%. At the high dose of 150 mg/kg bw radiolabel concentration peaked between one and eight hours after dosing, with relatively high levels in liver (45–54 ppm), kidney (30–49 ppm), and adrenal (30–37 ppm). The mean elimination half-lives in organs and tissues were determined to be 7–22 hours in males, and 12–48 hours in females, except in the GI tract. At 168 hours after administration the total radioactive residue was 0.1–0.2% AD. No notable sex-related difference was observed in C_{max} or in the mean elimination $t_{½}$ values at either dose. The longer half-lives at the high dose may be due to the increased time taken to complete the absorption phase (Nagahori, 2016a).

In a second study investigating the potential for bioaccumulation, groups of male and female Wistar Hannover rats were treated once daily for 14 consecutive days (days 0–13) by gavage with [*pyrazolyl-4-*^{14}C]inpyrfluxam at 1 mg/kg bw per day. Excretion of radiolabel and its concentration in plasma and blood were determined for seven days after administration of the last dose; radioactive residues and metabolic profiles were similarly recorded. Four rats/sex per group were used for these analyses.

The administered labelled inpyrfluxam was rapidly excreted in urine and faeces, with urinary excretion accounting for 33.0% of administered radioactivity (AR) in males, and 51.6% in females, and faecal excretion for 61.5% AR in males, and 44.8% in females. Total recovery of radiolabel was almost complete, accounting for 94.7% and 96.4% of the total dose in male and female rats respectively. It was noted that urinary excretion slightly more dominant in female rats.

Blood and plasma were collected before the first administration, at days 1, 2, 4, 13, and at 0.25–168 hours after the last dose. Plasma concentrations were around 0.012 µg equiv./g in males and 0.018 µg equiv./g in females for sampling on days 2 and 4, while predosing levels prior to the last dose were 0.022 µg equiv./g in males and 0.024 µg equiv./g in females. Maximum plasma concentrations of 0.198 and 0.214 µg equiv./g (ppm) were achieved at one and two hours after the last administration in male and female rats respectively. After the C_{max} was achieved, concentrations of radioactivity decreased rapidly, with mean elimination half-lives of 12 and 9 hours respectively for males and females. The plasma AUC values after the last administration were 2.04 and 2.10 µg equiv./g·hour in male and female rats respectively. Mean blood : plasma radioactivity concentration ratios for males and females were between 0.8 and 1.7.

Radiolabel tissue residues on the seventh day after the last administration (day 20) were low in both male and female rats. The highest residue level, with the exception of the GI tract, was detected in the liver (0.035–0.020 ppm). The total percentage of radioactivity distributed in tissues after 14 consecutive administrations amounted to 0.2% in males, and 0.1% in females, with no notable sex-related differences in accumulation for any of the organs or tissues examined (Nagahori, 2016b).

(b) Dermal route

No data on absorption, distribution and excretion following exposure via the dermal route was submitted.

1.2 Biotransformation

Inpyrfluxam was extensively metabolized, and 44, 44 and 43 metabolites were detected in urine, bile and faeces respectively. In addition to the parent, a total of 12 metabolites, including two conjugates, were identified and quantified. At the low dose, 1′-COOH-S-2840 and N-desMe-1′-COOH-S-2840 were the predominant metabolites in males and females, respectively. No unknown metabolite exceeded 5% of the dose. The parent compound was only detected in low amounts in faeces. There were no metabolites unique one of the labels. The identified metabolites were qualitatively similar in male and female rats at both the low and high doses, although there were some differences in quantity of metabolites between male and female rats. In biliary excretion, the glucuronides of 1′-CH_2OH-S-2840 and N-desMe-1′-CH_2OH-S-2840 were the predominant metabolites in both males and females.

The parent compound, 1′-COOH-S-2840, 1′-CH_2OH-S-2840, and N-desMe-S-2840 were detected as the major metabolites in plasma, liver and kidney. Although concentrations of inpyrfluxam and its major metabolites in liver and kidney were higher than in plasma, the profiles of metabolites were similar in tissues and plasma.

Racemization of inpyrfluxam was not observed in the course of metabolism in rats.

The main metabolic reactions of inpyrfluxam were identified as:

- *N*-demethylation,
- oxidation of the 1′,1′-dimethyl group of the indane ring, followed by further oxidation to carboxylic acid, and
- glucuronide conjugation.

As minor metabolic reactions, hydroxylation at the 3′- and 7′ positions of the indane group were also identified (Nagahori, 2016a).

In a second study to investigate the potential for bioaccumulation, groups of male and female Wistar Hannover rats were treated once daily for 14 consecutive days (days 0–13) by gavage with [*pyrazolyl-4-*^{14}C]inpyrfluxam at 1 mg/kg bw per day. The concentrations of excreted radiolabel in plasma and blood were determined for seven days after administration of the last dose; radioactive residues and metabolic profiles were similarly recorded. Four rats/sex per group were used for these analyses.

Metabolites in urine and faeces on days 0–3 and 11–14 were analysed by high-performance liquid chromatography (HPLC). In addition to the parent, a total of 12 metabolites were identified and quantified. Data on the quantity of different metabolites are presented in Table 2 below. Metabolite profiles were almost the same between days 0–3 and 11–14 in both males and females. The predominant

metabolite was 1′-COOH-S-2840 in males and N-desMe-1′-COOH-S-2840 in females. The parent inpyrfluxam was detected only in faeces, at no more than 0.3% of AD. The proposed metabolic pathway for inpyrfluxam is set out in Fig. 2.

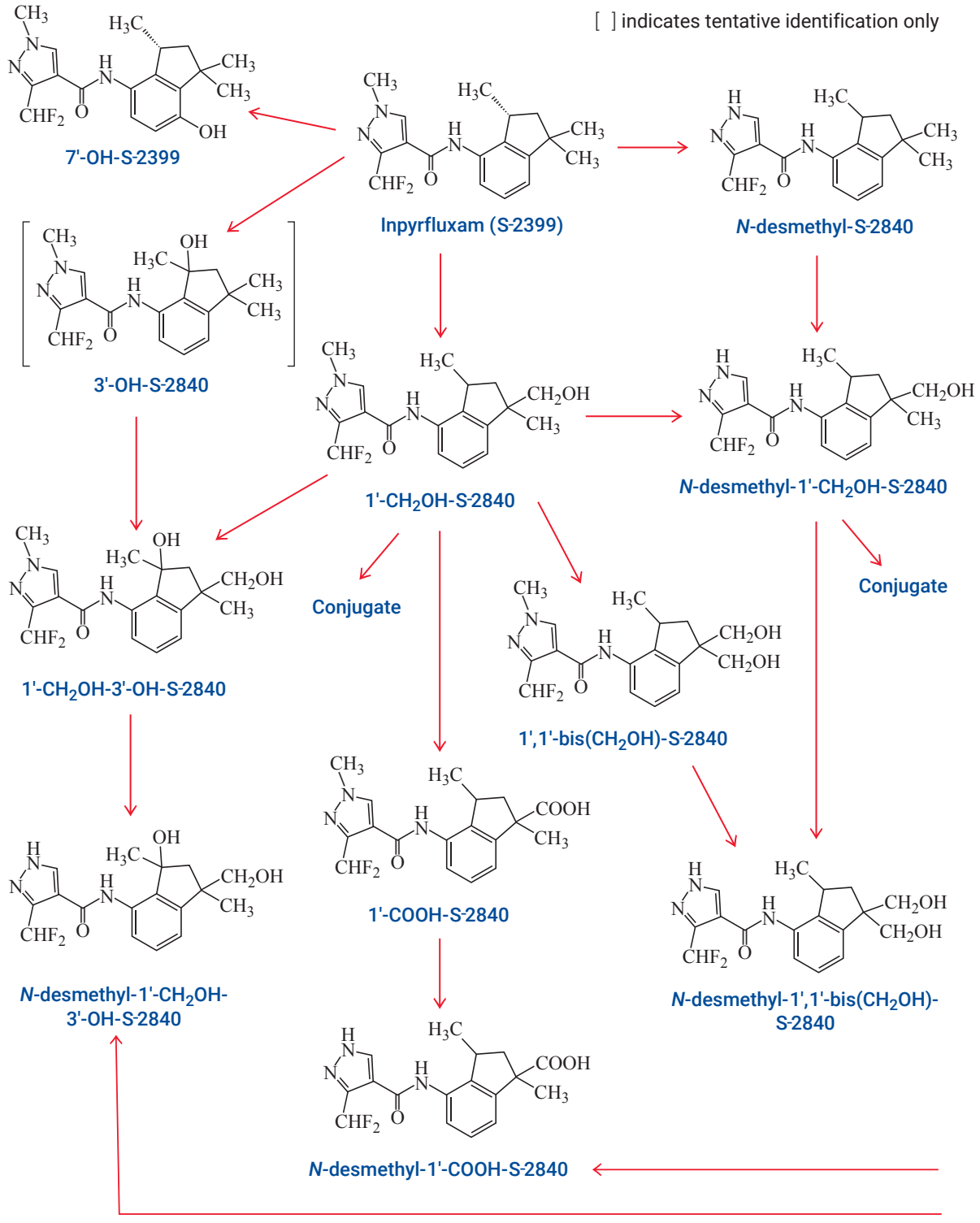
Table 2. Quantification of metabolites from pyrazolyl-labelled inpyrfluxam

Matrix	Metabolite	% of total radioactivity in pooled urine and faeces			
		Male		Female	
		Days 0–3	Days 11–14	Days 0–3	Days 11–14
Urine	N-desMe-1′1′-*bis*(CH_2OH)-S-2840	1.7	1.9	4.8	4.0
	1′1′-bis(CH_2OH)-S-2840	4.6	4.0	3.3	3.5
	Glucuronide of N-desMe-1′-(CH_2OH)-S-2840	1.5	0.5	3.6	4.3
	Glucuronide of 1′-CH_2OH-S-2840	0.8	1.1	1.1	ND
	N-desMe-1′-CH_2OH-3′-OH-S-2840	3.2	2.9	2.4	4.2
	N-desMe-1′-CH_2OH-S-2840	2.4	3.0	2.0	3.0
	1′-CH_2OH-3′-OH-S-2840				
	N-desMe-1′-COOH-S-2840	5.3	6.3	15.3	14.0
	1′-COOH-S-2840	10.4	9.2	8.3	7.5
	Others	7.5	12.5	14.8	14.2
	Total of urine	37.3	41.4		
Faeces: methanol extracts	N-desMe-1′1′-*bis*(CH_2OH)-S-2840	2.7	2.2	3.7	3.9
	1′1′-bis(CH_2OH)-S-2840	3.0	2.4	2.2	ND
	Glucuronide of N-desMe-1′-(CH_2OH)-S-2840	0.8	1.2	4.9	7.6
	Glucuronide of 1′-CH_2OH-S-2840	5.4	7.8	8.4	9.3
	N-desMe-1′-CH_2OH-3′-OH-S-2840	3.4	2.0	10.0	6.2
	N-desMe-1′-CH_2OH-S-2840	9.1	5.3		
	1′-CH2OH-3′-OH-S-2840				
	N-desMe-1′-COOH-S-2840	3.8	6.9	6.0	7.8
	1′- CH_2OH-S-2840	4.4	2.9	ND	1.0
	1′-COOH-S-2840	3.8	3.9	0.8	1.3
	7′-OH-S-2399	0	1.4		
	N-desMe-S2840	0,6	0.9	3.0	1.5
	Inpyrfluxam (parent)	0.9	1.2	0.7	ND
	Others	13.4	10.4	0	1.2
	Subtotal	53.1	48.4	39.7	39.9
Faeces: acidic methanol extracts		2.6	2.7	1.7	1.8
Faeces: basic methanol extracts		2.4	2.4	1.2	1.3
Unextractable		4.6	5.1	1.8	2.3
Faeces	**total**	**52.7**	**58.6**	**44.5**	**45.4**

ND: Not determined

Source: Nagahori, 2016a

Figure 2: Proposed metabolic pathways of inpyrfluxam (S-2399) in the rat



(Redrawn from Nagahori, 2016b)

The main metabolic reactions of inpyrfluxam were identified as:

- *N*-demethylation,
- oxidation of the 1′,1′-dimethyl group of the indane ring, followed by further oxidation to carboxylic acid, and
- glucuronide conjugation.

With regard to minor metabolic reactions, hydroxylations at the 3′- and 7′ positions of the indane group were also identified (Nagahori, 2016b).

To investigate the in vitro metabolism of inpyrfluxam in rats and humans, pyrazolyl-labelled inpyrfluxam was incubated at 10 µM for 15 minutes with liver microsomes from rats (male and female) and from humans (male and female). After incubation, the metabolites were quantified by HPLC radio-chromatography, and identified by HPLC co-chromatography and liquid chromatography–mass spectrometry (LC-MS) analysis.

Major metabolites were 1′-CH_2OH-S-2840 and N-desMe-S-2840, which were detected in both species. Metabolite 1′-CH_2OH-S-2840 was the most abundant metabolite in humans and in the male rat, accounting for 36% and 41% respectively of the total radioactivity on the radiochromatogram. Metabolite *N*-desMe-S-2840 was the most abundant metabolite in the female rat, accounting for 20%. The other four metabolites, N-desMe-1′-CH_2OH-S-2840, 3′-OH-S-2840, 7′-OH-S-2399 and 1′-CH_2OH-3’-OH-S-2840, occurred at 10% or less. Four unidentified metabolites accounted for 1–2% or more of total radioactivity on the radiochromatogram, and were detected only in male rats.

The main metabolic reaction of [*pyrazolyl-4-*^{14}C]inpyrfluxam were identified as *N*-demethylation and oxidation of the 1′,1′-dimethyl group of the indane ring. In addition, hydroxylations at the 3′ and 7′ positions of the indane ring were identified as minor metabolic reactions. The same metabolic pathways were noted in human and rat microsomal incubation, and there were no unique metabolites detected with human microsomes (Nagahori, 2017).

A second study to investigate the metabolism of inpyrfluxam in vitro was conducted in dogs. During the study pyrazolyl-labelled inpyrfluxam was incubated at 10 µM for 15 minutes with liver microsomes from male and female dogs. After incubation the metabolites were quantified by HPLC radiochromatography, and identified by LC-MS analysis. The major metabolites were 1′-CH_2OH-S-2840 and N-desMe-S-2840, which were detected at 11% or more of the total radioactivity on the radiochromatogram. Metabolite N-desMe-S-2840 was the most abundant metabolite in males and females, accounting for 19% and 17%, respectively. Metabolite 1′-CH_2OH-S-2840 was also an abundant metabolite in males and females, accounting for 16% and 11% respectively. The other two metabolites, 3′-OH-S-2840 and 7′-OH-S-2399, were present at 5% of the total radioactivity on the radiochromatogram or less. Chemical structures of the major metabolites are set out in Appendix 1.

The main metabolic reactions of [*pyrazolyl-4-*^{14}C]inpyrfluxam were identified as *N*-demethylation and oxidation of the 1′,1′-dimethyl group of the indane ring. As minor metabolic reactions, hydroxylations at the 3′ and 7′ positions of the indane ring were also demonstrated (Abe, 2018).

2. Toxicological studies

2.1 Acute toxicity

The results of acute oral, dermal and inhalation toxicity studies with inpyrfluxam, along with the results of dermal and eye irritation, skin sensitization and in vitro phototoxicity studies, are summarized in Table 3.

Table 3. Acute toxicity of inpyrfluxam

Species	Strain	Sex	Route	Purity	Result	Reference
Rat	RccHan:WIST	F	Oral	95.0%	$50 < LD_{50} < 300$ mg/kg bw	Hirano, 2015a
Rat	RccHan:WIST	F	Oral	95.0%	LD_{50} 180 mg/kg bw	Hirano, 2017a
Rat	RccHan:WIST	M+F	Dermal	95.0%	$LD_{50} > 2000$ mg/kg bw	Hirano, 2015b
Rat	RccHan:WIST	M+F	Inhalation	95.0%	$LC_{50} > 2.61$ mg/L analytically determined [a]	Hirano, 2015c
Rabbit	NZW	M	Skin irritation	95.0%	Non-irritating	Morimoto, 2015a
Rabbit	NZW	M	Eye irritation	95.0%	Minimally irritating	Morimoto, 2015b
Guinea pig	Slc:Hartley	F	Skin sensitization	95.0%	Not sensitizing	Morimoto, 2015c
Balb/3T3 cells	clone A31	NA	In vitro phototoxicity	95.0%	Not phototoxic	Nakagawa, 2016

M: Male; F: Female; LD_{50} Median lethal dose; LC_{50} Median lethal concentration; NZW: New Zealand White

[a] maximal attainable concentration;

It was concluded that inpyrfluxam has high acute oral toxicity to rats, but low acute toxicity when administered dermally or via inhalation. It is not a skin or eye irritant, nor a skin sensitizer, and is not phototoxic.

(a) Lethal doses

In an acute oral toxicity study performed according to the toxic class method, inpyrfluxam (purity 95.0%) was administered by gavage to fasted, female RccHan:WIST rats in 0.5% (w/v) aqueous methylcellulose at dose levels of 50 mg/kg bw (two groups, three rats per group), and 300 mg/kg bw (three rats), all at a dosing volume of 10 mL/kg bw. Mortality and clinical signs were recorded during the subsequent 14 days. At 300 mg/kg bw (first group), two of the three animals died within one day of administration. At 50 mg/kg bw (second and third groups), none of the six animals died. Decreased spontaneous activity was observed at both dose levels. In addition, ataxic gait was observed in all animals at 300 mg/kg bw, with prone position, lateral position, and loss of righting reflex observed at 300 mg/kg bw in the animals that subsequently died. Body weight of surviving animals was not affected by treatment. No abnormalities were detected at necropsy, neither of animals that died nor of animals that were euthanized at study termination. The acute oral median lethal dose (LD_{50}) for inpyrfluxam in female rats was greater than 50 mg/kg bw but lower than 300 mg/kg bw (Hirano, 2015a).

In another acute oral toxicity study, performed according to the up-and-down procedure, inpyrfluxam, (purity 95.0%) was administered by gavage to fasted, female RccHan:WIST rats in 0.5% (w/v) aqueous methylcellulose at a dosing volume of 10 mL/kg bw. The test substance was administered to two, three and one rats at dose levels of 57, 180 and 570 mg/kg bw respectively. All surviving animals were observed for 14 days after administration. At 57 mg/kg bw none of the animals died. At 180 mg/kg bw two of the three animals died. At 570 mg/kg bw the single animal died. Decreased spontaneous activity was observed at 180 and 570 mg/kg bw. In addition, ataxic gait was observed at 180 mg/kg bw. Body weight and body weight gain of surviving animals was not affected by treatment. Abnormalities were detected at necropsy in neither of the animals that died, nor at necropsy of animals which were euthanized at study termination. Based on the above results, the acute oral LD_{50} of inpyrfluxam was 180 mg/kg bw with a 95% confidence interval of 30.1 mg/kg bw (lower) to 735 mg/kg bw (upper) (Hirano, 2017a).

In an acute dermal toxicity study, inpyrfluxam, (purity 95.0%) was applied to the clipped skin of five male and five female RccHan:WIST rats at the limit dose of 2000 mg/kg bw, and covered with an occlusive dressing. Following the 24-hour exposure period, skin was washed and animals were observed for 14 days for mortality and clinical signs. No mortality occurred and no treatment-related clinical signs or dermal responses were observed during the study. The acute dermal LD_{50} for inpyrfluxam in male and female rats was greater than 2000 mg/kg bw (Hirano, 2015b).

In an acute inhalation toxicity study, five male and five female RccHan:WIST rats were exposed nose-only to a mist aerosol of inpyrfluxam (purity 95.0%) for four hours at the target concentration of 2 mg/L. This was the maximal attainable concentration for which the mean analytically determined concentration was 2.61 mg/L. The mean values for mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were 3.59 µm and 2.35, respectively. Mortality and clinical signs were recorded at least daily for 14 days. One female died during exposure. Clinical signs of toxicity were observed in a female on the day of exposure (day 0; ataxic gait and lateral position), and on the day following exposure (decreased spontaneous activity). These clinical signs disappeared on day 2. No macroscopic abnormalities attributable to treatment were observed at necropsy. The acute inhalation median lethal concentration (LC_{50}) for inpyrfluxam in male and female rats was 2.61 mg/L, the maximum attainable concentration (Hirano, 2015c).

(b) Dermal and ocular irritation, and dermal sensitization

In a primary skin irritation study, 0.5 g of inpyrfluxam (purity 95.0%), moistened with corn oil, was placed on a lint patch (2.5 cm × 2.5 cm) and applied to the shorn skin of three male New Zealand White rabbits. The exposure lasted four hours under semi-occlusive application. No skin irritation reactions were observed in any rabbits during the observation period of 72 hours, and there were no signs of toxicity or ill health. Inpyrfluxam was not irritating to the skin of rabbits (Morimoto, 2015a).

In a primary eye irritation study, 67 mg (the test substance occupying 0.1 mL) of inpyrfluxam, (purity 95.0%) was applied into the lower everted lid of one eye of six male New Zealand White rabbits. Three animals were used for the unwashed group, and three animals for the washed group. Treated eyes were irrigated for 30 seconds with saline, beginning 30 seconds after application. In the unwashed group, redness (grade 1) in the conjunctiva of all three rabbits was observed, and chemosis (grade 1) and discharge (grade 2) in the conjunctiva in one of three rabbits also seen after application. These reactions disappeared 48 hours after application. In the washed group, irritation reactions were not observed in any animals during the observation period of 72 hours following application. Inpyrfluxam was minimally irritating to the eyes of rabbits (Morimoto, 2015b).

The skin sensitizing potential of inpyrfluxam (purity 95.0%) was investigated using the Magnusson & Kligman maximization method. Twenty female albino Slc:Hartley Guinea pigs were tested according to the following dosing regimen: (a) induction by intradermal injection at 0.2% inpyrfluxam in corn oil and in an emulsion of Freund's complete adjuvant/distilled water; (b) induction by topical application of inpyrfluxam at 50% in propan-2-one (acetone); then (c) topical challenge with inpyrfluxam at 25% in propan-2-one. On the day before topical induction, the treatment site was pretreated with 0.5 mL of 10% sodium dodecyl sulfate to create skin irritation. Five animals were similarly treated with α-hexylcinnamaldehyde (HCA) as a positive control. Negative control groups were also included in the study, consisting of 10 and five animals respectively for the inpyrfluxam and positive controls. No skin reactions were seen in any of the inpyrfluxam TG-sensitized or inpyrfluxam TG control group animals challenged with inpyrfluxam TG at 25%. Skin reactions were seen in all of the five animals in the positive control group compared with none in the relevant controls. inpyrfluxam was not sensitizing to skin of Guinea pigs in the Magnusson & Kligman test (Morimoto, 2015c).

The phototoxic potential of inpyrfluxam (purity 95.0%) was evaluated in vitro using Balb/3T3 (clone A31) cells. This assesses cytotoxicity, expressed as a reduction in the uptake of the vital dye neutral red, in the presence and absence of exposure to a noncytotoxic dose of UVA light. A dose-finding test was conducted at 1.17, 2.34, 4.69, 9.38, 18.8, 37.5, 75 and 150 µg/mL, both with (+UV) and without (–UV) irradiation. No inhibition of cell growth greater than 50% was observed under either condition. At 150 µg/mL, with and without irradiation, precipitation was observed at the beginning of the treatment, at the beginning of irradiation and at the end of the treatment. Based on these results, the main test was performed at 0, 0.586, 1.17, 2.34, 4.69, 9.38, 18.8, 37.5 and 75 µg/mL, with and without irradiation. Cell growth was not inhibited more than 50% under any treatment conditions. The mean photo effect (MPE) was less than 0.1 (0.002). Vehicle and positive controls met acceptability criteria. It was therefore concluded that, under the conditions employed, inpyrfluxam was not phototoxic in this in vitro test system (Nakagawa, 2016).

2.2 Short-term studies of toxicity

(a) Oral administration

Mouse

In a 90-day oral toxicity study, CD1 mice (10 mice/sex per group) received inpyrfluxam (purity 95.0%) in basal diet at concentrations of 0, 200, 800, 3500 or 7000 ppm (equal to 27, 111, 491 and 973 mg/kg bw per day for males, 32, 130, 559 and 1097 mg/kg bw per day for females) over 91 consecutive days for males, and 92 days for females. All animals were observed daily for mortality and general clinical signs, and their body weights and feed consumption were recorded weekly. Haematology, blood biochemistry, organ weight measurement and necropsy on all animals were performed after 13 weeks of treatment. Histopathological examination was performed on the systemic organs of males and females receiving 7000 ppm, and on the liver, adrenals and thyroid of animals of both sexes, and of ovary of females, of all dose levels.

No mortality occurred. No treatment-related changes were observed in general clinical observations, body weight or haematology in any treated groups of either sex.

Feed consumption was decreased with statistical significance during the first week of treatment in animals of both sexes at 7000 ppm, and in females at 3500 ppm. Food consumption did not decrease at any other time point, and overall consumption throughout the study was not low.

Key effects from this study are presented in Tables 4a and b. In blood chemistry, animals of both sexes treated at 7000 ppm showed an increase in globulin and a decrease in the albumin : globulin ratio (A : G), with a decrease in albumin also recorded in females only. In addition, females also showed an increase in total cholesterol. An increase in globulin in males and decreases in albumin and A : G in females were noted at 3500 ppm. At necropsy, dark-coloured liver in 4/10 males and 9/10 females and enlargement of the liver in 3/10 males and 2/10 females were noted at 7000 ppm. Organ weight data showed increases in absolute and relative weights of the liver in animals of both sexes at 7000 ppm, and of relative liver weight in both sexes and of absolute liver weight in males only at 3500 ppm. Centrilobular hepatocellular hypertrophy was observed at histopathology in all males and diffuse hepatocellular hypertrophy in all females at 7000 ppm, and in 8/10 males or 4/10 females at 3500 ppm, suggesting an adaptive response. Centrilobular hepatocellular fatty change (confirmed by Oil Red-O stain) was also observed in 4/10 males at 7000 ppm and 3/10 males at 3500 ppm; it was thought to be related to lipid metabolism and was not considered to be adverse.

In the thyroid, histopathology revealed a statistically significant increase in the incidence of follicular cell hypertrophy in males at 7000 ppm, with a single instance observed in females. Since liver hypertrophy was observed in the present study, the thyroid lesion was considered to be possibly secondary to the effects on liver, and associated with hepatic uridine diphosphate glucuronosyltransferase (UDP-GT) induction.

Following dietary treatment for 13 consecutive weeks, inpyrfluxam had effects on the liver in both sexes treated at 3500 ppm or greater. Effects on the thyroid were observed in males with possible effects in females in the 7000 ppm group. The no-observed-adverse-effect level (NOAEL) was 3500 ppm (equal to 491 mg/kg bw per day), on the basis of follicular cell hypertrophy in the thyroid at 7000 ppm (equal to 973 mg/kg bw per day), (Shutoh, 2016a).

Table 4a. Key dose-related findings in the 90-day study in mice; clinical chemistry and organ weights

	Sex and dose level (ppm)									
Parameter	**Males**					**Females**				
[HC; n=125]	**0**	**200**	**800**	**3500**	**7000**	**0**	**200**	**800**	**3500**	**7000**
	Significant changes in clinical chemistry; mean ± SD [% of controls]									
Albumin (g/dL)	2.73 ± 0.21	2.80 ± 0.18 [103]	2.72 ± 0.15 [100]	2.72 ± 0.14 [100]	2.66 ± 0.22 [97]	3.04 ± 0.18	3.06 ± 0.16 [101]	3.01 ± 0.18 [99]	2.80 ± 0.14** [92]	2.76 ± 0.10** [91]
Globulin (g/dL)	1.86 ± 0.12	1.84 ± 0.16 [99]	1.90 ± 0.15 [102]	2.04 ± 0.15* [110]	2.09 ± 0.18** [112]	1.43 ± 0.06	1.47 ± 0.15 [103]	1.14 ± 0.11 [99]	1.57 ± 0.15 [110]	1.63 ± 0.05** [114]
A:G ratio	1.48 ± 0.10	1.53 ± 0.15 [103]	1.44 ± 0.13 [97]	1.34 ± 0.12 [91]	1.29 ± 0.15** [87]	2.13 ± 0.15	2.10 ± 0.15 [99]	2.14 ± 0.12 [100]	1.80 ± 0.19** [85]	1.70 ± 0.09** [80]
Total cholesterol (mg/dL)	111 ± 26	129 ± 30 [116]	122 ± 16 [110]	127 ± 21 [114]	135 ± 28 [122]	86 ± 15	83 ± 15 [97]	89 ± 16 [103]	93 ± 23 [108]	107 ± 19* [124]
	Significant changes in organ weight; mean ± SD [% of controls]									
Terminal body weight (g)	43.6 ± 2.6	46.1 ± 3.7 [106]	45.8 ± 4.8 [105]	42.8 ± 2.7 [98]	40.9 ± 2.4 [94]	34.1 ± 2.9	33.6 ± 3.1 [99]	34.4 ± 2.0 [101]	32.9 ± 2.4 [96]	32.7 ± 2.9 [96]
Liver weight; absolute (g)	2.31 ± 0.27	2.50 ± 0.22 [108]	2.52 ± 0.30 [109]	2.67 ± 0.19** [116]	2.89 ± 0.25** [125]	1.82 ± 0.20	1.76 ± 0.25 [97]	1.83 ± 0.17 [101]	1.95 ± 0.28 [107]	2.26 ± 0.35** [124]
Liver weight; (% relative to body weight)	5.29 ± 0.44	5.42 ± 0.26 [102]	5.51 ± 0.31 [104]	6.24 ± 0.25** [118]	7.06 ± 0.44** [133]	5.34 ± 0.41	5.23 ± 0.35 [98]	5.31 ± 0.40 [99]	5.91 ± 0.48* [111]	6.90 ± 0.49** [129]

Statistically significant usingDunnett's (or Dunnett-type) test: * $p \leq 0.05$; **: $p \leq 0.01$ Source: Shutoh, 2016a

Table 4b. Key dose-related findings of the 90-day feeding study in mice – histopathology

	Sex and dose level (ppm)									
	Males					Females				
Organ and lesion	**0**	**200**	**800**	**3500**	**7000**	**0**	**200**	**800**	**3500**	**7000**
Liver – Number examined	10	10	10	10	10	10	10	10	10	10
Centrilobular hepatocyte fatty change	0	0	0	3	4	0	0	0	0	0
Centrilobular hepatocyte hypertrophy	0	0	0	8**	10**	0	0	0	0	0
Diffuse hepatocyte hypertrophy	0	0	0	0	0	0	0	0	4	10**
Thyroid – Number examined	10	10	10	10	10	10	10	10	10	10
Follicular cell hypertrophy	0	0	1	0	5*	0	0	0	0	1

Statistically significant usingDunnett's (orDunnett-type) test: * $p \leq 0.05$; **:$p \leq 0.01$ Source: Shutoh, 2016a

Rat

In a non-GLP dose range-finding 28-day toxicity study, Wistar Hannover rats (six rats/sex per group) received inpyrfluxam (purity 99.2%) in basal diet for one month at concentrations of 0, 500, 1000, 3000 or 5000 ppm (equal to 44, 86, 246 and 406 mg/kg bw per day for males, 47, 91, 263 and 378 mg/kg bw per day for females). Clinical observations, measurements of motor activity, body weight, feed consumption, urinalysis, ophthalmology, haematology, blood biochemistry, organ weight, necropsy and histopathology were performed.

There were no deaths or treatment-related clinical signs. No treatment-related changes were observed at ophthalmology nor at motor activity assessment. In high-dose males a statistically significant increase in red blood cell (RBC) count and decrease in mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) were observed.

Body weight gain during the first nine days of treatment was statistically significantly less in both sexes fed a diet with 3000 ppm or more of inpyrfluxam, and this resulted in lower body weights. Body weight gain was also reduced in males at 3000 ppm and above, and in females at 3000 ppm on day 26. Apart from these time periods, however, body weight showed no statistically significant differences from the control values. Throughout the study, however, total body weight gain remained lower; at least 24% (not statistically significant) in males, and at least 26% in females at 3000 ppm and above. This resulted in statistically significant decreases in body weight at 3000 ppm and above in both sexes (10% in males and 7% in females at 3000 ppm, and 20% in males and 13% in females at 5000 ppm). Decreases in food consumption were seen only during the early stage of administration. Additionally, feed efficiency was lower in males and females from the 5000 ppm group.

Slight increases in erythrocyte count, and slight decreases in MCV and MCH were seen in males at the highest dose. Blood biochemistry changes are presented in Table 5 below, and revealed increases in total cholesterol, phospholipid and γ-glutamyl transpeptidase (GGTP) in both sexes at 3000 and 5000 ppm. An increase in albumin and calcium as well as decreases in glucose and chloride in males, and increases in triglycerides and alkaline phosphatase (ALP) in females were observed at 5000 ppm. Glucose was also decreased in males at 3000 and 1000 ppm, while increases in triglycerides and ALP in females were also seen at 3000 ppm. At urinalysis lower pH was recorded for males at 5000 ppm.

At necropsy, males and females at 5000 ppm showed enlarged and dark-coloured livers. Organ weight data indicated increases in liver weights in both sexes at 3000 and 5000 ppm, decreases in kidney (5000 ppm only) and thymus weights in males, and decreases in ovary and uterus weights (5000 ppm only) in females at 3000 and 5000 ppm. Histopathology revealed diffuse hepatocyte hypertrophy in the livers of males and females at 3000 and 5000 ppm; smooth endoplasmic reticulum proliferation in hepatocytes of the livers of animals treated at 5000 ppm was observed through electron microscopic examination. Follicular cell hypertrophy in the thyroid was observed in 4/6 males at 3000 ppm, and in 6/6 males and 5/6 females at 5000 ppm. Fine vacuolation of cortical cells in the zona fasciculata or glomerulosa of the adrenal was noted in 4/6 males at 1000 ppm and 5/6 males at 3000 ppm, and all males and 5/6females at 5000 ppm. In addition, males at 5000 ppm displayed hyaline droplets in the proximal tubules of the kidney that produced a positive reaction for α2u-globulin by immunohistochemical

staining, and atrophy in the thymus. Fatty infiltration in the bone marrow was observed in females at 3000 ppm, and in males and females at 5000 ppm. Females at 3000 and 5000 ppm showed vacuolation in interstitial gland of the ovary and atrophy in the uterus.

Following dietary treatment for one month, adverse effects of inpyrfluxam were observed in body weight at 3000 ppm and above in males. Effects on the adrenals were seen in males at and above 1000 ppm and in females at and above 5000 ppm. While effects were seen in the liver, these were considered adaptive and were not considered adverse. Additionally, effects on the reproductive organs were observed in females at 3000 and 5000 ppm. The NOAEL was 500 ppm (equal to 44 mg/kg bw per day) on the basis of decreased plasma glucose levels and fine vacuolation in cortical cells in the adrenal in males at 1000 ppm (equal to 86 mg/kg bw per day), (Hirano, 2014).

Table 5a. Key findings of the 28-day dietary toxicity study in rats

	Sex and dose level (ppm)									
	Males					Females				
Parameter	**0**	**500**	**1000**	**3000**	**5000**	**0**	**500**	**1000**	**3000**	**5000**
	Significant changes at clinical chemistry (mean ± SD)									
Albumin (g/dL)	2.2 ± 0.15	2.3 ± 0.05	2.3 ± 0.05	2.3 ± 0.08	2.5 ± 0.08**	2.4 ± 0.10	2.5 ± 0.12	2.4 ± 0.16	2.4 ± 0.10	2.4 ± 0.10
Glucose (mg/dL)	154 ± 12.5	141 ± 12.5	129 ± 13.1**	134 ± 9.0*	132 ± 16.0*	121 ± 7.0	122 ± 16.5	113 ± 5.0	115 ± 8.6	120 ± 16.2
Total cholesterol (mg/dL)	56 ± 10.6	62 ± 5.7	68 ± 11.7	84 ± 11.2*	114 ± 26.0*	51 ± 8.6	52 ± 6.2	56 ± 12.7	102 ± 17.7*	110 ± 26.9*
PL (mg/dL)	111 ± 16.1	119 ± 10.9	124 ± 21.4	145 ± 21.1*	185 ± 33.8**	106 ± 13.1	114 ± 7.9	116 ± 26.4	181 ± 23.8*	188 ± 38.0*
Trigycerides (mg/dL)	64 ± 29.9	85 ± 29.6	71 ± 45.2	88 ± 28.0	86 ± 31.1	17 ± 6.0	27 ± 10.6	29 ± 19.1	54 ± 16.1**	72 ± 16.7**
Alkaline phosphatase (U/L)	428 ± 120.0	391 ± 59.1	371 ± 41.3	426 ± 58.2	434 ± 114.0	196 ± 75.7	175 ± 31.6	248 ± 76.8	294 ± 60.4*	270 ± 64.5#
GGTP (U/L)	0 ± 0.4	1 ± 0.5	1 ± 1.2	3 ± 1.0*	6 ± 1.5**	1 ± 0.5	1 ± 0.5	1 ± 1.0	4 ± 1.9*	6 ± 1.8*
Calcium (mg/dL)	10.0 ± 0.13	10.0 ± 0.18	10.2 ± 0.26	10.2 ± 0.15	10.4 ± 0.13**	9.4 ± 1.06	9.9 ± 0.14	9.9 ± 0.23	10.1 ± 0.26	10.1 ± 0.27
Chloride (m equiv./L)	110 ± 1.3	109 ± 1.0	110 ± 1.6	110 ± 1.6	107 ± 1.8**	112 ± 1.2	113 ± 2.1	112 ± 1.3	111 ± 2.4	110 ± 1.4

GGTP: γ-glutamyl transpeptidase; Source: Hirano, 2014

Statistically significant usingDunnett's test: * $p \leq 0.05$; **: $p \leq 0.01$

Table 5b. Key findings of the 28-day dietary toxicity study in rats

	Sex and dose level (ppm)									
	Males					Females				
	0	**500**	**1000**	**3000**	**5000**	**0**	**500**	**1000**	**3000**	**5000**
Parameter	**Significant changes in organ weight (mean ± SD)**									
Terminal body weight (g)	280 ± 13.2	267 ± 18.0	268 ± 18.8	250 ± 16.5*	222 ± 10.8**	168 ± 2.6	168 ± 5.4	164 ± 5.1	154 ± 10.1 **	145 ± 7.5**
Liver weight; absolute (g)	8.41 ± 0.352	8.24 ± 0.934	7.89 ± 0.924	8.75 ± 0.795	9.06 ± 0.609	4.76 ± 0.272	4.99 ± 0.231	4.89 ± 0.259	5.46 ± 0.584**	5.68 ± 0.380**
Liver weight; relative (%)	3.01 ± 0.062	3.08 ± 0.184	2.95 ± 0.241	3.50 ± 0.252**	4.09 ± 0.129**	2.83 ± 0.174	2.97 ± 0.103	2.98 ± 0.131	3.54 ± 0.230**	3.93 ± 0.114**

	Sex and dose level (ppm)									
	Males					Females				
	0	500	1000	3000	5000	0	500	1000	3000	5000
Kidney weight; absolute (g)	2.14 ± 0.312	2.00 ± 0.189	1.93 ± 0.127	1.92 ± 0.141	1.71 ± 0.094**	1.35 ± 0.062	1.36 ± 0.103	1.27 ± 0.067	1.19 ± 0.060**	1.16 ± 0.078**
Kidneys weight; relative (%)	0.77 ± 0.091	0.75 ± 0.052	0.73 ± 0.041	0.77 ± 0.031	0.77 ± 0.030	0.80 ± 0.034	0.81 ± 0.052	0.77 ± 0.050	0.77 ± 0.041	0.80 ± 0.046
Thymus weight; absolute (g)	0.57 ± 0.060	0.48 ± 0.115	0.49 ± 0.091	0.38 ± 0.066**	0.33 ± 0.049**	0.37 ± 0.072	0.38 ± 0.021	0.36 ± 0.053	0.34 ± 0.031	0.32 ± 0.046
Thymus weight; relative (%)	0.20 ± 0.017	0.18 ± 0.035	0.18 ± 0.027	0.15 ± 0.021**	0.15 ± 0.022**	0.22 ± 0.044	0.23 ± 0.014	0.22 ± 0.027	0.22 ± 0.023	0.22 ± 0.029
Ovary weight; absolute (mg)	-	-	-	-	-	73 ± 7.4	78 ± 8.0	66 ± 10.0	59 ± 8.0*	58 ± 11.0*
Ovary weight; relative (%)	-	-	-	-	-	43.2 ± 3.82	46.2 ± 3.58	40.2 ± 5.36	38.5 ± 5.76	40.1 ± 8.40
Uterus weight; absolute (g)	-	-	-	-	-	0.40 ± 0.108	0.44 ± 0.154	0.42 ± 0.183	0.33 ± 0.154	0.27 ± 0.098
Uterus weight; relative (%)	-	-	-	-	-	0.24 ± 0.064	0.27 ± 0.097	0.26 ± 0.107	0.21 ± 0.091	0.18 ± 0.073
Parameter	**Histopathological effects**									
Thyroid: *N*	6	6	6	6	6	6	6	6	6	6
Follicular cells hypertrophy;										
total	1	2	2	4	6**	1	1	1	3	5*
slight	1	2	2	3	4	1	1	1	3	4
mild	0	0	0	1	2	0	0	0	0	1
Adrenal: *N*	6	6	6	6	6	6	6	6	6	6
Fine vacuolation, zona fasciculata										
slight	0	0	0	2	5**	0	0	0	1	4*
Fine vacuolation, zona glomerulosa										
total	1	1	4	5*	6**	-	-	-	-	-
mild	0	0	1	2	3	-	-	-	-	-
slight	1	1	3	3	3	-	-	-	-	-

Relative weights: Relative to body weight, expressed as percentage; *N*: Number of individuals; Source: Hirano, 2014
Statistically significant using Dunnett's test: * $p \leq 0.05$; **: $p \leq 0.01$

In a 90-day oral toxicity study, Wistar Hannover rats (10 rats/sex per group) received inpyrfluxam (purity 95.0%) in basal diet over 92 consecutive days at concentrations of 0, 150, 500, 2000 or 4000 ppm (mean substance intakes were 0, 9.72, 31.7, 123 and 255 mg/kg bw per day for males, 0, 11.5, 37.5, 144 and 292 mg/kg bw per day for females). All surviving animals were observed daily for mortality and general clinical signs, and their body weights and feed consumption recorded weekly. Detailed clinical observations were performed once prior to initiation of treatment and once a week during the treatment period. Functional observations were carried out on all surviving animals at 11 weeks of treatment.

Ophthalmological examinations were performed on all animals before initiation of treatment and in animals of the control and high-dose groups at week 13 of treatment. Haematology, clinical chemistry and urinalysis evaluations were performed on all animals at termination. Gross necropsy and histopathology were conducted on all animals, and absolute and relative organ weights determined.

No treatment-related mortality occurred. One high-dose female was terminated in extremis in week 9. Histopathological examination of this individual revealed malignant lymphoma which was not considered related to treatment. No treatment-related changes were observed in any treated groups of

either sex from general or detailed clinical observations, nor from ophthalmology.

A statistically significant decrease in mean body weight was continuously observed in males at 4000 ppm and females at 2000 ppm or greater. In addition, statistically significant decreases in body weight gain were observed in these same groups, accompanied by treatment-related decreases in food consumption. Males and females at 4000 ppm showed markedly lower feed efficiency at week 1 and slightly lower feed efficiency for several weeks, with an average feed efficiency during the treatment period of 88% and 69% of controls in males and females respectively. Additionally, females treated at 4000 ppm showed a statistically significant decrease in forelimb grip strength in functional observation, and there were decreases in open field rearing scores in females at 2000 ppm and above. The change was considered to be treatment-related because it was observed in the high-dose group, but it was also possibly related to the significant decrease in body weight recorded in females of this group.

Females at 4000 ppm showed a statistically significant prolongation in prothrombin time and statistically significant decreases in MCV and MCH. No significant changes were seen in any other groups. The changes in total leucocyte count, lymphocyte and neutrophil counts seen in males of the 500 ppm group were considered incidental to treatment.

Both males and females showed a statistically significant increase in GGTP. Males at 4000 ppm showed a statistically significant increase in inorganic phosphorus and females showed a statistically significant decrease in glucose and total bilirubin. A increase in potassium levels, not statistically significant, was seen in females. At 2000 ppm both sexes showed a statistically significant increase in GGTP; females at this dose showed increases in ALP and potassium, and decreases in total bilirubin. The change in glucose level was considered to reflect malnutrition, a conclusion suggested by the changes in body weight and food consumption. Decreases in total bilirubin levels were not considered to be of toxicological significance. Urinalysis revealed a low pH, statistically significant in rats of both sexes treated at 4000 ppm.

Several organs showed treatment-related changes, seen from blood biochemistry, necropsy, organ weight, and/or histopathology.

In the liver, GGTP and ALP levels were increased (attaining statistical significance in some cases) in males and/or females at 2000 ppm or more. At necropsy, females at 4000 ppm showed a statistically significant increase in the incidence of darkening in colour, as did three males at 4000 ppm. At 2000 ppm or more, absolute and/or relative liver weights were increased (statistically significant) in rats of both sexes, and increased incidences of diffuse hepatocellular hypertrophy were noted at histopathology. In addition, haematology investigation revealed females at 4000 ppm to have a statistically significant prolongation in prothrombin time, which was also considered possibly related to the observed liver effects. An increase in relative liver weight was seen at 500 ppm, without any associated clinical chemical or histopathological changes. This was therefore not considered to be of toxicological significance, and was regarded as an adaptive change.

In the thyroid, a statistically significant increase in the incidence of follicular cell hypertrophy was observed in females at 4000 ppm and a non-statistically significant increase in females at 2000 ppm. Since liver hypertrophy was observed in the present study, it is possible that the lesion was associated with hepatic enzyme (UDP-GT) induction and therefore secondary to the effects on liver.

In the kidney, statistically significant increases in relative weight and in the incidence of increased deposition of hyaline droplets in the proximal tubular cells in males were observed at 2000 ppm and/or 4000 ppm. The hyaline droplets were shown by immunohistochemistry to contain α2μ-globulin, so the changes, including increased kidney weight, were considered to be unique to male rats.

In the adrenal, males at 4000 ppm and females at 2000 ppm and above showed increased cortical cell vacuolation, with the incidence of the lesion attaining statistical significance in females at 4000 ppm only.

In the ovary, statistically significant increases in relative weight at 4000 ppm and in the incidence of interstitial gland vacuolation at 2000 ppm or more were observed.

Other statistically significant changes in organ weight (brain, heart, spleen, testes, and epididymides at 2000 and/or 4000 ppm) were considered secondary to the significantly decreased final

body because without any related histopathological changes.

Following dietary treatment for 13 consecutive weeks, effects of inpyrfluxam were mainly observed in overall body weight, and on weights of liver, ovary or adrenal. The NOAEL was 500 ppm (31.7 mg/kg bw per day) based on increased cortical cell vacuolation in the adrenal and interstitial gland vacuolation in the ovaries of females at 2000 ppm In addition, at 2000 ppm increased relative kidney weights, increased deposition of hyaline droplets in the proximal tubule cells were observed in males, however as this is a specific effect in male rats it was not considered of human relevance (Ohtsuka, 2016).

Table 6a. Key findings of the 90-day dietary toxicity study in rats; organ weights

	Dose (ppm)									
	Males					Females				
Parameter	**0**	**150**	**500**	**2000**	**4000**	**0**	**150**	**500**	**2000**	**4000**
	Significant changes in organ weight; mean ± SD [% of controls]									
Terminal body weight (g)	404 ± 35	407 ± 15 [101]	394 ± 27 [98]	384 ± 32 [95]	336 ± 17** [83]	234 ± 11	239 ± 11 [102]	224 ± 15 [96]	211 ± 15** [90]	193 ± 12** [82]
Brain weight relative (% of bw)	0.49 ± 0.04	0.50 ± 0.02 [102]	0.50 ± 0.03 [102]	0.52 ± 0.04 [106]	0.58 ± 0.02* [118]	0.77 ± 0.03	0.78 ± 0.04 [101]	0.82 ± 0.05 [106]	0.86 ± 0.05** [112]	0.94 ± 0.05** [122]
Heart weight absolute (mg)	1014 ± 90	1095 ± 95 [108]	1050 ± 90 [104]	1018 ± 80 [100]	951 ± 61 [94]	712 ± 35	723 ± 52 [102]	673 ± 54 [95]	678 ± 53 [95]	626 ± 32** [88]
Heart weight relative (% of bw)	0.25 ± 0.02	0.27 ± 0.02 [108]	0.27 ± 0.01 [108]	0.27 ± 0.03 [108]	0.28 ± 0.02** [112]	0.30 ± 0.02	0.30 ± 0.02 [100]	0.30 ± 0.02 [100]	0.32 ± 0.02 [107]	0.33 ± 0.02 [110]
Liver weight absolute (g)	9.79 ± 1.21	10.28 ± 0.42 [105]	10.26 ± 0.83 [105]	10.30 ± 1.12 [105]	10.09 ± 0.68 [103]	5.89 ± 0.51	6.16 ± 0.52 [105]	5.80 ± 0.50 [98]	6.28 ± 0.46 [107]	6.57 ± 0.47* [112]
Liver weight relative (% of bw)	2.42 ± 0.14	2.53 ± 0.16 [105]	2.60 ± 0.12* [107]	2.69 ± 0.19** [111]	3.00 ± 0.11** [124]	2.51 ± 0.21	2.58 ± 0.16 [103]	2.59 ± 0.07 [103]	2.98 ± 0.18** [119]	3.42 ± 0.28** [136]
Kidneys weight absolute (mg)	2286 ± 194	2463 ± 144 [108]	2397 ± 181 [105]	2280 ± 192 [100]	2175 ± 145 [95]	1537 ± 95	1706 ± 224 [111]	1565 ± 181 [102]	1446 ± 149 [94]	1332 ± 54* [87]
Kidneys weight relative (% of bw)	0.57 ± 0.02	0.61 ± 0.05 [107]	0.61 ± 0.05 [107]	0.60 ± 0.60 [105]	0.65 ± 0.03** [114]	0.66 ± 0.04	0.71 ± 0.08 [108]	0.70 ± 0.06 [106]	0.68 ± 0.06 [103]	0.69 ± 0.03 [105]
Spleen weight absolute (mg)	592 ± 75	611 ± 72 [103]	621 ± 92 [105]	594 ± 70 [100]	506 ± 20* [85]	444 ± 56	483 ± 57 [109]	429 ± 60 [97]	434 ± 48 [98]	377 ± 55* [85]
Adrenals weight absolute (mg)	70.7 ± 8.3	74.7 ± 6.4 [106]	67.4 ± 8.5 [95]	64.6 ± 7.2 [91]	62.8 ± 5.6 [89]	75.7 ± 7.6	75.9 ± 8.5 [100]	78.9 ± 10.1 [104]	68.6 ± 6.6 [91]	65.1 ± 6.2* [86]
Testes weight relative (% of bw)	0.86 ± 0.08	0.87 ± 0.06 [101]	0.88 ± 0.05 [102]	0.88 ± 0.07 [102]	1.02 ± 0.02** [119]	-	-	-	-	-
Epididymides weight relative (% of bw)	0.30 ± 0.03	0.31 ± 0.02 [103]	0.31 ± 0.04 [103]	0.32 ± 0.04 [107]	00.35± 0.02** [117]	-	-	-	-	-

	Dose (ppm)									
	Males					Females				
Parameter	**0**	**150**	**500**	**2000**	**4000**	**0**	**150**	**500**	**2000**	**4000**
Ovaries weight relative (% of bw)	-	-	-	-	-	0.044 ± 0.010	0.044 ± 0.007 [100]	0.047 ± 0.008 [107]	0.051 ± 0.009 [116]	0.056 ± 0.006* [127]

Statistically significant using Dunnett's test: * $p \leq 0.05$; **: $p \leq 0.01$; Source: Ohtsuka, 2016

Table 6b. Key findings of the 90-day dietary toxicity study in rats; histopathology

	Males					Females				
Dose (ppm)	**0**	**150**	**500**	**2000**	**4000**	**0**	**150**	**500**	**2000**	**4000**
	Summary of histopathology (all animals)									
Liver; number examined	10	10	10	10	10	10	10	10	10	10
Diffuse hepatocyte hypertrophy	0	0	0	4	10**	0	0	0	7**	9**
Kidney; number examined	10	10	10	10	10	10	10	10	10	10
Increased deposition of hyaline droplets in proximal tubular cells	0	0	0	2	8**	0	0	0	0	0
Thyroid; number examined	10	10	10	10	10	10	10	10	10	10
Follicular cells hypertrophy	1	2	2	2	2	0	1	0	4	7**
Adrenal; number examined	10	10	10	10	10	10	10	10	10	10
Increased cortical cell vacuolation	1	0	0	1	5	0	0	0	3	5*
Ovary; number examined	-	-	-	-	-	10	10	10	10	10
Interstitial gland vacuolation	-	-	-	-	-	1	2	2	7*	8**

Statistically significant using Fisher's exact probability test: * $p \leq 0.05$; **: $p \leq 0.01$; Source: Ohtsuka, 2016

Dog

In a 90-day oral toxicity study, beagle dogs (four dogs/sex per group) received inpyrfluxam (purity 95.0%) in gelatin capsules at 0, 40, 160 or 700/500 mg/kg bw per day. During the treatment period males and females in the high-dose group showed considerable decreases in body weight and food consumption in addition to vomiting of food, until week 2. Therefore, the treatment was temporarily discontinued at week 3 for animal welfare reasons, and the high dose was decreased from 700 to 500 mg/kg bw per day at week 4. The high dose level therefore shown from here on as 700/500 mg/kg bw per day. The treatment period, including withdrawal period, was 16 weeks for the 700/500 mg/kg bw per day group. During the acclimatization and treatment periods, general clinical observation, mortality check, and food consumption measurement were conducted daily for all surviving animals. Detailed clinical observations and body weight measurements were performed once prior to initiation of treatment and once a week during the treatment period. Animals were also subject to ophthalmology, urinalysis, haematology, and blood biochemistry assessments once prior to initiation of treatment. During the treatment period, ophthalmology was conducted at week 13 (other than high-dose group) or 16 (high-dose group), and urinalysis, haematology, and blood biochemistry were conducted at weeks 4, 8 and 13, and for the high-dose group only at week 16. In addition, unscheduled haematological and blood biochemical examinations were performed on animals in the high-dose group on days 15 and 20 (males) and days 12 and 20 (females) to examine the animals' condition. Haematology and blood biochemistry were also conducted on dogs killed in extremis before euthanasia. All surviving animals after 13 (other than high dose group) or 16 weeks (high dose group) of treatment, and dogs killed in extremis during treatment period, were euthanized and subjected to necropsy, organ weight measurement, and histopathology.

Mortalities occurred at 700/500 mg/kg bw per day, where one male and two females were killed in extremis at weeks 8 or 9 of treatment. These three animals showed various changes reflecting liver

injury, polyarteritis, and/or decreased body weight and food consumption, all of which were suspected to be the cause of debility. Additionally, specific effects on kidneys, spleen, stomach, duodenum, and thymus, and some changes in haematology or blood biochemistry were observed in these animals.

In general clinical observations, vomiting of food was noted in males and females at 700/500 and 160 mg/kg bw per day, especially at week 1 of treatment. Vomiting of food was also observed at week 4 of treatment, when top-dose animals initially received 500 mg/kg bw per day after withdrawal. At 700/500 mg/kg bw per day, males and females displayed staggering gait and convulsions. Additionally, one male showed torticollis and ananastasia.

Males and females treated at 700/500 mg/kg bw per day showed low body weight and food consumption during both the 700 and 500 mg/kg bw per day treatment periods. One male at 160 mg/kg bw per day also showed a decrease in body weight at week 1 and decreases in weekly and average food consumption throughout the treatment period.

Urinalysis revealed a low urine pH in males at 700/500 mg/kg bw per day. At haematology, a decrease in reticulocyte counts in both sexes at 700/500 mg/kg bw per day and in males at 160 mg/kg bw per day, and prolongation of prothrombin time in males at 700/500 and 160 mg/kg bw per day were noted, with a trend towards prolongation in males at 40 mg/kg bw per day, dependent on results in one male. One female at 700/500 mg/kg bw per day showed relatively high haematocrit, haemoglobin concentration, and erythrocyte count. Blood biochemistry data showed increases in ALP, aspartate transaminase (AST), alanine aminotransferase (ALT), and GGTP, and decreases in total protein, albumin, A : G ratio, glucose, total cholesterol, and calcium in both males and females at 700/500 mg/kg bw per day, and increases in ALP, ALT, and GGTP, and decreases in total protein, albumin, total cholesterol, and calcium in both sexes at 160 mg/kg bw per day. One male at 160 mg/kg bw per day showed an increase in AST, and decreases in A : G ratio and glucose were also recorded in females and males at 160 mg/kg bw per day, respectively. An increasing trend in ALP in both sexes was seen at 40 mg/kg bw per day, with males also displaying an increasing trend in ALT and GGTP at all examination points.

At necropsy, dark-coloured and enlarged livers were observed in males and females at 700/500 and 160 mg/kg bw per day. At 700/500 mg/kg bw per day calculi was observed in males and tar-like bile in the gallbladders of females; females also displayed a coarse surface, darkening, and enlargement of the spleen. One male at 160 mg/kg bw per day showed biliary sludge in the gallbladder. Organ weight data showed an increase in the liver weight of dogs of both sexes, and a decrease in prostate weight in males at 700/500 and 160 mg/kg bw per day. Increased spleen weight was recorded for one female 700/500 mg/kg bw per day. Other changes in heart weight were considered secondary to the low body weight. In addition, significant increases in absolute and relative liver weights in males at 40 mg/kg bw per day were seen.

Histopathological examination of dogs treated at 700/500 mg/kg bw per day revealed diffuse hepatocellular hypertrophy, hepatocellular cytoplasmic eosinophilic inclusion bodies, and deposition of brown pigment (determined to be haemosiderin) in the Kupffer cells in the liver, calculi in the gallbladder, congestion in the spleen, proximal tubular cell vacuolation in the kidney (2/4 males, 1/4 females), zona fasciculata cell vacuolation in the adrenals (2/4 males, 2/4 females), and degeneration of the optic nerve fibre in males and females. Males showed hypertrophy and cytoplasmic eosinophilic inclusion bodies in the proximal tubular cells of the kidney; follicular cell hypertrophy in the thyroid was also observed in males, and diffuse hepatocellular single cell necrosis and inflammation in the extrahepatic bile duct in the liver in females. At 160 mg/kg bw per day the following were observed: diffuse hepatocellular hypertrophy in the livers of both sexes, diffuse hepatocellular single cell necrosis and brown pigment deposition in the Kupffer cells in the liver, calculi in the gallbladder, hypertrophy and cytoplasmic eosinophilic inclusion bodies in the proximal tubular cell in the kidney, and zona fasciculata cell vacuolation in the adrenal in males (2/4 males), and hepatocellular cytoplasmic eosinophilic inclusion bodies in the liver, follicular cell hypertrophy in the thyroid and degeneration in the optic nerve fibre in females. At 160 mg/kg bw per day the liver changes observed were considered to be adaptive.

At 40 mg/kg bw per day, proximal tubule cell vacuolation (grade one) was observed in the

kidney of one male, however there were no associated changes in urinalysis or clinical chemistry that would suggest renal effects. Liver changes, including increased liver weight, increasing ALP and diffuse hepatocellular hypertrophy were also seen at this dose, however they were considered to be adaptive changes and not of toxicological significance.

The NOAEL of 40 mg/kg bw per day was identified based on vomiting, changes in clinical chemistry, including changes in liver enzymes and decreased total protein and albumin, decreased prostrate weight and histopathological changes in the liver and zona fasciculata cell vacuolation in the adrenals in males at 160 mg/kg bw per day (Takahashi, 2016). Key results for this 13 week (90 day) study in dogs are shown on Tables 7a and 7b.

Table 7a. Key results from 13-week study in dogs; food consumption (percentage of control)

	Sex and dose level (mg/kg bw per day)							
	Males				Females			
Week	**0**	**40**	**160**	**700/500**	**0**	**40**	**160**	**700/500**
Before treatment	(100)	100	100	100	(100)	100	100	100
1	(100)	100	97	86	(100)	100	99	87*
2	(100)	100	89	45*	(100)	100	100	53*
3	(100)	100	90	97 [b]	(100)	97	100	98 [b]
4	(100)	100	91	78*	(100)	95	100	68*
5	(100)	107	100	58	(100)	100	100	62*
6	(100)	102	97	76	(100)	99	100	61*
7	(100)	100	97	68*	(100)	96	100	47*
8	(100)	100	96	68*	(100)	98	100	37*
9	(100)	100	96	66*	(100)	99	100	56
10	(100)	100	97	87	(100)	98	100	80 [a]
11	(100)	100	100	88	(100)	99	100	67 [a]
12	(100)	100	100	90**	(100)	95	100	74 [a]
13	(100)	100	98	85**	(100)	95	100	92 [a]

[a] Excluded from statistical evaluation because of insufficient numbers; Source: Takahashi, 2016

[b] Excluded from statistical evaluation because of withdrawal period;

Statistically significant using Dunnett's test: * $p \leq 0.05$; **: $p \leq 0.01$;

Table 7b. Key results from 13-week study in dogs; significant changes in organ weight at terminal kill; mean ± standard deviation [% of controls]

	Sex and dose level (mg/kg bw per day)							
	Males				Females			
Parameter	**0**	**40**	**160**	**700/500**	**0**	**40**	**160**	**700/500**
	Significant changes in organ weight [mean ± SD (% of controls)]							
Number of animals	4	4	4	3	4	4	4	2
Terminal body weight (kg)	9.9 ± 0.6	10.1 ± 1.0 [102]	10.2 ± 1.1 [103]	8.7 ± 1.0 [88]	9.2 ± 0.8	9.6 ± 0.3 [104]	9.3 ± 0.9 [101]	8.2 [a] [89]
Liver weight absolute (g)	243 ± 12	313 ± 16* [129]	371 ± 18** [153]	363 ± 57** [149]	241 ± 34	290 ± 21 [120]	339 ± 59* [141]	338 [a] [140]
Liver weight relative (% of bw)	2.47 ± 0.18	3.11 ± 0.21* [126]	3.69 ± 0.47** [149]	4.18 ± 0.28** [169]	2.63 ± 0.31	3.02 ± 0.13 [115]	3.64 ± 0.28** [138]	4.14 [a] [157]
Heart weight absolute (g)	74.9 ± 2.8	76.8 ± 5.0 [103]	70.4 ± 4.6 [94]	64.8 ± 6.9* [87]	71.4 ± 7.1	70.6 ± 3.7 [99]	69.2 ± 2.9 [97]	59.6 [a] [83]

	Sex and dose level (mg/kg bw per day)							
	Males				Females			
Parameter	**0**	**40**	**160**	**700/500**	**0**	**40**	**160**	**700/500**
Heart weight relative (% of bw)	0.76 ± 0.08	0.77 ± 0.11	0.70 ± 0.06	0.75 ± 0.05	0.78 ± 0.07	0.73 ± 0.04	0.75 ± 0.06	0.73 [a]
Prostate weight absolute (g)	6.0 ± 1.8	5.6 ± 1.7 [93]	2.9 ± 0.8* [48]	2.9 ± 0.4* [48]	-	-	-	-
Prostate weight relative (% of bw)	0.060 ± 0.016	0.055 ± 0.018 [92]	0.029 ± 0.011* [48]	0.034 ± 0.008 [57]	-	-	-	-

[a] Excluded from statistical evaluation because of insufficient numbers; Source: Takahashi, 2016

Statistically significant using Dunnett's test: * $p \leq 0.05$; **: $p \leq 0.01$

In a 52-week oral toxicity study, beagle dogs (four dogs/sex per group) received inpyrfluxam (purity 95.0%) in gelatin capsules doses of 0, 2, 6, 30 or 160 mg/kg bw per day. During the treatment period, general clinical observations, mortality check, and feed consumption measurement were carried out daily for all animals. Detailed clinical observations were conducted once prior to initiation of treatment and once a week during the treatment period. Body weight was measured once prior to initiation of treatment, once a week from weeks 1–13, and once every four weeks from week 16 to 52. Animals were also subjected to ophthalmology, urinalysis, haematology and blood biochemistry investigations once prior to initiation of treatment. During the treatment period, ophthalmological assessment was conducted at week 52, and urinalysis at weeks 13, 26 and 52. Haematology and blood biochemistry was conducted at weeks 13, 26 and 52. Toxicokinetic parameters, using plasma samples, were determined for all animals at day 1, weeks 13, 26, and 52 (before administration and at 2, 4, 7 and 24 hours after administration). After 52 weeks of treatment, all animals were euthanized and subjected to necropsy and organ weight measurement. Histopathological examination was performed on organs and tissues from all animals.

Inpyrfluxam was detected in the plasma of animals of both sexes of all treatment groups except for the 2 mg/kg bw per day group. Additionally, C_{max} and AUC_{0-t} values increased broadly in a dose-proportional manner in both sexes. There was no difference in the systemic exposure of male and female dogs and no accumulation after repeated oral (capsule) administrations.

No treatment-related changes were observed in mortality, detailed clinical observations, body weight, food consumption, ophthalmology, urinalysis, or haematology.

At 160 mg/kg bw per day, an increased incidence of vomiting of food was observed in males and females during the study. An increased incidence of food vomiting was also observed in one female at 30 mg/kg bw per day.

Examination of blood biochemistry revealed statistically significant increases or increasing tendencies in ALP, ALT and GGTP at weeks 13, 26, and 52 in both sexes at 160 mg/kg bw per day, and in ALP and GGTP for males at 30 mg/kg bw per day. Males at 160 mg/kg bw per day also showed statistically significant decreases or decreasing tendencies in total protein, albumin, A : G ratio, total cholesterol and calcium at weeks 13, 26, and 52, and females also showed statistically significant decreases or decreasing tendencies in triglyceride, total cholesterol and potassium at week 26 and/or 52. Females at 6 mg/kg bw per day showed an increasing trend in ALP at week 52, however this was considered not to be of toxicological significance.

At necropsy, all males and three females at 160 mg/kg bw per day displayed dark-coloured livers; in addition, hepatic enlargement was observed in one male and two females. Liver weights, both absolute and relative to body weight, were significantly increased in both sexes at 160 mg/kg bw per day and in males at 30 mg/kg bw per day. In females at 160 mg/kg bw per day a statistically significant increase in the relative weight of the adrenal was also recorded, with an increase in absolute adrenal weight of 17% observed but not obtaining statistical significance. The body weight of females at 160 mg/kg bw per day was 11% lower than controls. Histopathologically, hepatocellular cytoplasmic eosinophilic inclusion bodies were noted in one animal of each sex at 160 mg/kg bw per day. Three animals of each sex at 160 mg/kg bw per day and one male at 30 mg/kg bw per day showed diffuse hepatocellular hypertrophy

in the liver. The observed liver changes may be considered to be adaptive and not of toxicological significance.

Vacuolation of the zona fasciculata cells in the adrenal was observed in one male and two females at 160 mg/kg bw per day, and in two males and one female at 30 mg/kg bw per day. In the eye, degeneration in the optic nerve fibre was detected in two females at 160 mg/kg bw per day, in one male and one female at 30 mg/kg bw per day, and in one female at 6 mg/kg bw per day. However, as a single instance of degeneration of the optic nerve fibre was also observed in one control animal of each sex the ocular changes were not considered to be related to treatment.

The NOAEL for inpyrfluxam to male and female dogs for 12 months was therefore 6 mg/kg bw per day based on histopathological changes in the adrenal gland at 30 mg/kg bw per day (Motomura, 2017). Key results from this study are summarized in Tables 8a and 8b. It was further noted that similar effects were seen in the 90-day study in dogs at 160 mg/kg bw per day, but not at 40 mg/kg bw per day in that study.

Table 8a. Key results from the one-year oral study in dogs; males

	Dose (mg/kg bw per day)				
	Males				
Parameter	**0**	**2**	**6**	**30**	**160**
	Significant changes in organ weight; mean ± standard deviation [% of controls]				
Terminal body weight (kg)	11.8 ± 1.1	12.2 ± 1.5	12.0 ± 1.0	12.3 ± 1.3	11.3 ± 0.8
Liver weight absolute (g)	274 ± 29	272 ± 18 [99]	316 ± 46 [115]	361 ± 36* [132]	410 ± 73** [150]
Liver weight relative (% of bw)	2.35 ± 0.29	2.24 ± 0.15 [95]	2.63 ± 0.19 [112]	2.92 ± 0.28* [124]	3.61 ± 0.43** [154]
Adrenal weight absolute (mg)	998 ± 57	1027 ± 109 [103]	1056 ± 30 [106]	1180 ± 198 [118]	1097 ± 176 [110]
Adrenal weight relative (% of bw)	0.0086 ± 0.0013	0.0084 ± 0.0005 [98]	0.0089 ± 0.0010 [103]	0.0096 ± 0.0021 [112]	0.0097 ± 0.0010 [113]

Statistically significant using Dunnett's (or Dunnett's type) test: * $p \leq 0.05$; **: $p \leq 0.01$ Source: Motomura, 2017

Table 8a. Key results from the one-year oral study in dogs; females

	Females				
Parameter	**0**	**2**	**6**	**30**	**160**
	Significant changes in organ weight; mean ± standard deviation [% of controls]				
Terminal body weight (kg)	11.3 ± 1.7	11.4 ± 1.9	11.2 ± 1.0	10.7 ± 1.4	10.2 ± 0.6
Liver weight absolute (g)	280 ± 32	312 ± 31 [111]	293 ± 23 [105]	322 ± 43 [115]	367 ± 67* [131]
Liver weight relative (% of bw)	2.52 ± 0.43	2.77 ± 0.46 [110]	2.60 ± 0.33 [103]	3.01 ± 0.47 [119]	3.54 ± 0.50* [140]
Adrenal weight absolute (mg)	1116 ± 130	1049 ± 164 [94]	1196 ± 61 [107]	979 ± 93 [88]	1351 ± 146 [121]
Adrenal weight relative (% of bw)	0.0100 ± 0.0013	0.0093 ± 0.0019 [93]	0.0106 ± 0.0009 [106]	0.0091 ± 0.0003 [91]	0.0131 ± 0.0012** [131]

Statistically significant using Dunnett's (or Dunnett's type) test: * $p \leq 0.05$; **: $p \leq 0.01$; Source: Motomura, 2017

(b) Dermal application

In a 28-day dermal toxicity study, Crl:CD(SD) rats (10 rats/sex per group; eight weeks old) received six hour occlusive dermal applications of inpyrfluxam technical grade (S-2399 TG, purity 95.0%), at dose levels of 100, 300 or 1000 mg/kg bw for 28 consecutive days. All surviving animals were observed daily for mortality and general clinical signs, and their body weight and food consumption recorded weekly. Detailed clinical observations were performed once prior to initiation of treatment and once per week during the treatment period.

Ophthalmological examinations were performed on all animals before initiation of treatment and on animals of the control and high-dose groups at week 4 of treatment. Haematology, clinical chemistry and urinalysis evaluations were performed on all animals at termination. Gross necropsy and histopathology were conducted on all animals; absolute and relative organ weights were determined.

No deaths occurred and no treatment-related changes were noted in males or females in clinical signs, detailed clinical observations, body weight, food consumption, ophthalmology, urinalysis, haematology, blood biochemistry, necropsy, organ weight, or histopathology. High values of mean corpuscular haemoglobin concentration (MCHC) were noted in females at 100 mg/kg bw per day, however levels were within historical control data values and were considered not to be treatment related. Low blood sodium values were detected in males at 300 mg/kg bw per day and above. These were slightly above historical control values, and were not associated with any other changes seen in other examinations; they were therefore considered not to be of toxicological significance. High values of relative heart weight were seen in males in the 1000 mg/kg bw per day group, which were not considered of toxicological significance as they were within the historical control range of the test facility.

The NOAEL inpyrfluxam technical grade (S-2399 TG) was therefore 1000 mg/kg bw per day, the highest dose tested (Iida, 2015).

(c) Exposure by inhalation

No short-term inhalation toxicity studies were submitted.

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

In a carcinogenicity study, groups of 52 male and 52 female CD1 mice received inpyrfluxam (purity 95.0%) in basal diet at concentrations of 0, 700, 2000 or 7000/5000 ppm (equal to 0, 77, 224 and 775 mg/kg bw per day for males, 0, 69, 210 and 701 mg/kg bw per day for females) for 78 weeks. The top dose level was initially set at 7000 ppm however, due to severe effects on body weight it was reduced to 5000 ppm at week 53 for males and week 52 for females. This dose level will be presented as 7000/5000 ppm within this summary. A further 12 animals/sex per group were treated at the same dose levels for 52 weeks (the satellite group). All animals were observed daily for mortality and clinical signs during the study, and their body weight, body weight gain and feed consumption measured periodically. The test substance concentration in plasma was analysed from four animals/sex per group of the satellite group on one occasion after 52 weeks of treatment. All animals were subjected to necropsy. Organ weight measurement was performed on all surviving animals after 52 weeks of treatment for the satellite group and 10 animals/sex per group after 78 weeks of treatment for the main group. After 78 weeks of treatment a differential leukocyte count was carried out on all surviving animals. Differential leukocyte counts were also carried out with blood smears from all animals killed in extremis during the study. Systemic organs and tissues from all animals killed in extremis or found dead during the study, and all animals subjected to terminal kill in the control and 7000/5000 ppm groups of the main and satellite groups were examined microscopically. In addition, for the satellite group, the liver (both sexes) and kidneys (males only), as well as any gross lesions, were also examined from animals treated at 700 and 2000 ppm. For the main group, all gross lesions and the liver (males and females), kidneys (males), heart (males), adrenals (males), thyroids (males), forestomach (males), duodenum (males), mesenteric lymph nodes (males), extra-orbital lacrimal glands (males) and cervical lymph nodes and glandular stomach (females) were also examined microscopically from animals treated at 700 and 2000 ppm and killed at termination.

Table 9a. Key results of the 78-week carcinogenicity study in mice; summary of plasma concentrations and body weight gain at selected time points

	Sex and diet concentration (ppm)							
	Males				Females			
Parameter	0	700	2000	7000/ 5000	0	700	2000	7000/ 5000
Mean plasma conc. of inpyrfluxam, week 52 (mg/L) [*N*]	<LOQ [4]	<LOQ [4]	0.006 [4]	0.092[a] [4]	<LOQ [4]	<LOQ [4]	0.004 [4]	0.017 [4]
Body weight gain, weeks 0–52 (g) satellite group	-	114	95	77	-	101	81	65**
Body weight gain, weeks 0–78 (g) main group	-	104	94	63**	-	95	90	76**

Statistical significance: ** $p \leq 0.01$ by Dunnett's test; *N*: number sampled; Source: Kitazawa, 2017

[a] 1 male mouse had a much higher plasma concentration; when removed, average plasma concentration was 0.024 mg/L

<LOQ: below the limit of quantitation (<0.004); the <LOQ was computed as zero for average calculations

Table 9b. Key results of the 78-week carcinogenicity study in mice; summary of significant changes in organ weight; mean ± SD [% of controls]

	Sex and diet concentration (ppm)							
	Males				Females			
Parameter	0	700	2000	7000/ 5000	0	700	2000	7000/ 5000
	Satellite group							
Terminal body weight (g)	49.1 ± 4.5	51.8 ± 5.8 [105]	48.5 ± 3.4 [99]	46.0 ± 5.6 [94]	56.2 ± 9.3	56.5 ± 9.7 [101]	50.4 ± 6.1 [90]	45.2 ± 5.6** [80]
Heart: Number examined	9	11	10	11	12	11	12	10
Absolute weight (mg)	234 ± 32	234 ± 25 [100]	218 ± 16 [93]	228 ± 28 [97]	198 ± 49	176 ± 15 [89]	180 ± 14 [91]	168 ± 24* [85]
Relative weight (%)	0.48 ± 0.06	0.46 ± 0.07	0.45 ± 0.04	0.50 ± 0.10	0.37 ± 0.16	0.32 ± 0.03	0.36 ± 0.02	0.37 ± 0.05
Liver: Number examined	9	11	10	11	12	11	12	10
Absolute weight (g)	2.60 ± 0.51	3.03 ± 0.86 [117]	2.75 ± 0.59 [106]	3.69 ± 0.96** [142]	2.37 ± 0.20	2.33 ± 0.25 [98]	2.35 ± 0.31 [99]	2.48 ± 0.30 [105]
Relative weight (%)	5.26 ± 0.59	5.83 ± 1.33 [111]	5.65 ± 1.04 [107]	8.03 ± 1.95** [153]	4.31 ± 0.70	4.18 ± 0.52 [97]	4.69 ± 0.56 [109]	5.52 ± 0.67** [128]
Kidneys: Number examined	9	11	10	11	12	11	12	10
Absolute weight (mg)	891 ± 99	876 ± 82 [98]	868 ± 102 [97]	845 ± 97 [95]	538 ± 56	527 ± 56 [98]	529 ± 59 [98]	471 ± 48* [88]
Relative weight (%)	1.83 ± 0.21	1.70 ± 0.20	1.79 ± 0.21	1.86 ± ± 0.24	0.97 ± 0.14	0.95 ± 0.13	1.05 ± 0.09	1.05 ± 0.09
Adrenals: Number examined	9	11	10	11	12	11	12	10
Relative weight (%)	0.010 ± 0.003	0.010 ± 0.003 [100]	0.010 ± 0.002 [100]	0.014 ± 0.005 [140]	0.020 ± 0.005	0.018 ± 0.006 [90]	0.021 ± 0.008 [105]	0.027 ± 0.007* [135]

Parameter	Sex and diet concentration (ppm)							
	Males				Females			
	0	700	2000	7000/ 5000	0	700	2000	7000/ 5000
	Main group							
Terminal body weight (g)	51.8 ± 6.7	50.2 ± 5.5 [97]	49.5 ± 7.6 [96]	42.4 ± 4.0** [82]	52.0 ± 8.9	53.5 ± 7.2 [103]	47.8 ± 7.2 [92]	45.8 ± 6.3 [88]
Brain: Number examined	10	10	10	10	10	10	10	10
Relative weight (%)	0.98 ± 0.09	1.03 ± 0.12 [105]	1.04 ± 0.17 [106]	1.20 ± 0.14** [122]	1.03 ± 0.19	1.01 ± 0.14 [98]	1.14 ± 0.17 [111]	1.16 ± 0.17 [113]
Heart: Number examined	10	10	10	10	10	10	10	10
Relative weight (%)	0.49 ± 0.09	0.51 ± 0.08 [104]	0.45 ± 0.05 [92]	0.57 ± 0.07* [116]	0.40 ± 0.12	0.40 ± 0.07 [100]	0.49 ± 0.10 [123]	0.45 ± 0.10 [113]
Liver: Number examined	10	10	10	10	10	10	10	10
Absolute weight (g)	3.26 ± 0.96	3.68 ± 1.84 [113]	3.21 ± 1.37 [98]	3.32 ± 1.22 [102]	2.87 ± 1.57	2.54 ± 0.27 [89]	2.78 ± 0.40 [97]	2.92 ± 0.38* [102]
Relative weight (%)	6.49 ± 2.50	7.42 ± 3.87 [114]	6.30 ± 1.71 [97]	8.03 ± 3.93 [124]	5.63 ± 2.93	4.82 ± 0.80 [86]	5.85 ± 0.68 [104]	6.43 ± 0.91* [114]
Adrenal: Number examined	10	10	10	10	10	10	10	10
Absolute weight (mg)	131	141	134	126	100	122	155	127
Relative weight (%)	0.015 ± 0.008	0.013 ± 0.003 [87]	0.014 ± 0.003 [93]	0.015 ± 0.003 [100]	0.020 ± 0.008	0.023 ± 0.005 [115]	0.024 ± 0.005 [120]	0.028 ± 0.008* [140]

Statistically significant using Dunnett's (or Dunnett's type) test: * $p \leq 0.05$; **: $p \leq 0.01$ Source: Kitazawa, 2017

Table 9c. Key results of the 78-week carcinogenicity study in mice; summary of statistically significant non-neoplastic lesions

Parameter	Sex and diet concentration (ppm)							
	Males				Females			
	0	700	2000	7000/5000	0	700	2000	7000/ 5000
	Satellite group							
Terminal kill								
Liver: Number examined	9	11	10	11	12	11	12	10
Hypertrophy, hepatocyte, centrilobular	0	0	3	6*	0	0	0	0
Hypertrophy, hepatocyte, diffuse	0	0	0	0	0	0	0	4*
Kidney: Number examined	9	11	10	11	12	0	2	10
Cyst, cortical	4	1	2	0*	1	-	2	1
Dilation, luminal, proximal tubule, diffuse	0	0	1	5*	0	-	0	0
Killed in extremis or found dead								
Kidney: Number examined	3	1	2	1	0	1	0	2
Dilation, luminal, proximal tubule, diffuse	1	0	0	0	-	1	-	0

	Sex and diet concentration (ppm)							
	Males				Females			
Parameter	0	700	2000	7000/5000	0	700	2000	7000/ 5000
	Main group							
All animals								
Liver: Number examined	52	52	52	52	52	52	52	52
Hypertrophy, hepatocyte, centrilobular	0	0	3	7*	0	0	0	0
Hypertrophy, hepatocyte, diffuse	0	0	0	0	0	0	0	11**
Fatty change, hepatocyte, centrilobular	8	6	6	1*	0	0	0	0
Kidney: Number examined	52	52	52	52	52	29[a]	26[a]	52
Dilation, luminal, proximal tubule, diffuse	4	6	4	14*	4	1	2	2
Nephropathy, amyloid	5	3	9	16*	14	12	19	20
Terminal kill								
Liver: Number examined	35	28	27	29	34	28	31	30
Hypertrophy, hepatocyte, centrilobular	0	0	3	7**	0	0	0	0
Hypertrophy, hepatocyte, diffuse	0	0	0	0	0	0	0	11**
Kidney: Number examined	35	28	27	29	34	5[a]	5[a]	30
Dilation, luminal, proximal tubule, diffuse	1	1	1	8**	1	0	0	0

Statistically significant using two-tailed Fisher's exact probability test: * $p \leq 0.05$; **: $p \leq 0.01$; Source: Kitazawa, 2017

[a] Organ examined only in animals that showed macroscopic lesions at terminal kill and/or on all animals killed in extremis or found dead during the study; not subjected to statistical analysis

Table 9d. Key results of the 78-week carcinogenicity study in mice; summary of amyloid and neoplastic lesions (number of affected animals/number of animals examined)

	Sex and diet concentration (ppm)							
	Males				Females			
Parameter	0	700	2000	7000/ 5000	0	700	2000	7000/ 5000
	Main group: amyloid lesions (all animals examined)							
Lesion: amyloidosis								
Lymph node (cervical)	-	-	-	-	5/52	12/52	15/52*	17/52**
Lymph node (mesenteric)	8/52	9/52	13/52	18/52*	-	-	-	-
Heart	7/52	6/52	13/52	17/52*	-	-	-	-
Forestomach	5/52	7/52	12/52	16/52 F*, M**	-	-	-	-
Glandular stomach	-	-	-	-	9/52	18/52	21/52*	19/52*
Duodenum	6/52	8/52	11/52	16/52*	-	-	-	-
Liver	5/52	5/52	10/52	15/52*	-	-	-	-
Kidney								
Nephropathy, amyloid	5/52	3/52	9/52	16/52**	14/52	12/29	19/26[a]	20/52
Thyroid	7/52	7/52	13/52	17/52*	-	-	-	-
Adrenal	5/52	4/52	8/52	14/52*	-	-	-	-
Extraorbital lacrimal gland	9/52	11/52	17/52	21/52*	-	-	-	-

	Sex and diet concentration (ppm)							
	Males				Females			
Parameter	0	700	2000	7000/ 5000	0	700	2000	7000/ 5000
	Main group: neoplastic lesions (killed in extremis or found dead)							
Organ and lesion								
Malignant lymphoma	0/17	3/24	0/25	2/23	6/18	5/24	0/21**	5/22
Lung - Adenoma, benign	2/17	2/24	5/25	1/23	4/18	3/24	0/21*	3/22

Statistically significant: *: p≤0.05; **: p≤0.01 Source: Kitazawa, 2017

[F] Fisher's exact probability test; [M] Mann-Whitney's U test; when not indicated, same level of significance at both tests;

[a] Examined only in animals that showed macroscopic lesions at terminal kill and on all animals killed in extremis or found dead during the study

Mortality rate was not affected by treatment in either the satellite or the main group. The plasma concentration of inpyrfluxam, measured at week 52, is presented in Table 8 above. It was noted that one male mouse in 7000/5000 ppm group displayed a much higher concentration than any other male or female, at 0.297 mg/L.

In males receiving 7000/5000 ppm, increased incidences of emaciation, pale-coloured skin, and pale colour of the eye were observed in the main group. Body weight, body weight gain and food consumption were decreased compared to controls throughout the study in animals of both sexes in the main and satellite groups. Feed efficiency during the first 13 weeks of treatment was decreased compared to controls in animals of both sexes of the main group, and in females of the satellite group. At necropsy, the incidences of dark-coloured liver were significantly increased in males of the main and satellite groups (5/11 and 8/52 animals respectively), and an increased tendency was also noted in females of the main group (5/52 animals).

A decreased incidence of hypertrophy of seminal vesicles and the coagulating gland was seen in males from the main group. Organ weight data revealed significant increases and/or increasing tendencies in the absolute and/or relative liver weights for males and females of the main and satellite groups, compared to controls. Relative testis weight was increased in the main group, with a slight trend in the satellite group. Other statistically significant changes in organ weight (brain, heart, adrenals and kidney) were considered secondary to decreased final body weight. At histopathological examination, significant increases in the incidence of centrilobular hepatocellular hypertrophy in males, and diffuse hepatocellular hypertrophy in females were noted in the animals subjected to terminal kill and all animals examined of the main group and interim kill of the satellite group. Significant increases in the incidence of diffuse luminal dilatation of the proximal tubules of the kidneys were noted in males subjected to terminal kill and all animals examined of the main group and in males of the satellite group subjected to interim kill. Systemic amyloidosis was increased. The incidences of amyloidosis in the following organs and groups of animals treated at 7000/5000 ppm attained statistical significance: lymph nodes (mesenteric) and extraorbital lacrimal glands in all males examined; heart, forestomach, liver, duodenum, kidneys, thyroids and adrenals in males killed in extremis or found dead, and in all males examined; lymph nodes (cervical) in females killed in extremis or found dead and all females examined; and glandular stomach in all females examined. There was a decreased retention of secreted material in the seminal vesicles and coagulating gland.

At 2000 ppm, there was an increase in absolute and relative testis weight in males in the main group, with a slight trend in the satellite group; these were not associated with any histopathological changes. Histopathological examination revealed an increasing tendency in the incidences of centrilobular hepatocellular hypertrophy, which was observed in three males each of the main and satellite groups. The incidence of amyloidosis was significantly increased in the lymph nodes (cervical) and glandular stomach in females of the main group.

There were no significant changes in the incidences of neoplastic lesions except for significant decreases in the incidences of systemic malignant lymphoma and adenoma of the lung observed in main group females dosed at 2000 ppm that were killed in extremis or found dead. Histopathological examinations did not show any evidence of a rare tumour suspected to be related to treatment, enhancement

of multifocal development of any neoplastic lesion, or an earlier occurrence of spontaneous tumours, than would be expected in controls. Therefore, it was concluded that inpyrfluxam (technical grade) has no carcinogenic potential in mice. The NOAEL for carcinogenicity was 7000/5000 ppm (equal to 701 mg/kg bw per day), the highest dose level tested

The NOAEL for toxicity was 700 ppm (equal to 69 mg/kg bw per day), on the basis of an increase in amyloid nephropathy in both sexes, centrilobular hepatocellular hypertrophy in males and an increase in the incidence of amyloidosis in cervical lymph nodes and glandular stomach in females at 2000 ppm. The compound was not carcinogenic up to 7000/5000 ppm (equal to 701 mg/kg bw per day), the highest dose tested (Kitazawa, 2017).

Rat

Groups of Wistar Hannover rats received inpyrfluxam (purity 95.0%) for a period of 52 or 104 weeks in basal diet at dose levels of 0, 150, 500 or 1500/1000 ppm for females (the dose level was reduced at week 46, see below) or 2000 ppm for males (equal to 0, 5.85, 19.4 and 78.4 mg/kg bw per day for males, 0, 7.45, 25.5 and 65.9 mg/kg bw per day for females). Each dose group consisted of 21 males and 21 females for the chronic toxicity phase, and 51 males and 51 females for the carcinogenicity phase of the study.

All animals were observed daily for mortality and general clinical signs, and their body weights and food consumption were recorded periodically. Detailed clinical observations were performed on the toxicity group once prior to initiation of treatment and once a week during the treatment period.

For the toxicity group animals, functional observations were carried out on 10 animals/sex per group at week 49. Ophthalmological examinations were conducted on all animals before initiation of treatment and on all surviving animals in the control and high-dose groups at week 52. The concentration of inpyrfluxam in plasma samples was determined on four animals/sex per group at 14, 26 and 51 weeks of treatment. Urinalysis (at 13, 25, and 51 weeks of treatment), haematology and blood biochemistry analysis (after 14, 26, and 52 weeks of treatment) were each performed on 10 animals/sex per group.

In the carcinogenicity group animals, haematology was limited to total and differential leukocyte counts and these were performed on all surviving animals at 104 weeks.

All animals were subjected to necropsy. Organ weights were determined for all surviving animals of the toxicity phase after 52 weeks of treatment, and for 10 animals/sex per dose group after 104 weeks of treatment for the carcinogenicity phase. Histopathological examinations were performed on organs and tissues from all animals killed in extremis or found dead during the treatment period, and all animals subjected to terminal kill in the control and high-dose groups. In addition the spleen from females of all groups in the toxicity phase, and all gross lesions in both sexes subjected to terminal kill were also examined microscopically.

Table 10a. Key results of the combined study for chronic toxicity and carcinogenicity in rats; plasma concentrations of inpyrfluxam (mg/L; mean value from four rats shown)

	Sex and dose level (ppm)							
	Males				Females			
Time point	**0**	**150**	**500**	**2000**	**0**	**150**	**500**	**1500/1000**
Week 14	<LOQ	<LOQ	<LOQ	0.06	<LOQ	0.02	0.15	0.24
Week 26	<LOQ	<LOQ	<LOQ	0.07	<LOQ	0.03	0.17	0.31
Week 51	<LOQ	<LOQ	<LOQ	0.04	<LOQ	0.02	0.11	0.22

<LOQ: Below the limit of quantitation (<0.01 mg/L); computed as 0 for average calculations Source: Ohtsuka, 2017

Table 10b. Key results of the combined study for chronic toxicity and carcinogenicity in rats; carcinogenicity group, summary of body weight gain at selected time points (% of control)

	Dose (ppm)							
	Males				Females			
Time point	**0**	**150**	**500**	**2000**	**0**	**150**	**500**	**1500/1000**
Weeks 0–1	(100)	96	100	89**	(100)	105	100	84**
Weeks 1–2	(100)	100	96	87**	(100)	105	100	80**
Weeks 2–3	(100)	100	97	86**	(100)	94	94	83**
Weeks 3–4	(100)	97	97	83**	(100)	93	93	73**
Weeks 4–5	(100)	96	96	76**	(100)	109	91	91
Weeks 5–6	(100)	90	90	75**	(100)	100	100	89
Weeks 6–7	(100)	100	100	87*	(100)	89	89	67**
Weeks 7–8	(100)	100	92	83*	(100)	86	86	71
Weeks 8–9	(100)	100	100	83*	(100)	100	100	100
Weeks 13–16	(100)	105	89	89	(100)	111	100	56**
Weeks 16–20	(100)	100	100	95	(100)	91	82	55**
Weeks 20–24	(100)	100	100	90	(100)	100	78	56**
Weeks 24–28	(100)	112	100	94	(100)	113	113	50**
Weeks 28–32	(100)	94	106	100	(100)	110	90	40**
Weeks 32–36	(100)	118	100	109	(100)	78	89	33**
Weeks 36–40	(100)	108	117	92	(100)	90	90	10**
Weeks 40–44	(100)	118	109	82	(100)	111	89	33**
Weeks 56–60	(100)	110	100	70	(100)	122	100	44**
Weeks 64–68	(100)	250**	150	250*	(100)	92	92	62*
Weeks 68–72	(100)	92	58*	58**	(100)	100	86	43*
Weeks 76–80	(100)	200	133	33*	(100)	100	0**	100
Weeks 80–84	(100)	160	140	80	(100)	100	73	45*
Weeks 92–96	(100)	3	1	–5	(100)	4	5*	6*
Weeks 0–104	(100)	103	99	82**	(100)	102	96	73**

Statistically significant using Dunnett's (or Dunnett's type) test: * $p \leq 0.05$; **: $p \leq 0.01$ Source: Ohtsuka, 2017

Table 10c. Key results of the combined study for chronic toxicity and carcinogenicity in rats; Summary of significant haematology changes at week 52 (toxicity group) or week 104 (carcinogenicity group); mean ± standard deviation [% of controls]

	Sex and dose level (ppm)							
	Males				Females			
Parameter	**0**	**150**	**500**	**2000**	**0**	**150**	**500**	**1500/1000**
				Toxicity group				
Differential leukocyte count								
Number of rats	10	10	10	10	10	10	10	10
Neutrophils ($10^3/\mu L$)	0.89 ± 0.29	1.09 ± 0.30 [122]	0.96 ± 0.34 [108]	1.13 ± 0.21 [127]	0.66 ± 0.28	0.67 ± 0.26 [102]	0.51 ± 0.13 [77]	0.39 ± 0.10** [59]
Monocytes ($10^3/\mu L$)	0.16 ± 0.05	0.21 ± 0.05 [131]	0.20 ± 0.07 [125]	0.18 ± 0.05 [113]	0.10 ± 0.04	0.11 ± 0.03 [110]	0.10 ± 0.02 [100]	0.06 ± 0.02* [60]

Parameter	Sex and dose level (ppm)							
	Males				Females			
	0	150	500	2000	0	150	500	1500/1000
	Carcinogenicity group							
Number of rats	42	46	41	47	34	33	36	42
WBC (10^3/µL)	6.20 ± 2.03	5.52 ± 1.60 [89]	5.53 ± 1.97 [89]	5.22 ± 2.19 [84]	3.61 ± 1.61	3.42 ± 1.28 [95]	3.44 ± 1.95 [95	2.78 ± 1.15* [77]
Differential leukocyte count								
Neutrophils (10^3/µL)	2.53 ± 1.24	2.21 ± 0.93 [87]	2.27 ± 1.39 [90]	2.02 ± 1.43* [80]	1.59 ± 1.08	1.38 ± 0.69 [87]	1.40 ± 0.96 [88]	1.03 ± 0.56 [65]
Monocytes (10^3/µL)	0.36 ± 0.14	0.32 ± 0.11 [89]	0.31 ± 0.13 [86]	0.29 ± 0.16* [81]	0.18 ± 0.08	0.16 ± 0.07 [89]	0.16 ± 0.07 [89]	0.13 ± 0.04* [72]

Statistically significant using Dunnett's (or Dunnett's type) test: * $p \leq 0.05$; **: $p \leq 0.01$ Source: Ohtsuka, 2017

***Table 10d. Key results of the combined study for chronic toxicity and carcinogenicity in rats; Summary of significant changes at clinical chemistry ; mean ± standard deviation* [*% of controls*]**

Parameter	Time point (week)	Sex and dose level (ppm)							
		Males				Females			
		0	150	500	2000	0	150	500	1500/1000
		Toxicity group: significant changes in clinical chemistry [mean ± SD (% of controls)]							
N		10	10	10	10	10	10	10	10
AST (U/L)	26	71 ±10	76±12 [107]	76±18 [107]	82±15 [115]	150 ±106	143±70 [95]	91±24 [61]	66±11** [44]
	52	75 ±17	80±23 [107]	64±14 [85]	76±16 [101]	137 ±61	173±101 [126]	100±25 [73]	78±30** [57]
ALT (U/L)	14	27 ±3	29±5 [107]	30±7 [111]	27±4 [100]	26 ±7	34±24 [131]	27±9 [104]	19±2* [73]
	26	30 ±6	33±8 [110]	31±10 [103]	33±5 [110]	58 ±37	56±29 [97]	46±25 [79]	20±4** [34]
	52	42 ±16	40±15 [95]	31±10 [74]	38±11 [90]	63 ±25	78±50 [124]	62±41 [98]	32±12** [51]
GGTP (U/L)	14	0.7 ±0.1	0.7±0.1 [100]	0.6±0.1 [86]	1.8±0.6** [257]	0.8 ±0.2	0.8±0.2 [100]	0.9±0.2 [113]	1.8±0.7** [225]
	26	0.8 ±0.1	0.8 ±0.1 [100]	0.6 ±0.2 [75]	1.6 ±0.5** [200]	0.8 ±0.2	0.8 ±0.2 [100]	0.9 ±0.3 [113]	1.2 ±0.5 [150]
	52	1.2 ±0.2	1.2±0.4 [100]	1.1±0.3 [92]	2.1±1.2 [175]	1.0 ±0.3	1.0±0.3 [100]	0.9±0.2 [90]	0.9±0.3 [90]
BUN (mg/dL)	52	14.2 ±2.0	14.4 ±1.0 [101]	14.1 ±1.1 [99]	14.9 ±1.8 [105]	14.2 ±1.3	16.1 ±2.8 [113]	15.1 ±1.9 [106]	17.1 ±1.6** [120]
Globulin (g/dL)	14	2.04 ±0.16	2.08 ±0.14 [102]	1.90 ±0.23 [93]	1.80 ±0.22* [88]	1.72 ±0.17	1.67 ±0.21 [97]	1.71 ±0.23 [99]	1.71 ±0.14 [99]
	26	2.25 ±0.22	2.17 ±0.11 [96]	2.12 ±0.19 [94]	1.96 ±0.22** [87]	1.76 ±0.14	1.74 ±0.25 [99]	1.81 ±0.19 [103]	1.79 ±0.14 [102]

Parameter	Time point (week)	Sex and dose level (ppm)							
		Males				Females			
		0	150	500	2000	0	150	500	1500/1000
A:G ratio	14	2.10 ±0.23	2.08 ±0.18 [99]	2.25 ±0.23 [107]	2.44 ±0.30* [116]	2.93 ±0.46	3.07 ±0.51 [105]	3.03 ±0.49 [103]	2.76 ±0.24 [94]
	26	1.87 ±0.22	1.91 ±0.14 [102]	1.98 ±0.14 [106]	2.16 ±0.25** [116]	3.05 ±0.27	3.03 ±0.59 [99]	2.81 ±0.43 [92]	2.81 ±0.24 [92]
Toxicity group: significant changes in organ weights; mean ± standard deviation [% of control]									
Terminal body weight (g)		552 ±62	568 ± 65 [103]	556 ± 55 [101]	520 ± 51 [94]	338 ±40	326 ± 44 [96]	322 ± 38 [95]	258 ± 19** [76]
Brain: *N*		21	21	21	20	21	21	21	21
Relative weight (% of body weight)		0.39 ±0.04	0.37 ±0.04 [95]	0.38 ±0.03 [97]	0.40 ±0.04 [103]	0.58 ±0.07	0.60 ±0.09 [103]	0.61 ±0.07 [105]	0.75 ±0.07** [129]
Thyroid: *N*		21	21	21	20	21	21	21	21
Absolute weight (mg)		28.7 ±4.3	29.0 ±3.1 [101]	29.1 ±4.4 [101]	27.9 ±4.3 [97]	24.1 ±4.5	25.2 ±4.7 [105]	22.0 ±4.6 [91]	20.7 ±3.1* [86]
Relative weight (% of body weight)		0.0052 ± 0.0006	0.0052 ±0.0008 [100]	0.0053 ±0.0006 [102]	0.0054 ±0.0009 [104]	0.0072 ± 0.0013	0.0077 ±0.0012 [107]	0.0068 ±0.0010 [94]	0.0080 ±0.0010* [111]
Heart: *N*		21	21	21	20	21	21	21	21
Relative weight (% of body weight)		0.21 ±0.01	0.21 ±0.02 [100]	0.21 ±0.01 [100]	0.23 ±0.02** [110]	0.25 ±0.02	0.25 ±0.02 [100]	0.26 ±0.02 [104]	0.31 ±0.03** [124]
Liver: *N*		21	21	21	20	21	21	21	21
Absolute weight (g)		12.12 ±1.83	12.77 ±1.98 [105]	12.86 ±1.78 [106]	12.62 ±1.10 [104]	7.10 ±0.88	6.86 ±1.09 [97]	7.14 ±0.95 [101]	6.34 ±0.44** [89]
Relative weight (% of body weight)		2.19 ±0.15	2.24 ±0.19 [102]	2.31 ±0.21 [105]	2.43 ±0.13** [111]	2.10 ±0.15	2.10 ±0.16 [100]	2.22 ±0.12* [106]	2.46 ±0.15** [117]
Kidneys: *N*		21	21	21	20	21	21	21	21
Absolute weight (mg)		2839 ±324	2805 ±378 [99]	2868 ±299 [101]	2803 ±327 [99]	2019 ±175	1943 ±294 [96]	1981 ±161 [98]	1817 ±311** [90]
Relative weight (% of body weight)		0.51 ±0.04	0.50 ±0.05 [98]	0.52 ±0.04 [102]	0.54 ±0.04 [106]	0.60 ±0.07	0.60 ±0.06 [100]	0.62 ±0.08 [103]	0.71 ±0.13** [118]
Spleen: *N*		21	21	21	20	21	21	21	21
Absolute weight (mg)		822 ±127	842 ±163 [102]	835 ±126 [102]	790 ±83 [96]	590 ±65	559 ±82 [95]	566 ±74 [96]	493 ±53** [84]
Relative weight (% of body weight)		0.15 ±0.02	0.15 ±0.02 [100]	0.15 ±0.02 [100]	0.15 ±0.02 [100]	0.18 ±0.03	0.17 ±0.02 [94]	0.18 ±0.02 [100]	0.19 ±0.02* [106]

Parameter	Time point (week)	Sex and dose level (ppm)							
		Males				Females			
		0	150	500	2000	0	150	500	1500/1000
Adrenals: *N*		21	21	21	20	21	21	21	21
Relative weight (% of body weight)		0.011 ± 0.001	0.010 ±0.001 [91]	0.011 ±0.001 [100]	0.011 ±0.001 [100]	0.022 ± 0.004	0.021 ±0.003 [95]	0.022 ±0.004 [100]	0.026 ±0.004** [118]
Testes: *N*		21	21	21	20	-	-	-	-
Relative weight (% of body weight)		0.69 ±0.06	0.66 ±0.15 [96]	0.69 ±0.05 [100]	0.75 ±0.07* [109]	-	-	-	-
Uterus: *N*		-	-	-	-	21	21	21	21
Relative weight (% of body weight)		-	-	-	-	0.29 ±0.11	0.28 ±0.12 [97]	0.28 ±0.10 [97]	0.43 ±0.15** [148]
Carcinogenicity group: significant changes in organ weights [mean ± SD (% of controls)]									
Terminal body weight (g)		641 ±74	651±84 [102]	583±75 [91]	562±49 [88]	385 ±52	388±68 [101]	398±38 [103]	316±52* [82]
Brain: *N*		10	10	10	10	10	10	10	10
Relative weight (% of body weight)		0.35 ±0.05	0.34 ±0.05 [97]	0.39 ±0.04 [111]	0.39 ±0.04 [111]	0.53 ±0.06	0.52 ±0.09 [98]	0.51 ±0.04 [96]	0.64 ±0.11* [121]
Thyroid: *N*		10	10	10	10	10	10	10	10
Absolute weight (mg)		109.5 ± 224.4	34.6 ±12.3 [32]	36.6 ±7.2 [33]	32.0 ±9.5* [29]	26.0 ±6.3	24.1 ±4.2 [93]	29.0 ±7.0 [112]	21.1 ±3.4 [81]
Thyroid: *N*[#]		9	10	10	10	10	10	10	10
Absolute weight (mg)		38.6 ±7.3	34.6 ±12.3 [90]	36.6 ±7.2 [95]	32.0 ±9.5 [83]	26.0 ±6.3	24.1 ±4.2 [93]	29.0 ±7.0 [111]	21.1 ±3.4 [81]
Relative weight (% of body weight)		0.0060 ± 0.0007	0.0053 ±0.0014 [88]	0.0063 ±0.0013 [105]	0.0056 ±0.0012 [93]	0.0061 ± 0.0011	0.0063 ±0.0008 [103]	0.0072 ±0.0014 [118]	0.0068 ±0.0014 [111]
Kidneys: *N*		10	10	10	10	10	10	10	10
Absolute weight (mg)		4112 ± 1278	3613 ±457 [88]	3945 ±1238 [96]	3245 ±773** [79]	2338 ±316	2343 ±321 [100]	2380 ±220 [102]	1989 ±114** [85]
Adrenals: *N*		10	10	10	10	10	10	10	10
Absolute weight (mg)		81.8 ±16.7	69.7 ±15.0 [85]	76.9 ±24.7 [94]	62.6 ±7.3** [77]	80.3 ±11.9	74.4 ±16.2 [93]	77.1 ±10.0 [96]	70.6 ±15.3 [88]

N: Number evaluated; AST: Aspartate transaminase; ALT: Alanine transaminase; Source: Ohtsuka, 2017
GGTP: γ-Glutamyl transpeptidase; BUN: Blood urea nitrogen; A:G: Albumin to globulin ratio;
N[#]: Values recalculated to exclude extremely high outlier value attributed to a thyroid mass in animal No. 2
Statistically significant using Dunnett's (or Dunnett's type) test: * $p \leq 0.05$; **: $p \leq 0.01$

Table 10e. Key results of the 104-week carcinogenicity study in rats; Summary of neoplastic and non-neoplastic lesions

	Sex and diet concentration (ppm)							
	Males				Females			
Organ and lesion	**0**	**150**[a]	**500**[a]	**2000**	**0**	**150**[a]	**500**[a]	**1500/1000**
Toxicity group; statistically significant non-neoplastic lesions (number of affected animals)								
Spleen: Number examined	21	0	0	20	21	21	21	21
Deposition, brown pigment, increased	0	-	-	0	0	0	0	5*
Carcinogenicity group; statistically significant neoplastic lesions (number of affected animals)								
Mammary gland: number examined	51	5	11	51	51	30	22	51
Fibro-adenoma (benign)	0	0	1	0	16	17	7	4**
Pituitary – No. examined	51	18	18	51	51	40	41	51
Adenocarcinoma, anterior (malignant)	1	0	0	0	5	1	1	0*
Terminal kill								
Mammary gland: number examined	42	0	1	47	34	12	7	42
Fibro-adenoma (benign)	0	-	1	0	13	8	4	2**
Killed in extremis or found dead (ke/fd)								
Mammary gland: number examined	9	5	10	4	17	18	15	9
Fibro-adenoma (benign)	0	0	0	0	3	9*	3	2

Statistically significant using two-tailed Fisher's exact probability test: * $p \leq 0.05$; **: $p \leq 0.01$; Source: Ohtsuka, 2017

[a] Organ examined only in animals that showed macroscopic lesions at terminal kill and/or on all animals killed in extremis or found dead during the study; not subjected to statistical analysis

No significant changes were noted in the mortality rates of any treated group of either sex compared to the controls, neither in the carcinogenicity nor toxicity groups.

Inpyrfluxam was detected in the plasma of females from all treated groups, whereas all or three of four males treated at 150 and 500 ppm yielded concentrations under the limit of quantitation (< LOQ was 0.01 mg/L). It was noted that plasma concentrations in females in the 1500/1000 ppm group was higher than that in males at 2000 ppm. Additionally there was no detectable inpyrfluxam in the plasma of rats of the control group.

In males receiving 2000 ppm, body weight gain and food consumption were significantly decreased compared to controls. Decreased feed efficiency was observed mainly within the initial 10 weeks of treatment. Significant decreases in neutrophil count and monocyte count were observed in haematological investigations. Significant increases in GGTP and the A : G ratio, and a significant decrease in globulin were noted from blood biochemistry examinations. Relative liver weight was significantly increased. An extremely high thyroid weight was recorded in one control male, which it was noted showed thyroid mass(es) at necropsy. Excluding this value, the apparently significant decrease in relative thyroid weight recorded for males at 2000 ppm disappeared. Therefore this change was considered unrelated to treatment. There were a number of other organ weight changes in top-dose animals that were considered to be secondary to the significant decreases in group mean body weight and/or body weight gain.

The initial top dose level for females was set at 1500 ppm, however, body weight fell to about 80% of the control value at week 40 in the carcinogenicity and toxicity groups. The dose level in females in the toxicity and carcinogenicity groups was reduced to 1000 ppm at week 46; data for this group is presented as 1500/1000 ppm. Body weight, body weight gain and food consumption were significantly decreased compared to controls. Decreased feed efficiency was observed during the treatment period. Significant decreases in total leukocyte count, neutrophil and monocyte counts were noted in haematology examinations. In females treated at 1500/1000 ppm there were statistically significant decreases in AST and ALT after 14, 26 and/or 52 weeks of treatment. The changes were not in the direction of change

that would be considered adverse, and these changes were therefore considered not to be toxicologically adverse. Although a significant increase in blood urea nitrogen (BUN) was observed in females treated at 1500/1000 ppm after 52 weeks, the values of BUN were almost comparable to those in the historical control data (HCD) provided by the testing facility. Therefore BUN changes was considered to be incidental. In the toxicity group animals the incidence of greater deposition of brown pigment in the spleen, determined to be haemosiderin, was significantly increased.

There were no treatment-related changes in either sex receiving 150 or 500 ppm.

No treatment-related increase in the incidence of neoplastic lesions was observed in any treated groups of either sex. Two male rats in the high-dose group had a benign Schwannoma in the heart tissue. This is a relatively rare tumour, however, historical control data from the testing facility using the same strain of rat, had previously identified single occurrences in four testing years. On this basis, the occurrence of two incidences, particularly as this was not associated with other neuronal tumours, or with any signs of hyperplasia at any dose, and was only seen in one sex at the high dose, was considered of doubtful toxicological relevance to inpyrfluxam exposure. There were a small number of ovarian tumours, observed at the high dose, with a single incidence at the medium dose; these were not considered to be related to treatment. It was concluded that inpyrfluxam (technical grade) had no carcinogenic potential in rats. The NOAEL for carcinogenicity was 2000 ppm in males (78.4 mg/kg bw per day) and 1500/1000 ppm in females (65.8 mg/kg bw per day), the highest dose levels tested (Ohtsuka, 2017).

The NOAEL for toxicity was 500 ppm (19.4 mg/kg bw per day), based on decreased body weight gain and feed efficiency, and haematological changes in both sexes, and decreased body weight in females at 1500/1000 ppm. The compound was not carcinogenic up to the highest dose tested, 1500/1000 ppm (equal to 65.8 mg/kg bw per day) (Ohtsuka, 2017).

2.4 Genotoxicity

Inpyrfluxam was shown to be negative for genotoxic potential in three in vitro assays (chromosome aberration and gene mutation in bacterial and mammalian cells) in both the presence and absence of metabolic activation, and also negative in one in vivo assay (mouse bone marrow micronucleus).

Table 11. Results of genotoxicity studies performed with inpyrfluxam

End-point	Test object	Concentration/dose	Purity	Results	Reference
		In vitro			
Point mutations	*Salmonella typhimurium* (TA100, TA98, TA1535, and TA1537) and, *Escherichia coli* (WP2uvrA)	1.5–5000 µg/plate (± S9)	95.0%	Negative [a]	Kitamoto, 2014a
Chromosome aberration	Chinese hamster lung cells (CHL/IU)	Short-term treatment: –S9; 32.5–130 µg/mL +S9; 42.5–170 µg/mL Long-term treatment –S9: 0.188–1.5 µg/mL	95.0%	Negative [b, c]	Kitamoto, 2014b
Gene mutation	Chinese Hamster V79 cells (*HPRT* locus)	4 hour treatment: –S9; 6.5–39.0 µg/mL +S9; 6.5–78.0 µg/mL 24 hour treatment: –S9; 13.0–78.0 µg/mL	95.0%	Negatived [e]	Wollny, 2014

End-point	Test object	Concentration/dose	Purity	Results	Reference
		In vivo			
Induction of micronuclei	Mouse bone marrow	200, 400, 800 mg/kg bw, once by oral gavage to five CD-1 mice per sex	95.0%	Negative [f, g]	Kitamoto, 2015

HPRT: Hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × g supernatant fraction from rat liver homogenate; OECD TG: Organisation for Economic Co-operation and Development test guideline

[a] The study meets OECD TG No. 471 Bacterial reverse mutation test.

[b] The study meets OECD TG No. No. 473 In vitro Mammalian Chromosome Aberration Test, adopted 21 July 1997.

[c] Short term treatment included six hour exposure, following by 18 hours in fresh medium. Long term treatment included exposure for 24 hours in the absence of metabolic activation.

[d] The study meets the requirements of the OECD TG No. 473 In vitro mammalian chromosome aberration test, adopted 21 July 1997.

[e] Two experiments conducted, the first for four hours with and without S9, the second for four hours with S9, and 24 hours without S9. Cytotoxicity was seen at doses above 78 µg/mL without metabolic activation, and above 65 µg/mL in the presence of metabolic activation. Positive controls induced an increase in mutant colonies.

[f] The study meets the requirements of the current OECD TG No. 474 Mammalian erythrocyte micronucleus test.

[g] Mortality (800 mg/kg bw in the female animals) and severe clinical signs (e.g. ataxia, hunched posture) were observed in both male and female animals. A significant decrease in the ratio of polychromatic erythrocytes to the whole erythrocytes was observed in the male animals (48 hours, 800 mg/kg bw). Based on these findings it was considered that the test animals were systemically exposed to inpyrfluxam and that the substance reached the bone marrow.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Rat

A preliminary reproduction study was conducted with inpyrfluxam, (purity 95.0%) in order to select suitable dose levels of the technical grade substance for a definitive reproduction toxicity study in rats. In this preliminary dose range-finding study, groups of eight-week-old Wistar Hannover rats (eight per sex) received inpyrfluxam in a basal diet. Dietary concentrations of 0, 300, 1000, 2000 or 4000 ppm were administered for three weeks before mating and until necropsy of F1 weanlings at 26 days of age. Groups of F1 females were examined until they reached sexual maturation. The entire treatment period lasted approximately 15 weeks. Treatment had no effect on the reproductive performance of parental animals. At 4000 ppm body weights, body weight gains, and food consumption of parent animals of both sexes were significantly decreased during almost the entire treatment period. At termination of the males, their liver weights were seen to have increased, and livers that were darkly coloured were observed in three males. At 2000 ppm body weights, body weight gains, and food consumption were significantly decreased during the premating, gestation and lactation periods in parental females. At this dose the absolute and relative liver weights were significantly increased in males.

Mean body weights of male and female pups on and after lactation day (LD) 4 were significantly decreased in the 2000 and 4000 ppm groups. Absolute and relative weights of the spleen and thymus were also significantly decreased in pups of both sexes at 4000 ppm, the accompanying reduced body weights probably influencing the relative values. Completion of vaginal opening of pups was significantly delayed at 2000 and 4000 ppm. The delay was considered due to the delayed growth of F1 females, indicated by the lower body weight during the lactation period and subsequent growth period, because by the time of vaginal opening there were no body weight differences among the groups. Based on these results, a dose level of less than 2000 ppm (judged as about 1500 ppm) was considered a suitable high-dose level for the subsequent definitive reproduction toxicity study in rats (Hojo, 2015a).

The main two-generation rat reproduction study was conducted with inpyrfluxam (purity 95.0%; Lot no. 13CG0617G) in a basal diet at constant dietary concentrations over two consecutive generations (OECD Test Guideline 416). Based on results of the dose range-finding study, tested dietary concentrations were 0, 150, 500 and 1250 ppm for parental females, or 2000 ppm for parental males; the top dose

level is presented as 1250/2000 ppm hereafter. Each group consisted of 24 male and 24 female rats. Inpyrfluxam TG was administered to parental (F0) animals from the initiation of rearing to necropsy, after weaning of F1 pups. Similarly, parental F1 animals received the treated diets from the initiation of rearing, after selection, to necropsy after weaning of the F2 pups. The duration of dosing for F0 and F1 parental animals was approximately 18–19 weeks (depending on duration of the mating period). Mean daily intake of the inpyrfluxam was calculated and is shown below in Table 12.

Table 12. Mean daily intake (mg/kg bw per day) of inpyrfluxam in a reproductive toxicity study in rats

	Dietary concentration (ppm)							
	Males				Females			
	0	150	500	2000	0	150	500	1250
F0, premating	0	9.38	31.3	124	0	10.9	35.5	86
F1, premating	0	11.6	38.7	156	0	12.2	41.4	103
F0, breeding	0	6.61	22.0	93	0	16.4	55.3	138
F1, breeding	0	16.4	23.9	103	0	16.2	55.3	138

Source: Hojo, 2017

Treatment had no effect on the reproductive performance of parental animals in either generation, including fertility index, gestation index, duration of gestation, number of pups delivered, sex ratio or viability index. While the number of implantation sites was decreased in F1 females at the top dose, this did not affect the number of pups delivered or the viability index, and was therefore considered incidental.

At 1250/2000 ppm, significantly decreased body weights (except for F0 males), body weight gains, and food consumption were recorded in both sexes for parental animals during the treatment period. A delay in completion of preputial separation was observed in F1 males, and this was considered due to the decreased body weight of these males at the start of the second generation. Treatment-related changes were noted in organ weights and at histopathology. In the liver, increased liver weight and significantly increased incidences of diffuse hepatocellular hypertrophy were observed in F0 and F1 males. Liver weights were significantly increased in F0 and F1 females, and in addition, thyroid weights and the incidence of follicular cell hypertrophy in the thyroid were also significantly increased in F0 and F1 females. The changes affecting both the liver and thyroid of females were considered likely to be associated with hepatic enzyme induction. In the kidneys of F1 parental males, statistically significant increases in relative weight and incidence of hyaline droplet deposition, (which was determined to be α2μ-globulin, peculiar to male rats), in proximal tubular cells were observed. At 500 ppm, absolute and relative liver weights of F1 males and F2 females were increased, however this was considered to be an adaptive change, based on the lack of corresponding histopathological changes seen at this dose.

Mean body weights of F1 and F2 male and female pups on and after LD 14 were significantly decreased at 1250 ppm; no other effects were observed.

Based on these results, the systemic NOAEL for parental toxicity was 500 ppm (equal to 31 mg/kg bw per day) based on decreased body weights in females, and decreased body weight gain in both sexes at the LOAEL of 1250 ppm in females (equal to 86 mg/kg bw per day). The increased incidences of diffuse hepatocellular hypertrophy in males, as well as increased incidence of follicular cell hypertrophy in the thyroid of females were considered to be adaptive, or secondary to adaptive changes in the liver. The NOAEL for reproductive toxicity was 1250/2000 ppm (93 mg/kg bw per day), the highest dose level(s) tested as no reproductive effects were observed at any dose. The NOAEL for offspring toxicity was also 500 ppm, estimated as approximately 22 mg/kg bw per day, based on the decreased mean body weight of pups at 1250 ppm. It was noted that there were no observations of any malformations, including cyclopia, in the pups. (Hojo, 2017). A summary of the significant effects seen in this study is shown below in Table 13.

Table 13. Summary of key results of the two-generation reproduction study in rats

	Dietary concentration (ppm)							
	Males				Females			
Parameter	**0**	**150**	**500**	**2000**	**0**	**150**	**500**	**1250**
Thyroid, hypertrophy, follicular cell								
F0 Parents	0/24	0/0	0/0	0/24	3/24	1/23	0/24	16/24**
F1 Parents	0/21	0/0	0/0	0/22	0/21	0/22	0/22	10/22**
Liver, hypertrophy, hepatocyte, diffuse								
F0 Parents	0/24	0/23	0/24	7/24**	0/24	0/0	0/0	0/24
F1 Parents	0/21	0/21	0/22	11/22**	0/21	0/0	0/0	0/22
Kidney, deposition, hyaline droplet, proximal tubular cell, increased								
F0 Parents	0/24	0/23	0/24	2/24	0/24	0/0	0/0	0/24
F1 Parents	1/21	0/21	0/22	7/22*	0/21	0/0	0/0	0/22

Statistically significant: * $p \leq 0.05$; **: $p \leq 0.01$; Source: Hojo, 2017

(b) Developmental toxicity

Rat

In a preliminary teratogenicity study in rat, inpyrfluxam (purity 95.0%; Lot no. 13CG0617G) was administered orally, via gavage, to groups of seven or eight pregnant female Wistar Hannover rats once per day from gestation day (GD) 6 to 19 at dosages of 0, 20, 40 or 80 mg/kg bw per day to evaluate the potential effects on maternal rats and their fetuses. Dams were terminated on GD 20, and uterine contents examined. Mean body weights of dams were reduced during the treatment period, and body weight gains, food consumption and the adjusted body weights were significantly lower in dams treated at 80 mg/kg bw per day than those of the control group. Examination of the ovary and uterus at caesarean section revealed no differences in the mean gravid uterine weight between the treated and control groups, nor the mean numbers of corpora lutea, implantations or the percentage pre-implantation losses. There were no treatment-related effects on the mean number of live fetuses, percentage incidence of resorptions and fetal deaths, or sex ratio, and no abnormal fetuses were identified in any group at external examination. At 80 mg/kg bw per day mean body weights of male fetuses were lower to a statistically significant extent than controls, and those of female fetuses were also reduced. Based on these results, 80 mg/kg bw per day of inpyrfluxam TG was considered a suitable high dose for the main teratogenicity study in rats (Hojo, 2015b).

In the main teratogenicity study, inpyrfluxam (purity 95.0%; Lot no. 13CG0617G) was administered orally, via gavage, to groups of 22–24 pregnant female Wistar Hannover rats once per day from GD 6 to GD 19 at doses of 0, 10, 25 or 80 mg/kg bw per day. Dams were terminated on GD 20 and uterine contents examined.

No adverse clinical signs were observed throughout the study. The mean body weights on GDs GD 12, 18 and 20, body weight gains and food consumption during GDs GD 6–9 and thereafter, and the adjusted body weights, were slightly (but statistically significant) lower in dams treated at 80 mg/kg bw per day than in the control group, although the decrease in body weight was by less than 10% in comparison to controls. Examination of the ovary and uterus at caesarean section revealed no differences in mean gravid uterine weight between the treated and control groups, nor differences in the mean numbers of corpora lutea, implantations or the percentage of pre-implantation losses. There were no treatment-related effects on the mean number of live fetuses, percentage incidence of resorptions and fetal deaths, nor on the sex ratio. At 80 mg/kg bw per day mean body weights of fetuses of both sexes were significantly lower than controls.

Although several malformations and variations were observed in all groups, including the controls, there were no test substance-related differences in the incidence of abnormalities in any of the treated groups. Cyclopia with proboscis was observed in a single fetus of the 80 mg/kg bw per day

group. Although cyclopia is spontaneously observed, the incidence of cyclopia is quite low and this malformation had not appeared in the HCD of the testing facility. In addition, cyclopia was observed only in the high-dose group. Therefore, an additional teratogenicity study in rats was conducted to determine whether cyclopia was likely to be treatment related. The NOAEL for maternal toxicity was 25 mg/kg bw per day (Endo, 2017b).

To further investigate any possible relationship between treatment with inpyrfluxam TG and cyclopia, observed in one high-dose fetus in the previous prenatal developmental toxicity study (Endo, 2017a), groups of 39 (control group) or 40 (treated group) pregnant female Wistar Hannover rats were treated via gavage with inpyrfluxam TG (purity 95.0%; Lot no. 13CG0617G) once per day from GD 6 to 19 at doses of 0 or 90 mg/kg bw per day. Dams were terminated on GD 20 and uterine contents examined.

The mean body weights on and after GD 9, body weight gains and feed consumption during GDs 6–9 and thereafter, and the adjusted body weight (without gravid uterus) were significantly lower in the treated group than in the control group. The decrease in body weight at 90 mg/kg bw per day was comparable to that seen at 80 mg/kg bw per day in the main study. Additionally, one maternal animal treated at 90 mg/kg bw per day showed staggering gait on GD 12. Necropsy at GD 20 revealed no treatment-related abnormalities in the dams treated at 90 mg/kg bw per day. Examination of the ovary and uterus at caesarean section revealed a significant decrease in mean gravid uterine weight at 90 mg/kg bw per day compared to the control. No differences between the treated and control groups were recorded for the mean numbers of corpora lutea, implantations, nor of the percentage of pre-implantation losses. There were no treatment-related effects in the mean number of live fetuses, percentage incidence of resorptions and fetal deaths, nor differences in the sex ratio. At 90 mg/kg bw per day mean body weights of fetuses of both sexes were significantly lower than for controls. Although some external malformations were observed in both the control and the treated groups, there were no test substance-related differences in the incidence of abnormalities, and cyclopia was not observed in any fetuses from any groups.

In this second study, the number of examined fetuses had increased to 533 compared to 309 in the previous study. The tested dose was increased from 80 to 90 mg/kg bw per day to confirm that a maximal tolerated dose had been achieved. In addition to decreases in body weight, body weight gain and feed consumption, clinical signs were also observed in one maternal animal (Endo 2017b).

Based on the results of these studies, it was concluded that 25 mg/kg bw per day of inpyrfluxam TG was the (NOAEL) for maternal animals, based on decreased body weight in dams at 80 mg/kg bw per day, and their fetuses, based on decreased mean body weights of fetuses at 80 mg/kg bw per day. Inpyrfluxam TG has no teratogenic properties (Endo, 2017a). The single incidence of cyclopia, observed in the first study (Endo, 2017a), was considered not likely to be related to treatment with inpyrfluxam TG (Endo, 2017b).

Table 14a. Key findings of the first teratogenicity study in rats

		Dose (mg/kg bw per day)			
		0	**10**	**25**	**80**
Number of females mated		24	24	24	24
Non-pregnant		1	0	2	0
Maternal body weight (g) day 20		348	344	345	322**
Gravid uterine weight (g)		70	71	70	65
Maternal weight gain (g),	days 6–9	10	8	9	0**
	days 6–12	22	21	21	11**
	days 6–15	37	36	36	25**
	days 6–18	71	71	70	51**
	days 6–20	98	98	97	72

		Dose (mg/kg bw per day)			
		0	10	25	80
Food consumption (g/rat per day),	days 6–9	17.2	18.5	17.6	12.4**
	days 9–12	18.0	19.2	17.6	14.3**
	days 12–15	18.5	19.6	19.1	16.1*
	days 15–18	19.6	21.4	20.7	16.1**
	days 18–20	21.4	22.9	21.7	17.4**
Pre-implantation loss (%)		8.7	9.0	8.8	9.0
Resorptions and fetal deaths (%)		8.4	4.9	3.8	7.7
Mean litter size (live fetuses)		12.3	12.8	12.6	12.9
Sex ratio		0.509	0.500	0.552	0.495
Mean fetal weight (mg): males		3736	3592*	3634	3335**
Mean fetal weight (mg): females		3546	3409*	3514	3168**
Number of females with malformed fetuses [%]		4 [17.4]	1 [4.2]	3 [13.6]	2 [8.3]
Number of females with fetuses with variations [%]		23 [100]	24 [100]	22 [100]	24 [100]

Significantly different from the vehicle control group: * $p \leq 0.05$; ** $p \leq 0.01$; Source: Endo, 2017a

Table 14b. Key findings of the first teratogenicity study in rats; summary of malformations and variations; number of affected litters [number of fetuses affected]

	Dose (mg/kg bw per day)			
	0	10	25	80
Number of litters	23	24	22	24
Malformations				
External examination (total number of fetuses)	283	306	277	309
Fetuses with malformations	1, [1]	0	1, [1]	1, [1]
Local oedema	0	0	1, [1]	0
Meningocele	1, [1]	0	0	0
Cyclopia with proboscis	0	0	0	1, [1]
Visceral examination (total number of fetuses)	148	158	145	162
Fetuses with malformations	1, [1]	0	1, [1]	0
Microphthalmia	1, [1]	0	0	0
Overriding aorta	0	0	1, [1]	0
Skeletal examination (total number of fetuses)	135	148	132	147
Fetuses with malformations	3, [3]	1, [1]	2, [3]	1, [1]
Sternoschisis	1, [1]	0	0	0
Fused sternebra	0	0	0	1, [1]
Short rib (true rib; rib cartilage not fused to sternum)	1, [1]	0	0	0
Fused rib cartilage (true rib)	0	1, [1]	0	1, [1]
Cervical hemivertebra	1, [1]	0	0	0
Fused cervical centrum cartilage	1, [1]	0	0	1, [1]
Misshapen cervical centrum	0	0	1, [1]	0
Dumbbell-shaped cartilage of cervical centrum	0	0	2, [2]	0
Split cartilage of cervical centrum	1, [1]	0	0	0
Fused cervical arch	1, [1]	0	0	0

	Dose (mg/kg bw per day)			
	0	**10**	**25**	**80**
Variations				
Visceral examination (total number of fetuses)	148	158	145	162
Fetuses with variations	15, [25]	18, [29]	17, [35]	17, [31]
Thymic remnant in the neck	1, [1]	0	0	0
Dilated renal pelvis	2, [2]	0	1, [1]	1, [1]
Left umbilical artery	14, [22]	18, [29]	17, [34]	16, [30]
Skeletal examination (total number of fetuses)	135	148	132	147
Fetuses with variations	23, [101]	24, [105]	22, [101]	24, [114]
Zygomatic bone fused with maxilla	3, [3]	2, [2]	3, [3]	2, [2]
Bipartite ossification of sternebra	0	0	0	1, [1]
Cervical rib	1, [1]	4, [5]	5, [7]	1, [1]
Supernumerary rib	22, [82]	20, [80]	21, [80]	24, [97]
Percentage of fetuses with above variation	61%	76%	80%	85%
Wavy rib	3, [3]	0	0	0
Discontinuous rib cartilage (false rib)	21, [57]	19, [47]	17, [43]	22, [60]
Branched rib cartilage (false rib)	3, [3]	9, [10]	1, [1]	5, [5]
Posteriorization of cervical vertebra	0	0	2, [2]	0
Dumbbell ossification of thoracic centrum	0	2, [2]	1, [1]	1, [1]
Lumbosacral transitional vertebra	10, [12]	5, [8]	3, [6]	11, [14]
27 presacral vertebrae	6, [8]	6, [12]	6, [14]	8, [13]

Significantly different from the vehicle control group: * $p \leq 0.05$; ** $p \leq 0.01$; Source: Endo, 2017a

Table 15a. Key findings from the additional teratogenicity study in rats

		Dose (mg/kg bw per day)	
		0	**90**
Number of females mated		40	40
Non-pregnant		1	0
Maternal body weight (g)	day 20	355	325**
Maternal weight gain (g),	days 6–9	10	–4**
	days 6–12 (g)	22	7**
	days 6–15 (g)	38	19**
	days 6–18 (g)	72	47**
	days 6–20 (g)	99	70**
Feed consumption (g/rat per day),	days 6–9	19.8	13.2**
	days 9–12	19.2	12.9**
	days 12–15	21.2	15.5**
	days 15–18	21.5	16.1**
	days 18–20	21.6	17.1**

	Dose (mg/kg bw per day)	
	0	90
Pre-implantation loss (%)	10.7	6.1
Resorptions and fetal deaths (%)	4.3	5.1
Mean litter size (live fetuses)	13.0	13.3
Sex ratio	0.498	0.520
Mean fetal weight (mg), males	3568	3230**
Mean fetal weight (mg), females	3383	3081**
Number of females with malformed fetuses (%)	2 (5.1)	1 (2.5)

Significantly different from the vehicle control group: ** $p \leq 0.01$; Source: Endo, 2017b

Table 15b. Additional teratogenicity study in rats; summary of malformations

	Dose (mg/kg bw per day)	
Fetal finding	**0**	**90**
Number of females mated	39	40
External examination,		
Total number of fetuses	506	533
Fetuses with malformations [litters]	2, [2]	1, [1]
Microphthalmia [litters]	0	1, [1]
Thread-like tail [litters]	1, [1]	0
Gastroschisis [litters]	1, [1]	0

Source: Endo, 2017b

Rabbit

In a range finding teratogenicity study, inpyrfluxam TG (purity 95.0%; Lot no. 13CG0617G) was administered orally, via gavage, to groups of eight pregnant female Japanese White rabbits, once per day from GD 6 to GD 27 at dose levels of 0, 15, 50 or 150 mg/kg bw per day to evaluate the potential effects on maternal animals and their fetuses. Does were terminated on GD 28 and uterine contents examined. No effects were observed on any investigated parameter on either the maternal animals or the fetuses (Hojo, 2015c).

As no effects were observed in the initial range-finding study, an additional range-finding teratogenicity study was conducted. inpyrfluxam TG (purity 95.0%) was administered orally, via gavage, to groups of eight pregnant female Japanese White rabbits, once per day, from GD 6 to GD 27 at dose levels of 0, 300, 500, or 1000 mg/kg bw per day to evaluate the potential effects on maternal animals and their fetuses. Does were terminated on GD 28 and uterine contents examined. Severe maternal toxicity occurred at all dose levels. Four females treated at 1000 mg/kg bw per day were found dead on GD 6 or GD 7; the remaining females in this group were terminated on animal welfare grounds on GDs 6–7. One female treated at 500 mg/kg bw per day was found dead on GD 9; the remaining animals in this group were terminated due to animal welfare concerns on GDs 17–18. Severe clinical signs, including neurotoxic signs, were observed in females treated at 300 mg/kg bw per day. In addition, three females of this group showed signs of abortion, and were therefore terminated. Body weights, body weight gains, food consumption and adjusted body weights were significantly reduced in females treated at 300 and 500 mg/kg bw per day compared to controls.

Treatment at 300 mg/kg bw per day had no effects on mean gravid uterine weight, mean numbers of corpora lutea and implantations, or the percentage of pre-implantation losses. There were no treatment-related effects in the mean number of live fetuses, percentage incidence of resorptions and fetal deaths, sex ratio, fetal body weights, or external examination of live fetuses at 300 mg/kg bw per day. Based on these results and the results of the previous range finding study (Hojo, 2015c), 150–300 mg/kg bw per day was considered to be appropriate as a high dose in the definitive teratogenicity study in rabbits (Hojo, 2015d).

In the main developmental study in rabbits, inpyrfluxam (purity 95.0%; Lot no. 13CG0617G) was administered orally, via gavage, to groups of 23–25 pregnant female Japanese White rabbits, once per day, from GD6 to GD 27 at dose levels of 0, 20, 60 or 200 mg/kg bw per day. Does were terminated on GD 28 and uterine contents examined.

Maternal toxicity was observed in does treated at 200 mg/kg bw per day. The mean body weight gains were lower than those in the control group throughout the study and significant differences were recorded during GDs 6–9, 6–12, and 6–21. Mean food consumption also decreased significantly during gestation days 6–9, 9–12, 12–15, and 15–18. In addition, two animals aborted during the dosing period (one animal each on GDs 21 and 23); this was considered to be due to decreased body weight and food consumption, which was particularly marked in these two does. Examination of the ovary and uterus at caesarean section revealed no differences in mean gravid uterine weight between the treated and control groups, nor in the mean numbers of corpora lutea and implantations, or the percentage of pre-implantation losses. There were no treatment-related effects on the mean number of live fetuses, percentage incidence of resorptions and fetal deaths, sex ratio or fetal weight. Although several malformations and variations were observed in all groups, including the control, there were no test substance-related differences in the incidence of abnormalities in any treated groups. No rare malformations or variations were detected.

The NOAEL for inpyrfluxam TG was 60 mg/kg bw per day for maternal toxicity, based on decreased body weight gains, decreased mean food consumption and maternal abortion seen at 200 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 200 mg/kg bw per day, the highest tested dose. Inpyrfluxam TG has no teratogenic properties in rabbits (Endo, 2017c).

2.6 Special studies

(a) Neurotoxicity

In a preliminary acute neurotoxicity study, an aqueous solution of inpyrfluxam (purity 95.0%), suspended in 0.5% methylcellulose was administered once by gavage to groups of Wistar rats 6–7 weeks old (three/sex per dose) at dose levels of 0, 10, 30, 100, 200 or 400 mg/kg bw. Animals were observed daily for mortality and clinical signs for seven days after treatment. In addition, all animals were subjected to detailed clinical observations every hour for eight hours after dosing on the day of administration. At the end of the seven-day observation period all animals were euthanized and subjected to necropsy. One of three females treated at either 200 or 400 mg/kg bw was found dead and necropsied on the day of administration. The following detailed clinical observations were noted in females at 400 mg/kg bw on the day of administration (all hours post dose): changes in body position/posture (prone position at 1–2 h); tremors (at 1 h and 4–5 h); co-ordination of movement (ataxia at 1–2 h); secretions/excretions (red nose at 2 h); skin colour (paleness at 2 h); hypothermia (at 1–2 h); ananastasia (at 2 h); respiratory pattern (bradypnoea/laboured respiration at 1–6 h); and gait (staggering at 2–5 h) as well as decreases in spontaneous motor activity (at 3 h), muscle tone (at 1–2 h), alertness (at 1–3 h) and exploration (at 1–8 h). Similar changes in respiratory pattern (at one hour post dose) and decreases in muscle tone (at 1–3 h) and alertness (at 1 h) were also observed in males of the same group. Abnormal gait and decreases in muscle tone, alertness and exploration also were observed in females treated at 200 mg/kg bw. No abnormal changes related to administration of the test substance were seen in clinical observations, body weight or necropsy at any dose levels in either sex, including necropsy findings from the animals found dead. Based on these results, the time to peak effect was estimated to be 1–5 hours after administration. In addition, dose levels for the acute neurotoxicity study in rats were recommended to be around 200 mg/kg bw, at which level neurotoxic signs, but not excessive mortality, might be expected to occur (Shutoh, 2015).

In the main acute oral neurotoxicity study, an aqueous solution of inpyrfluxam (purity 95.0%), suspended in 0.5% methylcellulose, was administered once, by gavage, to groups of six-week-old Wistar Hannover rats (10/sex per dose) at dose levels of 0, 30, 100 or 200 mg/kg bw at a dose volume of 10 mL/kg. In general health condition observations, a decrease in muscle tone was observed in females treated at 200 mg/kg bw; this sign was observed from one day after the administration and disappeared by day 2. In functional tests, a significant decrease in body temperature was detected and a significant decrease in motor activity recorded on the day of administration at 0–10 minutes, 10–20 minutes, 20–30 minutes, and the total (60 minutes) of measurement in females at 100 and 200 mg/kg bw. No abnormal changes relating to the administration of the test substance were apparent at the detailed clinical

observations, nor any effect seen in body weights, necropsy or histopathological findings at any dose levels in either sex. The treatment-related changes in neurofunction that were observed in females at 200 and 100 mg/kg bw were, however, considered to be related to systemic toxicity, and no neurostructural changes were detected. The NOAEL for systemic toxicity was 30 mg/kg bw, based on reduced motor activity and body temperature at 100 mg/kg bw. The NOAEL for neurotoxicity was 200 mg/kg bw, the highest dose tested (Shutoh, 2016b).

Table 16. Acute neurotoxicity study in rats; functional test results in females on day of dosing

Dose (mg/kg bw per day)	Number of animals		Time period of observation						
			0–10	10–20	20–30	30–40	40–50	50–60	Total
0	10	Mean	1324	543	296	39	5	10	2215
		SD	505	468	281	98	10	16	968
30	10	Mean	994	212	58	45	79	60	1448
		SD	367	264	170	134	196	177	1007
100	10	Mean	532**	48*	4**	2	16	22	624**
		SD	240	76	13	4	29	60	201
200	10	Mean	558**	101*	61*	155	71	26	972*
		SD	357	164	83	205	121	45	577

SD: Standard deviation; Source: Shutoh, 2016b

Data were statistically analyzed by Dunnett's test following one-way ANOVA, or Dunnett-type test following Kruskal–Wallis test; Significantly different from control: *, $p \leq 0.05$; **, $p \leq 0.01$

In a sub-chronic dietary neurotoxicity study, inpyrfluxam (purity 95.0%) was administered via the diet to six-week-old Wistar Hannover rats (10/sex per dose) for a period of 91 days (13 weeks). Dose levels were 0, 500, 2000 or 4000 ppm for males (mean substance intakes, 30.0, 118.9 and 240 mg/kg bw per day), and 0, 500, 1000 or 2000 ppm for females (mean substance intakes: 35.2, 68 and 133 mg/kg bw per day). During the treatment periods, all animals were observed daily for mortality and general health condition; body weight and food consumption were measured weekly. In addition, detailed clinical observations and functional tests were performed once prior to initiation of treatment and at weeks 2, 4, 8, and 13 during the treatment period. After 13 weeks of treatment, five males and five females from each group were euthanized and underwent necropsy and histopathology. The animals that were not subjected to necropsy were euthanized by exsanguination under deep anaesthesia and discarded.

No treatment-related changes were observed in mortality, general health condition, detailed clinical observations, necropsy or histopathology.

In functional tests, a statistically significant decrease in forelimb grip strength was recorded in males treated at 4000 ppm at week 13. This change was not considered to be indicative of specific neurotoxicity because there were no changes from the detailed clinical observations that related to the central nervous system, such as changes in muscle tone, and in addition there were no other changes in functional tests nor histopathological findings.

Mean body weight was reduced (with statistical significance) throughout the treatment period in males treated at 4000 ppm, at weeks 6–13 in females at 2000 ppm, and at weeks 8 and 13 in females at 1000 ppm. Statistically significant decreases in food consumption were also noted in males at 4000 ppm during the treatment period (except for weeks 9 and 10), and in females at 2000 ppm at weeks 1, 2, 4, 5, 8, 11, and 13. In addition, a statistically significant decrease in food consumption was also noted in females at 1000 ppm in week 1; food consumption for this group, however, was recorded throughout the treatment period as being lower than for controls. These food and weight-related changes were considered to be toxic effects of the test substance. Therefore, the decreases in body weight and food consumption in males at 4000 ppm and females at 2000 and 1000 ppm were regarded as being treatment-related.

As described above, there were no neurostructural or neurofunctional changes in males treated at 4000 ppm, or in females treated at 2000 ppm, and no treatment-related neurological abnormalities were observed in any dose group.

The NOAEL for systemic toxicity was 500 ppm (equal to 35.2 mg/kg bw per day) based on decreased body weight and food consumption at 1000 ppm in females. The NOAEL for neurotoxicity was 2000 ppm (equal to 133 mg/kg bw per day), the highest dose tested (Motomura, 2016).

(b) Immunotoxicity

No data were available on immunotoxicity.

(c) Pharmacological studies

The effects of inpyrfluxam TG on pentetrazol/pentylenetetrazole-induced convulsions in rats, the respiratory and cardiovascular systems (effects on the respiratory rate, tidal volume, minute volume, blood pressure, heart rate and electrocardiogram in dogs) and autonomic nervous system (effects on the isolated Guinea pig ileum) were evaluated.

Administration of inpyrfluxam TG at 6, 20 or 60 mg/kg bw to female SD rats showed neither antagonist nor synergist effects towards pentetrazol injected intraperitoneally at 45 or 90 mg/kg bw four hours after treatment. There were no statistically significant differences in the number of animals showing clonic, tonic flexion or tonic extension convulsions for 30 minutes after pentetrazol administration (antagonism study), or in the number of animals showing clonic convulsions for 30 minutes (synergism study).

No statistically significant differences compared to controls were observed at 1, 2, 4, 8 or 24 hours after administration on the respiratory rate, tidal volume, minute volume, blood pressure, heart rate or electrocardiograms of male dogs treated with inpyrfluxam TG at 100, 300 or 1000 mg/kg bw. As was observed in standard toxicity studies, video recordings showed vomiting in 1/4, 3/4 or 4/4 dogs in the 100, 300 or 1000 mg/kg bw groups respectively, one to four hours after administration. Staggering gait was also observed in two dogs at 1000 mg/kg bw one to four hours after administration.

Significant decreases compared to the controls were recorded in the percentage contraction of isolated ileum induced by acetylcholine, histamine and barium chloride at 0.3, 3 and 30 µg/mL, and by serotonin at 3 and 30 µg/mL. As the intestinal smooth muscle contraction was inhibited to almost the same extent with all these agonists, the effect was attributed to a nonselective inhibitory effect rather than any specific selective inhibition of particular neurotransmitters (Imaizumi, 2015).

(d) Mechanistic studies

A non-GLP study was conducted to analyze the mode of action (MOA) of mouse hepatic and thyroid alterations induced by inpyrfluxam TG (purity 95.0%). In this study, alterations in the early phase of treatment with inpyrfluxam TG were evaluated, mainly focusing on hepatic gene expression, liver enzyme induction, thyroid hormones and morphology. BrdU-containing osmotic pumps were also implanted for possible future analysis of cell proliferation. Groups of male and female Crlj:CD1 (ICR) mice were fed diets containing 0 (control) or 7000 ppm of inpyrfluxam TG for seven and 14 day treatment periods. The dose level was identical to the highest dose in the mouse 78-week bioassay.

Treatment with inpyrfluxam TG caused no deaths and did not show excess toxicity in any phase of the study. Therefore, under conditions of the present study, treatment with inpyrfluxam TG did not confound evaluation of the main target end-points such as hepatic gene expression, liver enzyme induction, thyroid hormones and morphology.

Liver

Increased liver weight was observed in both sexes after each phase of treatment with inpyrfluxam TG. This change was accompanied by increased incidence of enlarged liver and centrilobular hepatocellular hypertrophy. An increase in Cyp2b10 mRNA expression was statistically significant in both sexes after each treatment period. Thus, it was considered that the effects of inpyrfluxam TG on mouse liver are due to enzyme induction in the liver.

Thyroid

Increased hepatic UDP-GT mRNA expression was observed in both sexes after each treatment period with inpyrfluxam TG. After the 14-day treatment, liver T4-UDP-GT activity of the S9 fraction was increased to a statistically significant extent in treated females. Although thyroid follicular cell hypertrophy and alteration of thyroid stimulating hormone (TSH) level were not observed in either sex after any treatment period with inpyrfluxam TG, triiodothyronine (T3) and/or thyroxine (T4) decreased (with statistical significance) in males after the 14-day treatment and in females after both 7- and 14-day treatments. Moreover, in the 90-day study in mice using inpyrfluxam TG (see section 2.2), there was observed increased liver weight accompanied by hepatocellular hypertrophy at 3500 ppm and above in both sexes and thyroid follicular cell hypertrophy at 7000 ppm in both sexes. It was suggested that effects of inpyrfluxam TG on mouse thyroid were sufficiently slight that no morphological change or alteration in TSH level occurred within the treatment durations in the present study. Based on these results, it was suggested that inpyrfluxam TG affected mouse thyroid by perturbation of the hypothalamus–pituitary–thyroid axis via induction of hepatic UDP-GT. Thus, it was considered that the effects of inpyrfluxam TG on the mouse thyroid are secondary to perturbation of the thyroid hormone axis.

In conclusion, the present study demonstrated that treatment with inpyrfluxam in mice causes increased liver weight with increased incidence of hepatocellular hypertrophy and enlarged liver, and induction of Cyp2b and UDP-GT. Furthermore, inpyrfluxam TG showed decreased T3 and/or T4 accompanied by UDP-GT induction. In view of this, the effects of inpyrfluxam on mouse thyroid in the 90-day study can be considered secondary to perturbation of the thyroid hormone axis. Thus, it is reasonable to conclude that inpyrfluxam TG's effects on the liver and thyroid are produced via hepatic enzyme induction (Kondo, 2017a).

Table 17. Detailed summary of results (mean of 5–10 mice, unless otherwise specified)

	Dietary concentration (ppm)							
	Males				**Females**			
	7-day treatment		**14-day treatment**		**7-day treatment**		**14-day treatment**	
Parameter	**0**	**7000**	**0**	**7000**	**0**	**7000**	**0**	**7000**
Liver weight								
Absolute (g)	1.98	2.37**	2.12	2.44**	1.59	2.01**	1.68	2.08**
Relative to body weight (%)	5.25	6.24**	5.40	6.26**	4.95	6.32**	5.03	6.17**
Necropsy (number of affected animals/number examined)								
Liver; enlarged	0/10	3/10	0/10	3/10	0/10	3/10	0/10	3/10
Thyroid; not remarkable	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10
Histopathology (number of affected animals/number examined)								
Liver								
Hypertrophy, hepatocyte, centrilobular	0/10	10/10**	0/10	10/10**	0/10	2/10	0/10	5/10*
Thyroid								
Not remarkable	10/10	10/10	10/10	10/10	9/10	10/10	8/10	9/10
Ectopic thymus	0/10	0/10	0/10	0/10	1/10	0/10	2/10	1/10
Gene expression (% of control)								
Cyp2b10	100	3471**	100	3699**	100	212**	100	254**
Ugt1a1	100	131*	100	121*	100	141**	100	124**
Ugt2b1	100	151*	100	154**	100	134*	100	104

	Dietary concentration (ppm)							
	Males				Females			
	7-day treatment		14-day treatment		7-day treatment		14-day treatment	
Parameter	0	7000	0	7000	0	7000	0	7000
Enzyme activity								
S9 protein(mg/g liver)	216	233**	222	222	229	219*	226	234
UDP-GT (pmol/min/mg S9 protein)	0.27	0.23**	0.19	0.18	0.30	0.23**	0.19	0.19
UDP-GT (pmol/min/g liver)	57.70	52.63	43.07	39.92	68.22	49.67**	43.27	44.35
UDP-GT (pmol/min/liver)	114.51	125.28	91.46	97.13	108.20	99.50	72.31	90.78*
Serum hormones								
TSH (ng/mL)	4.3	4.4	3.8	3.7	10.6	3.1	5.5	2.9
T3 (ng/mL)	0.8	0.6	0.6	0.6	0.8	0.6*	0.7	0.6
T4 (µg/dL)	12.2	9.9	15.8	11.0**	13.5	8.9**	13.0	7.5**

Shadowed cells indicate biologically significant changes; min: minute Source: Kondo, 2017a

TSH: Thyroid-stimulating hormone; T3: Triiodothyronine; T4: Thyroxine

Statistically significant at: *, p≤ 0.05; **, p≤0.01

A non-GLP study was performed to investigate the effect of inpyrfluxam (purity 95.0%) on thyroid-related hormones and metabolizing enzymes in the liver. Hepatic gene expression, liver enzyme induction, thyroid hormones and morphology were monitored following exposure. BrdU-containing osmotic pumps were also implanted for possible future analysis of cell proliferation. Groups of male and female RccHan:WIST rats were fed diets containing 0 (male and female controls), 2000 or 1500 ppm of inpyrfluxam TG for males and female respectively. Dosing continued for periods of 7, 14 and 28 days. Dose levels were identical to the highest dose in the 104-week rat bioassay (Ohtsuka, 2017). In addition, as a positive control for enzyme induction, 1000 ppm sodium phenobarbital (NaPB) groups were used in each phase of the study.

Treatment with inpyrfluxam TG caused no deaths. Although suppression of body weight gain and food consumption were observed in the inpyrfluxam TG-treated groups, no excessive toxicity was observed in any phase of the study. Therefore, under conditions of the present study, treatment with inpyrfluxam TG did not confound evaluation of the main target end-points such as hepatic gene expression, liver enzyme induction, thyroid hormones and morphology.

Liver

Increased liver weight was observed after each phase of inpyrfluxam TG treatment. This change was accompanied by enlarged livers in males after 14 and 28-day treatments, and hepatocellular hypertrophy in females after the seven-day treatment and in both sexes after the 14-day treatment. Rats treated with NaPB displayed enlarged livers and increased liver weight after each phase of treatment, and hepatocellular hypertrophy after the 7- and 14-day treatments (not assessed after the 28-day treatment). Increased levels of CYP (CYP2B and CYP3A) and UDP-GT mRNA expression (indicators of enzyme induction) were also observed after each phase of treatment with inpyrfluxam TG and with NaPB. A statistically significant increase in the hepatic T4 and UDP-GT activity of the S9 fraction was observed in females from the inpyrfluxam-treated group, and in both sexes from the NaPB-treated group after the 28-day treatment. Therefore, it was considered that effects of inpyrfluxam TG on rat liver are induced by enzyme induction in the liver, in a similar way to that in which NaPB operates.

Thyroid

Increased hepatic UDP-GT mRNA expression was observed in both sexes after each treatment with inpyrfluxam TG or NaPB. After the 28-day treatment, UDP-GT induction was observed in females of the inpyrfluxam TG-treated group and in both sexes of the NaPB-treated group. Although T3 and T4 levels were not changed after any treatment with inpyrfluxam TG or NaPB, TSH showed some increase

in males of the inpyrfluxam TG-treated group, and in both sexes of the NaPB-treated group, and thyroid follicular cell hypertrophy was induced. In females of the inpyrfluxam TG-treated group, TSH, T3 and T4 were not changed after treatment of any duration, however three animals out of ten showed thyroid follicular cell hypertrophy. This alteration indicates that serum hormone should have been affected. Thus, it is likely that slight alteration in serum hormone levels could not be detected under the conditions of this study.

Taken together, these results indicated that inpyrfluxam TG affected the rat thyroid by perturbation of the hypothalamus–pituitary–thyroid axis via induction of hepatic UDP-GT. Therefore the effects of inpyrfluxam TG on rat thyroid were considered secondary to perturbation of the thyroid hormone axis, a process which is similar to that observed with enzyme inducers such as NaPB.

In conclusion, the present study demonstrated that treatment with inpyrfluxam TG in rat causes increased liver weight with hepatocellular hypertrophy and/or enlarged liver and induction of CYP2B, CYP3A and UDP-GT. Furthermore, the present study demonstrated that inpyrfluxam TG induced hepatic UDP-GT and secondarily perturbed the hypothalamus–pituitary–thyroid hormone axis, which resulted in thyroid follicular cell hypertrophy. These effects are similar to those caused by NaPB, an enzyme inducer. Therefore, it is reasonable to conclude that effects on liver and thyroid by inpyrfluxam TG treatment are due to hepatic enzyme induction (Kondo, 2017b).

Table 18a. Mechanistic study of the effect of inpyrfluxam on rat liver and thyroid function; detailed summary of results in male rats (mean of 6–10 rats, unless otherwise specified)

	Dietary concentration (ppm)								
	7-day treatment			14-day treatment			28-day treatment		
	Control	S-2399 TG	NaPB	Control	S-2399 TG	NaPB	Control	S-2399 TG	NaPB
Parameter	0	2000	1000	0	2000	1000	0	2000	1000
Liver weight (mean of 6–10 rats)									
Absolute (g)	9.32	9.61	12.02**	10.88	12.61**	14.83**	12.38	13.21	16.65**
Relative to body weight (%)	4.80	5.13**	6.11**	4.59	5.27**	6.02**	4.06	4.36**	5.32**
Necropsy (number of affected animals/number examined)									
Liver: enlarged	0/10	0/10	10/10**	0/10	3/10	10/10**	0/10	4/10	10/10**
Thyroid: Not remarkable	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10
Histopathology (number of affected animals/number examined)									
Liver; hypertrophy, hepatocyte,									
centrilobular	0/10	0/10	10/10**	0/10	0/10	10/10**	-	-	-
diffuse	0/10	0/10	0/10	0/10	10/10**	0/10	-	-	-
Thyroid; hypertrophy, follicular cell,									
diffuse	-	-	-	0/10	7/10**	10/10**	0/10	3/10	10/10**
Gene expression (percentage of control)									
Cyp2b1/2	100	324**	34696**	100	207**	17888**	100	365**	57894**
Cyp3a1	100	588*	1191**	100	704**	1039**	100	413**	804**
Cyp3a2	100	139**	225**	100	148**	240**	100	137	222*
Ugt1a1	100	194**	184**	100	180**	188**	100	235**	230**
Ugt2b1	100	320**	1101**	100	270**	1351**	100	640**	2021**

	Dietary concentration (ppm)								
	7-day treatment			14-day treatment			28-day treatment		
	Control	S-2399 TG	NaPB	Control	S-2399 TG	NaPB	Control	S-2399 TG	NaPB
Parameter	0	2000	1000	0	2000	1000	0	2000	1000
Enzyme activity									
S9 (mg/g liver)	-	-	-	-	-	-	166	162	167
UDP-GT (pmol/min/mg S9 protein)	-	-	-	-	-	-	0.17	0.19	0.42**
UDP-GT (pmol/min/g liver)	-	-	-	-	-	-	28.83	31.10	69.75**
UDP-GT (pmol/min/liver)	-	-	-	-	-	-	354.00	407.33	1151.80**
Serum hormones									
TSH (ng/mL)	6.8	6.3	9.4	5.3	7.0	10.0*	6.0	8.4	13.9*
T3 (ng/mL)	0.7	0.7	0.7	0.7	0.7	0.6	0.6	0.6	0.6
T4 (µg/dL)	3.91	3.75	3.63	4.12	4.13	4.49	4.31	4.20	4.76

Shadowed cells indicate biologically significant changes; min: minute Source: Kondo, 2017b

TSH: Thyroid-stimulating hormone; T3: Triiodothyronine; T4: Thyroxine; - : Not evaluated at time point;

Statistically significant at: *, p≤ 0.05; **, p≤0.01

Table 18b. Mechanistic study of the effect of inpyrfluxam on rat liver and thyroid function; detailed summary of results in female rats (mean of 6–10 rats, unless otherwise specified)

	Dietary concentration (ppm)								
	7-day treatment			14-day treatment			28-day treatment		
	Control	S-2399 TG	NaPB	Control	S-2399 TG	NaPB	Control	S-2399 TG	NaPB
Parameter	0	1500	1000	0	1500	1000	0	1500	1000
Liver weight (mean of 6–10 rats)									
Absolute (g)	6.51	6.51	9.28**	6.90	7.10	8.96**	6.98	7.24	9.86**
Relative to body weight (%)	4.42	4.65	5.78**	4.19	4.57*	5.20**	3.63	3.92*	4.76**
Necropsy (number of affected animals/number examined)									
Liver: enlarged	0/10	0/10	9/10**	0/10	0/10	7/10**	0/10	0/10	10/10**
Thyroid: Not remarkable	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10
Histopathology (number of affected animals/number examined)									
Liver; hypertrophy, hepatocyte,									
centrilobular	0/10	0/10	9/10**	0/10	0/10	10/10**	-	-	-
diffuse	0/10	3/10	0/10	0/10	3/10	0/10	-	-	-
Thyroid; hypertrophy, follicular cell,									
diffuse	-	-	-	-	-	-	0/10	3/10	10/10**

	Dietary concentration (ppm)								
	7-day treatment			14-day treatment			28-day treatment		
	Control	S-2399 TG	NaPB	Control	S-2399 TG	NaPB	Control	S-2399 TG	NaPB
Parameter	0	1500	1000	0	1500	1000	0	1500	1000
Gene expression (percentage of control)									
Cyp2b1/2	100	335	17853**	100	172*	16738**	100	268*	96471**
Cyp3a1	100	832*	1023**	100	700**	907**	100	882**	1041**
Cyp3a2	100	394	3193*	100	174	4270*	100	327**	4450**
Ugt1a1	100	223**	154*	100	196**	126	100	225**	136
Ugt2b1	100	128	273**	100	172**	392**	100	192**	356**
Enzyme activity									
S9 (mg/g liver)	-	-	-	-	-	-	160	166	158
UDP-GT (pmol/min/mg S9 protein)	-	-	-	-	-	-	0.12	0.36**	0.18*
UDP-GT (pmol/min/g liver)	-	-	-	-	-	-	19.12	60.77**	28.68*
UDP-GT (pmol/min/liver)	-	-	-	-	-	-	133.32	439.55**	28834**
Serum hormones									
TSH (ng/mL)	4.5	3.8	6.6*	4.1	4.3	6.8**	5.2	5.5	6.3
T3 (ng/mL)	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
T4 (µg/dL)	2.52	2.47	2.38	2.95	2.79	3.22	2.99	2.96	3.34

Shadowed cells indicate biologically significant changes; Source: Kondo, 2017b

TSH: Thyroid-stimulating hormone; T3: Triiodothyronine; T4: Thyroxine; - : Not evaluated at time point;

Statistically significant at: *, p≤ 0.05; **, p≤0.01

Summary of mode of action of inpyrfluxam

Mechanistic studies were performed in rats and mice to determine whether the postulated mode of action of thyroid effects secondary to those in the liver, was applicable to inpyrfluxam TG. While the studies demonstrated enzyme induction in both species, the studies did not adequately demonstrate whether the postulated MOA was supported.

(e) Endocrine disruption potential

Study 1

Inpyrfluxam (purity 99.9%) was studied in mammalian cell-based luciferase reporter gene assays developed for detecting estrogenic, anti-estrogenic, androgenic and anti-androgenic effects on human estrogen receptor alpha (hERα) and human androgen receptor (hAR)-mediated mechanisms. Initially, inpyrfluxam was evaluated for cytotoxicity (in the range 100 pM–100 µM) to set the maximum analysable concentration, and 10 µM and 100 µM of inpyrfluxam showed cytotoxicity (equal or less than 80% cell viability). As a result, the maximum concentration of inpyrfluxam in both the hERα and hAR assays was set at 1 µM. In the reporter gene assay for hERα, positive controls for hERα (agonist 17β-estradiol; antagonist 4-hydroxytamoxifen) showed agonistic and antagonistic activities, respectively as expected. Inpyrfluxam displayed neither agonistic nor antagonistic activity in the assays. In the reporter assay for hAR, positive controls for hAR (agonist dihydrotestosterone; antagonist hydroxyfultamide) showed agonistic and antagonistic activities, respectively as expected. Inpyrfluxam showed neither agonistic nor antagonistic activity. Inpyrfluxam did not show any agonistic or antagonistic effects on hERα or hAR, indicating that inpyrfluxam does not have any effect in vitro on hERα- or hAR-mediated transcriptional activation (Kitamoto, 2017a).

Study 2

Inpyrfluxam (purity: 99.2%) was evaluated for its potential to affect androgen or estrogen production in H295R cells in vitro. Cultured cells in 24-well plates were treated with inpyrfluxam for 48 hours in two independent assays (Run l and Run 2) and production of testosterone and 17β-estradiol was measured using enzyme-linked immunosorbent assay (ELISA) kits. Before the steroidogenesis assay, the test substance was evaluated by the preliminary solubility test, cell viability assay and chemical interference test to set the highest concentration for the assay. The test substance was dissolved in culture medium up to 100 µM; cytotoxicity (20% or more reduction in viability) was observed at and above 10 µM, and no interference with ELISA kits was observed up to 3 µM of inpyrfluxam. Therefore, the highest concentration of inpyrfluxam in the steroidogenesis assay was set at 3 µM.

In both runs, inpyrfluxam was used at concentrations of 300 pM, 3 nM, 30 nM, 300 nM and 3 µM. No significant change of testosterone or 17β-estradiol production was observed at any concentration of inpyrfluxam in either run. Concurrent positive controls with the known inducer forskolin and the known inhibitor prochloraz, showed marked increase and decrease in the production of each hormone, respectively as expected. In addition, all quality control parameters met the criteria of relevant protocol criteria. Inpyrfluxam did not cause any increase or decrease in the production of testosterone or 17β-estradiol under the conditions of the present study, indicating that inpyrfluxam has no potential to affect androgen or estrogen production in vitro (Kitamoto, 2017b).

(f) Toxicity studies with metabolites

A number of the major metabolites identified in residue studies have not been investigated separately as they are covered by the reference values for the parent, since they are major metabolites in the rat. Such metabolites include:

- 1′-CH_2OH-S-2840,
- 1′-CH_2OH-S-2840A,
- 1′-CH_2OH-S-2840B,
- 1′,1′-bis(CH_2OH)-S-2840, and
- 1′-CH_2OH-3′-OH-S-2840.

Toxicity studies have been performed with the crop metabolites 3′-OH-S-2840 and 1′-COOH-S-2840, and are presented below. It is noted that these are also major metabolites in the rat. Other crop and soil metabolites identified are common to other succinate dehydrogenase inhibitor (SDHI) fungicides and in order to avoid repetition of available vertebrate studies, no further animal studies were conducted on these metabolites.

Metabolite 3′-OH-S-2840

In an acute oral toxicity study performed according to the toxic class method, 3'-OH-S-2840 (purity 99.6 %) was administered by gavage to fasted female RccHan:WIST rats in 0.5% (w/v) aqueous methylcellulose at dose levels of 2000 mg/kg bw and a dosing volume of 10 mL/kg bw. None of the six animals died, and no clinical signs were noted. Body weight and body weight gain were not affected by treatment. No treatment-related findings were observed at gross pathological examination. The acute oral LD_{50} for 3′-OH-S-2840 in female rats was greater than 2000 mg/kg bw (Hirano, 2017b).

In a 90-day toxicity study, groups Wistar Hannover rats (10/sex per dose) received 3′-OH-S-2840 (purity 99.7%) in basal diet at concentrations of 0, 500, 2000 or 4000 ppm (mean substance intakes: 0, 32.2, 128 and 258 mg/kg bw per day for males, 0, 37.9, 157 and 291 mg/kg bw per day for females). All animals were observed daily for mortality and general clinical signs and their body weights and food consumption recorded weekly. Detailed clinical observation was performed once prior to initiation of treatment and once a week during the treatment period. Functional observations were carried out on all animals at 11 weeks of treatment. Ophthalmology was conducted on all animals before initiation of treatment and on all animals in the control and high-dose groups at 13 weeks of treatment. Urinalysis was conducted at 13 weeks of treatment. After 13 weeks of treatment all animals underwent haematology, blood biochemistry, necropsy, and organ weight measurement. Histopathological examination was

performed on the systemic organs of males and females in the 0 and 4000 ppm groups, and the adrenal and all gross lesions from males and females, plus the liver and ovary from females in the 500 and 2000 ppm groups.

There were no treatment-related changes of toxicological significance in mortality, nor from general and detailed clinical observations, functional observations, food consumption, feed efficiency, ophthalmology or urinalysis for any dose groups of either sex. Body weight and the cumulative body weight gain throughout the treatment period of males treated at 4000 ppm were slightly lower than for the control group. Haematology revealed a significant prolongation in activated partial thromboplastin time in females at 4000 ppm. Blood biochemistry showed a significant increase or increasing tendency in GGTP in both sexes at 4000 and 2000 ppm. Females of the same groups also showed a significant increase in total cholesterol. At necropsy, dark-coloured livers were observed in a few males at 4000 ppm. Organ weight measurement revealed significant increases in absolute and/or relative weights of the liver in both sexes at and above 2000 ppm and of the adrenal in females at 4000 ppm. The incidences of centrilobular hepatocellular hypertrophy in the liver, and interstitial gland vacuolation in the ovary were significantly increased in females at 4000 ppm; the same lesions were observed in a few females at 2000 ppm. An increasing tendency in the incidence of increased vacuolation in cortical cells of the adrenal was noted in both sexes at 4000 ppm.

The NOAEL in this study was 500 ppm for males and females (32.2 mg/kg bw per day) based on decreased body weight and histopathological changes in the liver, ovary and adrenal at 2000 ppm (Koyama, 2018).

The toxicity of this metabolite was considered to be adequately covered by the parent.

Table 19. Results of genotoxicity studies with inpyrfluxam metabolite 3'-OH-S-2840

End-point	Test object	Concentration	Purity	Results	Reference
Point mutations	*Salmonella typhimurium* strains TA100, TA1535, TA98 and TA1537 and *Escherichia coli* strain WP2 uvrA	1.5–5000 µg/plate (± S9)	99.6%	Negative (± S9 Mix)	Wada, 2017a [a]
Gene mutation	Chinese hamster V79 cells – *HPRT* locus	3.8–60 µg/mL (± S9)	99.6%	Negative (± S9 Mix)	Chang, 2017a [b]
Chromosome aberration	Chinese hamster CHL/IU lung cells	25–100 µg/mL, short-term treatment (–S9) 50–200 µg/mL, (short-term treatment (+S9) 12.5–50 µg/mL continuous treatment (–S9)	99.6%	Negative (± S9 Mix)	Wada, 2017b [c]

S9: 9000 × *g* supernatant fraction from rat liver homogenate;

[a] Precipitation of 3′-OH-S-2840 was observed at and above 313 µg/plate in the absence of S9 activation, and at and above 1250 µg/plate in the presence of S9 activation. No cytotoxicity was observed.

[b] Maximum test concentration determined based on precipitation seen in solubility pretest. No cytotoxicity observed after 4 hours treatment, however precipitation observed at 30 µg/mL and above. Positive controls were included.

[c] Concentration levels selected based on results of preliminary cell growth inhibition test. Precipitation was noted at the highest concentration in each test condition.

Metabolite 1'-COOH-S-2840

In an acute oral toxicity study performed according to the toxic class method, 1′-COOH-S-2840 (purity 99.8 %) was administered by gavage to fasted female RccHan:WIST rats in 0.5% (w/v) aqueous methylcellulose at dose levels of 2000 mg/kg bw and at a dose volume of 10 mL/kg bw. None of the six animals died, and no toxicologically significant clinical signs were noted. Body weight and body weight gain were not affected by treatment. No treatment-related findings were observed at gross pathological examination. The acute oral LD_{50} for 1′-COOH-S-2840 in female rats was greater than 2000 mg/kg bw (Hirano, 2017c).

Table 20. Results of genotoxicity studies with inpyrfluxam metabolite 1'-COOH-S-2840

End-point	Test object	Concentration	Purity	Results	Reference
Ames test	*Salmonella typhimurium* strains TA100, TA1535, TA98 and TA1537 and *Escherichia coli* strain WP2 urvA	156–5000 µg/plate (± S9)	99.8%	Negative (± S9)	Matsuyama, 2017 [a]
Mammalian cells gene mutation	Chinese hamster V79 cells – *HPRT* locus	Experiment I: 125–2000 µg/mL, (±S9) Experiment II: 125–1000 µg/mL, (±S9)	99.8%	Negative (± S9)	Chang, 2017b [b]
Chromosome aberration	Chinese hamster CHL/IU lung cells	500–2000 µg/mL short-term treatment, (±S9) 500–1250 µg/mL continuous treatment, (–S9)	99.8%	Negative (± S9)	Kitamoto, 2017c [c]

S9: 9000 × *g* supernatant fraction from rat liver homogenate;

[a] No cytotoxicity observed in any strains either with or without metabolic activation. Precipitation observed at and above 2500 µg/plate in the presence of S9 activation.

[b] Exposure for 4 hours with and without S9 mix. Appropriate reference mutagens produced an increase in mutant colonies.

[c] Conducted with appropriate negative and positive controls.

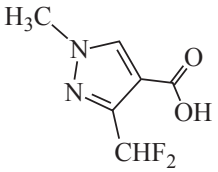
As this was identified as a major metabolite in plasma, as well as present in urine at >10% and to a lesser extent in faeces, it can be considered on this basis to be covered by the toxicity of the parent.

Metabolite DFPA:

Inpyrfluxam metabolite DFPA (SMILES code: FC(F)C1=NN(C)C=C1C(O)=O) is a plant-specific metabolite found in rotational crops (but not in bananas or potatoes). No data were submitted for this metabolite.

QSAR evaluation with OECD tool box plus simulated rat S9 metabolism in vivo, and simulated rat S9 metabolism in vitro, gave a positive alert for genotoxicity based on an acyl-halide group for two predicted metabolites. However, these alerts were also identified for the parent, inpyrfluxam. Since the parent was found to be negative in an adequate range of genotoxicity studies, DFPA is not considered to be genotoxic, and the human dietary exposure to DFPA should be limited to less than or equal to the Cramer Class III threshold of toxicity (TTC) value of 1.5 µg/kg bw per day.

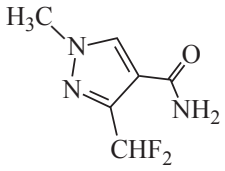
Figure 3. Chemical structure DFPA



Metabolite DFPA-CONH$_2$

Inpyrfluxam metabolite DFPA-CONH$_2$ (SMILES code: FC(F)C1=NN(C)C=C1C(N)=O) is a plant-specific metabolite found in rotational crops (but not in bananas or potatoes). No data were submitted for this metabolite. QSAR evaluation with OECD tool box plus simulated rat S9 metabolism in vivo and simulated rat S9 metabolism in vitro metabolism, gave a positive alert for genotoxicity based on an epoxide and azidine group for two predicted metabolites. However, these alerts were also identified for the parent inpyrfluxam. Since the parent was found to be negative in an adequate range of genotoxicity studies, DFPA-CONH$_2$ is not considered to be genotoxic, and the human dietary exposure to DFPA should be limited to less than or equal to the Cramer Class III TTC value of 1.5 µg/kg bw per day.

Figure 4. Chemical structure DFPA-CONH2



3. Observations in humans

Medical surveillance on manufacturing plant personnel

No information of effects on manufacturing plant personnel was submitted. Health checks on staff engaged in the synthesis, or for research and development, are routinely monitored at the manufacturing plants and no ill health related to inpyrfluxam TG has been detected by, or reported to medical staff (Nishimoto, 2018).

4. Microbiological aspects

The impact of inpyrfluxam residues on the human intestinal microbiome was evaluated through a decision-tree approach, adopted by the sixty-sixth meeting of the JECFA Committee for food-producing animal drugs, which complies with VICH GL36(R)2, and can also be used for pesticides (VICH, 2013).

Since there was no information/data found in the sponsor submission in this regard, and no information was available in the public domain, it was not possible for the Committee to determine if there is a need for the calculation of the microbiological acceptable daily intake (mADI) for inpyrfluxam. This conclusion also applies to the need to determine a microbiological acute reference dose (mARfD).

Comments

Biochemical aspects

Following administration of [^{14}C]inpyrfluxam to rats by gavage at a single dose of 1 or 150 mg/kg bw, or repeated doses of 1 mg/kg bw of radiolabelled material for 14 days, [^{14}C]inpyrfluxam was rapidly and nearly completely absorbed (c 97%). Concentrations in plasma then declined slowly with half-lives ranging between 12 and 17 hours. Most of the absorbed [^{14}C]inpyrfluxam was excreted in either urine (up to 59% in females) or bile (up to 73% in males) following all dosing regimens. In biliary excretion studies, less than 3% of the radioactivity was excreted in faeces. At 168 hours after oral administration, the tissue concentrations of radioactivity (including the carcass) were about 0.2% of the administered dose (AD) (Nagahori, 2016a).

Inpyrfluxam was extensively metabolized with over 40 metabolites detected respectively in urine, in bile and in faeces. The main routes of metabolism were *N*-demethylation, oxidation of the 1′,1′dimethyl group of the indane ring, followed by further oxidation to the carboxylic acid, and glucuronide conjugation (Nagahori, 2016b). Major rat metabolites are: 1′-CH_2OH-S-2840, 1′-CH_2OH-S-2840A, 1′CH_2OH-S-2840B, 1′,1′-*bis*(CH_2OH)-S-2840, 1′-CH_2OH-3′-OH-S-2840, as well as the *N*-des-Me form of 1′-CH_2OH-S-2840.

In vitro comparative metabolism studies conducted with liver microsomes from humans, male and female rats, and male and female dogs showed that there were no qualitative differences between species (although quantitative differences were apparent), and there was no unique human metabolite (Abe, 2018; Nagahori, 2016c).

Toxicological data

The acute oral median lethal dose (LD_{50}) for inpyrfluxam was 180 mg/kg bw, and the dermal LD_{50} was greater than 2000 mg/kg bw in rats. The inhalation median lethal concentration (LC_{50}) of inpyrfluxam was greater than 2.61 mg/L in rats. Inpyrfluxam was not a skin or eye irritant, was not skin sensitizing, nor phototoxic (Hirano, 2015a, b, c; Hirano, 2017a; Morimoto, 2015a, b, c; Nakagawa, 2016).

In mice and rats adaptive changes were seen in the liver. Thyroid effects were also observed. Adrenal effects were seen in rats and dogs, with dogs the most sensitive species.

In a 13-week dietary study in mice, inpyrfluxam was administered at dietary concentrations of 0, 200, 800, 3500 or 7000 ppm (equal to 0, 27, 111, 491 and 973 mg/kg bw per day for males, 0, 32, 130, 559 and 1097 mg/kg bw per day for females). The no-observed-adverse-effect level (NOAEL) was 3500 ppm (equal to 491 mg/kg bw per day) on the basis of follicular cell hypertrophy in the thyroid at 7000 ppm (equal to 973 mg/kg bw per day) (Shutoh, 2016a).

In a 13-week dietary study in rats, inpyrfluxam was administered at dietary concentrations of 0, 150, 500, 2000 or 4000 ppm (equal to 0, 9.72, 31.7, 123 and 255 mg/kg bw per day for males, 0, 11.5, 37.5, 144 and 292 mg/kg bw per day for females). The NOAEL was 500 ppm (equal to 37.5 mg/kg bw per day) based on decreased body weight, increased cortical cell vacuolation in the adrenals and interstitial gland vacuolation in the ovaries of females at 2000 ppm (equal to 292 mg/kg bw per day) (Ohtsuka, 2016).

In a 90-day oral capsule study in dogs, inpyrfluxam was administered at 0, 40, 160 or 700/500 mg/kg bw per day. The NOAEL of 40 mg/kg bw per day was based on zona fasciculata cell vacuolation in the adrenals of males at 160 mg/kg bw per day (Takahashi, 2016).

In a 12-month oral capsule study in dogs, inpyrfluxam was administered at 0, 2, 6, 30 or 160 mg/kg bw per day. The NOAEL was 6 mg/kg bw per day based on histopathological changes (zona fasciculata cell vacuolation) in the adrenal gland at 30 mg/kg bw per day (Motomura, 2017).

Mechanistic studies were performed in rats and mice which demonstrated hepatic enzyme induction (Kondo, 2017a, b).

In a carcinogenicity study in mice, inpyrfluxam was administered for 78 weeks at dietary concentrations of 0, 700, 2000 or 7000/5000 ppm (equal to 0, 77, 224 and 775 mg/kg bw per day

for males, 0, 69, 210 and 701 mg/kg bw per day for females). The top dose level was initially set at 7000 ppm, but due to severe effects on body weight it was reduced to 5000 ppm after one year. The NOAEL for toxicity was 700 ppm (equal to 69 mg/kg bw per day) based on an increase in centrilobular hepatocellular hypertrophy in males, amyloid nephropathy in both sexes, and an increase in the incidence of amyloidosis in cervical lymph nodes and glandular stomach in females at 2000 ppm (equal to 210 mg/kg bw per day). The compound was not carcinogenic up to 7000/5000 ppm, the highest dose tested (equal to 701 mg/kg bw per day) (Kitazawa, 2017).

In a 104-week dietary toxicity study, rats received inpyrfluxam at dietary concentrations of 0, 150, 500 or 1500/1000 in females (the top dose level being reduced at week 46). Males received 0, 150, 500 or 2000 ppm This was equal to 0, 5.85, 19.4 and 78.4 mg/kg bw per day for males, 0, 7.45, 25.5 and 65.9 mg/kg bw per day for females. The NOAEL for toxicity was 500 ppm (equal to 19.4 mg/kg bw per day), on the basis of decreased body weight gain and feed efficiency, haematological changes in both sexes, and decreased body weight in females at 1500/1000 ppm (equal to 65.9 mg/kg bw per day). The compound was not carcinogenic up to the highest dose tested, 1500/1000 ppm (equal to 65.9 mg/kg bw per day) (Ohtsuka, 2017).

The Meeting concluded that inpyrfluxam is not carcinogenic in mice or rats.

Inpyrfluxam was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found (Kitamoto, 2014a, b; Kitamoto, 2015; Wollny, 2014).

The Meeting concluded that inpyrfluxam is unlikely to be genotoxic.

In view of the lack of genotoxicity and the absence of carcinogenicity in mice and rats, the Meeting concluded that inpyrfluxam is unlikely to pose a carcinogenic risk to humans.

In a two-generation rat reproduction study, inpyrfluxam was administered over two consecutive generations at dietary concentrations of 0, 150, 500 or 1250 ppm for parental females (equal to 0, 10.9, 35.5 and 86 mg/kg bw per day), and 0, 150, 500 and 2000 ppm for parental males (equal to 0, 6.6, 22 and 93 mg/kg bw per day). The NOAEL for parental toxicity was 500 ppm (equal to 22 mg/kg bw per day), based on decreased body weights in females and decreased body weight gain in both sexes at the LOAEL of 1250 ppm (equal to 86 mg/kg bw per day). The NOAEL for reproductive toxicity was 1250 ppm (equal to 86 mg/kg bw per day) the highest dose tested. The NOAEL for offspring toxicity was also 500 ppm (estimated as approximately 22 mg/kg bw per day), based on decreased mean body weight of pups at 1250 ppm (estimated at 86 mg/kg bw per day) (Hojo, 2017).

In a developmental toxicity study in rats, inpyrfluxam was administered orally, via gavage, at doses of 0, 10, 25 or 80 mg/kg bw per day from gestation days (GDs) 6–19. The maternal NOAEL was 25 mg/kg bw per day, based on decreased adjusted body weight in dams at 80 mg/kg bw per day. The embryo/fetal NOAEL was 25 mg/kg bw per day, based on decreased mean body weights of fetuses at 80 mg/kg bw per day (Endo, 2017a). Based on a single incidence of cyclopia observed at 80 mg/kg bw per day a follow-up study was conducted at 0 and 90 mg/kg bw per day. This fetal abnormality was not evident in the second study at 90 mg/kg bw per day (Endo, 2017b).

In a developmental toxicity study in rabbits, inpyrfluxam was administered orally, via gavage, at doses of 0, 20, 60 or 200 mg/kg bw per day from GDs 6–27. The maternal NOAEL was 60 mg/kg bw per day based on decreased body weight gains, decreased mean food consumption and maternal abortion seen at 200 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 200 mg/kg bw per day, the highest dose tested (Endo, 2017c).

The Meeting concluded that inpyrfluxam is not teratogenic.

In an acute neurotoxicity study in rats inpyrfluxam was administered by gavage at doses of 0, 30, 100 or 200 mg/kg bw. The NOAEL for systemic toxicity was 30 mg/kg bw based on reduced motor activity and body temperature at 100 mg/kg bw The NOAEL for neurotoxicity was 200 mg/kg bw, the highest dose tested (Shutoh, 2016b).

A subchronic neurotoxicity study was carried out in rats in which inpyrfluxam was administered at dietary concentrations of 0, 500, 2000 or 4000 ppm for males (equal to 0, 30.0, 118.9 and 240 mg/kg bw per day), and 0, 500, 1000 or 2000 ppm for females (equal to 0, 35.2, 68 and 133 mg/kg bw per day). The NOAEL for neurotoxicity was 2000 mg/kg bw (equal to 133 mg/kg bw per day), the

highest dose tested. The NOAEL for systemic toxicity was 500 ppm (equal to 35.2 mg/kg bw per day) based on decreased body weight and food consumption at 1000 ppm (equal to 68 mg/kg bw per day) (Motomura, 2016).

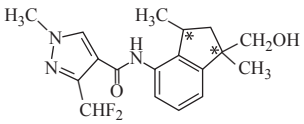
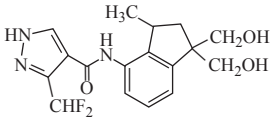
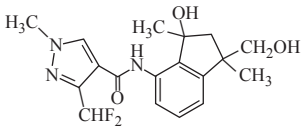
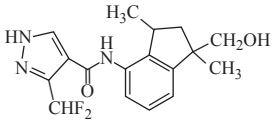
The Meeting concluded that inpyrfluxam is not neurotoxic.

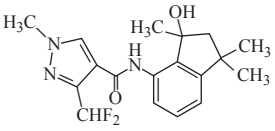
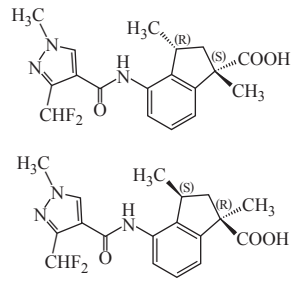
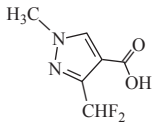
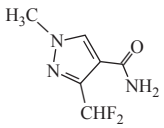
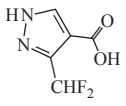
Inpyrfluxam's ability to interact with the estrogen, androgen and steroidogenesis pathways was investigated in two in vitro studies, and no effects were observed (Kitamoto, 2017a, b).

Toxicological data on metabolites and/or degradates

The metabolites of inpyrfluxam are summarized in the table below.

Summary overview of toxicological characterization of plant/livestock metabolites

Compound, codes and structure	**Rat ADME** Toxicity covered by toxicological properties of parent (> 10% AD in rat biofluids or 10%TRR)?	**Genotoxicity assessment** (data, QSAR, read-across)	**General toxicity**	**HBGV**
1′-CH_2OH-S-2840, 1′-CH_2OH-S-2840A, and 1′-CH_2OH-S-2840B  (All stereoisomers of above; differ only in disposition around two centres of chirality marked *)	Yes Present in bile at 29%, (glucuronide form) in males and 14.5% in females Detected in plasma, liver and kidney In vitro metabolism; 36% in human, 41% in male rat, 16% in male dog, 11% in female dog	Not genotoxic	Covered by parent	Covered by parent
1′,1′-bis(CH_2OH)-S-2840 	Yes Urine; 11% in males, 6.1% in females	No	Covered by parent	Covered by parent
1′-CH_2OH-3′-OH-S-2840 	Yes Bile as mixture with N-des-Me-1′-CH_2OH-S-2840 at up to 20.2% in males and 21.1% in females	No	Covered by parent	Covered by parent
N-des-Me-1′-CH_2OH-S-2840 	Yes Urine at 7.3% in females Bile as mixture with 1′-CH_2OH-3′-OH-S-2840 at up to 20.2% in males and 21.1% in females	No	Covered by parent	Covered by parent

Compound, codes and structure	Rat ADME Toxicity covered by toxicological properties of parent (> 10% AD in rat biofluids or 10%TRR)?	Genotoxicity assessment (data, QSAR, read-across)	General toxicity	HBGV
3′-OH-S-2840 	No Detected in plasma, liver and kidney in male rats Less than 10% in vitro metabolism in rats and humans, < 5% in dogs	Negative in a range of in vivo and in vitro tests (Chang, 2017a; Wada, 2017a; Wada, 2017b)	LD_{50} in rat > 2000 mg/kg bw (Hirano, 2017b); 90-day dietary study in rats, at 0, 500, 2000 or 4000 ppm (= 0, 32.2, 128 and 258 mg/kg bw/day for males, 0, 37.9, 157 and 291 mg/kg bw/day for females) (Koyama, 2018); NOAEL 500 ppm (32.2 mg/kg bw/day) based on decreased body weight and histopathological changes in liver, ovary and adrenal at 2000 ppm	Covered by parent
1′-COOH-S-2840 (cis and trans) 	Yes Urine at up to 15% in males, 10.5% in females Detected in plasma, liver and kidney	Negative in a range of in vivo and in vitro tests (Chang, 2017b; Kitamoto, 2017a; Matsuyama, 2017)	LD_{50} in rat > 2000 mg/kg bw Considered similar to parent (Hirano, 2017c)	Covered by parent
DFPA 	No	No QSAR alerts not produced by parent	No data	Cramer class III [a]
DFPA-$CONH_2$ 	No	No QSAR alerts not produced by parent	No data	Cramer class III [a]
N-des Me DFPA 	No	No QSAR alerts not produced by parent	No data	Cramer class III [a]

[a] Cramer class III: threshold of toxicological concern (TTC) is 1.5 µg/kg bw per day

Microbiological data

There was no information available in the public domain and no experimental data were submitted that addressed the possible impacts of inpyrfluxam residues on the human intestinal microbiome.

Human data

No information was provided on the health of workers involved in the manufacture or use of inpyrfluxam.

The Meeting concluded that the existing database on inpyrfluxam was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.06 mg/kg bw based on the NOAEL of 6 mg/kg bw per day in the one-year dog study and using a safety factor of 100.

The Meeting established an acute reference dose (ARfD) of 0.3 mg/kg bw based on the NOAEL of 30 mg/kg bw in the acute neurotoxicity study in rats and using a safety factor of 100.

Levels relevant to risk assessment of inpyrfluxam

Species	Study	Effect	NOAEL	LOAEL
Mouse	78-week carcinogenicity [a]	Toxicity	700 ppm, equal to 69 mg/kg bw/day	2000 ppm, equal to 210 mg/kg bw/day
		Carcinogenicity	7000/5000 ppm equal to 701 mg/kg bw/day [d]	-
Rat	104-week toxicity and carcinogenicity [a]	Toxicity	500 ppm equal to 19.4 mg/kg bw/day	1500/1000 ppm equal to 65.9 mg/kg bw/day
		Carcinogenicity	1500/1000 ppm equal to 65.9 mg/kg bw/day [d]	-
	Two-generation reproductive toxicity study [a]	Reproduction/fertility	1250 ppm equal to 86 mg/kg bw/day [d]	-
		Parental toxicity	500 ppm equal to 22 mg/kg bw/day	1250 ppm equal to 86 mg/kg bw/day
		Offspring toxicity	500 ppm estimated at 22 mg/kg bw per day	1250 ppm estimated at 86 mg/kg bw/day
	Developmental toxicity study [b]	Maternal toxicity	25 mg/kg bw/day	80 mg/kg bw/day
		Developmental toxicity	25 mg/kg bw/day	80 mg/kg bw/day
	Acute oral neurotoxicity study [b]	Systemic toxicity	30 mg/kg bw	100 mg/kg bw
		Neurotoxicity	200 mg/kg bw [d]	-
	90-day neurotoxicity study [b]	Systemic toxicity	500 ppm equal to 35.2 mg/kg bw/day	1000 ppm equal to 68 mg/kg bw/day
		Neurotoxicity	2000 ppm equal to 133 mg/kg bw/day [d]	-
Rabbit	Developmental toxicity study [b]	Maternal toxicity	60 mg/kg bw/day	200 mg/kg bw/day
		Developmental toxicity	200 mg/kg bw/day [d]	-
Dog	1-year toxicity study [c]	Toxicity	6 mg/kg bw/day	30 mg/kg bw/day

[a] Dietary administration; [b] Gavage administration; [c] Gelatine capsule administration; [d] Highest dose tested

*Acceptable daily intake (ADI) for inpyrfluxam**

0–0.06 mg/kg bw

*Acute reference dose (ARfD) for inpyrfluxam**

0.3 mg/kg bw

* Applies to inpyrfluxam, 1′-CH_2OH-S-2840 (free or conjugated), 1′-CH_2OH-S-2840A, 1′-CH_2OH-S-2840B, 1′,1′-bis(CH_2OH)-S-2840, 1′-CH_2OH-3′-OH-S-2840, 3′-OH-S-2840 and 1′-COOH-S-2840

Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure.

Critical end-points for setting guidance values for exposure to inpyrfluxam

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Fast; T_{max} ca 1 hour; almost entirely absorbed (c 97%)
Dermal absorption	No data available
Distribution	Distributed according to relative organ blood flow; highest amounts in liver, kidney, adrenals and heart
Rate and extent of excretion	Urinary and faecal elimination via the bile (c 97%); urinary excretion (up to 59%)
Potential for accumulation	No evidence of accumulation
Metabolism in mammals	Extensively metabolized. Main routes: *N*-demethylation, oxidation of the 1′,1′-dimethyl group of the indane ring followed by further oxidation to carboxylic acid, and glucuronide conjugation Major metabolites: 1′-CH_2OH-S-2840 (and its conjugates), 1′-CH_2OH-S-2840A, 1′-CH_2OH-S-2840B, 1′,1′-bis(CH_2OH)-S-2840, 1′-CH_2OH-3′-OH-S-2840
Toxicologically significant compounds (animals, plants, and the environment)	Inpyrfluxam, 1′-CH_2OH-S-2840 (free or conjugated), DFPA, DFPA-$CONH_2$, N-desmethyl-DFPA
Acute toxicity	
Rat LD_{50} oral	180 mg/kg bw
Rat LD_{50} dermal	> 2000 mg/kg bw
Rat LC_{50} inhalation	> 2.61 mg/L air analytically determined (maximum attainable concentration)
Rabbit, skin irritation	Non-irritating
Rabbit, eye irritation	Mildly irritating
Guinea pig, skin sensitization	Non-sensitizing (Magnusson & Kligman)
Short-term studies of toxicity	
Target/critical effect	Thyroid follicular cell hypertrophy (mouse) Vacuolization in adrenals (rat, dog) and ovaries (rat)
Lowest relevant oral NOAEL	6 mg/kg bw per day (dog)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Systemic amyloidosis (mouse) Reduced body weights, feed efficiency, haematological changes (rat)
Lowest relevant oral NOAEL	19.4 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic
Genotoxicity	Unlikely to be genotoxic
Reproductive toxicity	
Reproduction target/critical effect	Reduced body weight (gains) in parental animals and pups
Lowest relevant parental NOAEL	500 ppm (equal to 22 mg/kg bw per day) (rat)
Lowest relevant offspring NOAEL	500 ppm (approx. equivalent to 22 mg/kg bw per day) (rat)
Lowest relevant reproductive NOAEL	1250 ppm (equal to 86 mg/kg bw per day) (rat)

Developmental toxicity	
Developmental target/critical effect	**Maternal**: body weights (rat, rabbit) feed consumption and abortions (rabbit) **Embryo/fetal**: fetal body weights (rat)
Lowest relevant maternal NOAEL Lowest relevant embryo/fetal NOAEL	25 mg/kg bw per day (rat) 25 mg/kg bw per day (rat)
Neurotoxicity	
Acute neurotoxicity NOAEL	200 mg/kg bw, the highest dose tested (rat)
Subchronic neurotoxicity NOAEL	90-day NOAEL: 133 mg/kg bw per day, highest dose tested (rat)
Developmental neurotoxicity	No data
Other toxicological studies	
Endocrine disruption potential	No effect in vitro on estrogen, androgen or steroidogenesis pathways
Mechanism studies	Effects on liver and thyroid in rodents may be related to hepatic enzyme induction
Studies on toxicologically relevant metabolites	
3′-OH-S-2840 and 1′-COOH-S-2840	Acute oral LD_{50} > 2000 mg/kg bw (rat)
3′-OH-S-2840	90-day oral NOAEL: 500 ppm equal to 32.2 mg/kg bw per day (rat)
3′-OH-S-2840 and 1′-COOH-S-2840	Negative reverse mutation test in bacterial systems Negative mammalian cell gene mutation Negative chromosome aberration
Microbiological data	No data available
Human data	Limited information; inpyrfluxam is a new substance

Summary

	Value	Study	Safety factor
ADI [a]	0–0.06 mg/kg bw	One-year study (dog)	100
ARfD [a]	0.3 mg/kg bw	Acute neurotoxicity study (rat)	100

[a] Applies to inpyrfluxam, 1′-CH_2OH-S-2840 (free or conjugated), 1′-CH_2OH-S-2840A, 1′-CH_2OH-S-2840B, 1′,1′-bis(CH_2OH)-S-2840, 1′-CH_2OH-3′-OH-S-2840, 3′-OH-S-2840 and 1′-COOH-S-2840

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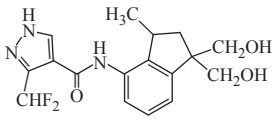
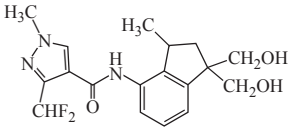
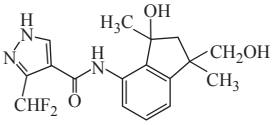
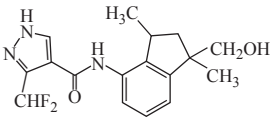
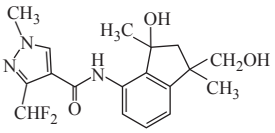
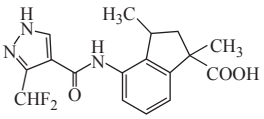
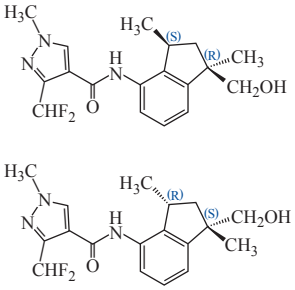
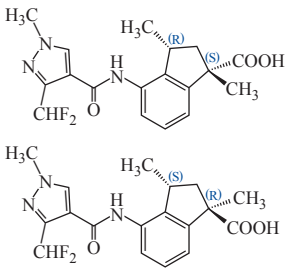
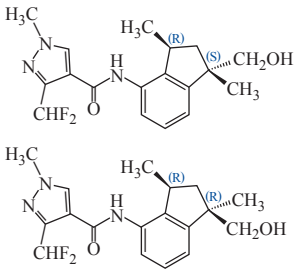
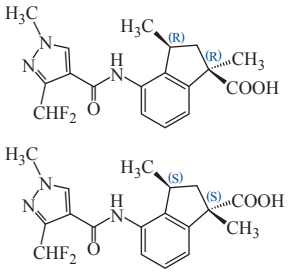
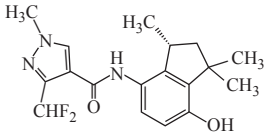
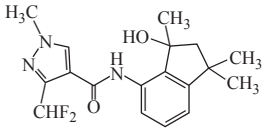
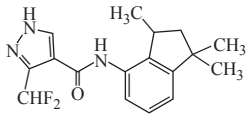
Annex 1 Metabolites of inpyrfluxam

Table 1. Metabolites identified in metabolism studies

Name and abbreviation	Chemical structure	Detected in:
N-des-Me-1′,1′-bis(CH_2OH)-S-2840		Urine (10.5% M, 7.2% F) Faeces Bile Liver Kidney
1′,1′-bis(CH_2OH)-S-2840		Urine (11% M, 6.3%F) Faeces Bile (male) Plasma Liver Kidney
N-des-Me-1′-CH_2OH-3′-OH-S-2840		Urine Faeces Bile Liver Kidney (male)
N-des-Me-1′-CH_2OH-S-2840		Urine Faeces Bile (free and glucuronide) (20.2%) Plasma Liver Kidney Rat and human microsomes
1′-CH_2OH-3′-OH-S-2840		Urine Faeces Bile (free and glucuronide) Plasma Liver Kidney Rat microsomes
N-des-Me-1′-COOH-S-2840		Urine (10.2% M, 21% F) Faeces Bile Plasma (male) Liver Kidney
1′- CH_2OH-S-2840A (two stereoisomers)		Urine Faeces Bile (free and glucuronide) (29%) Plasma Liver Kidney Rat, dog and human microsomes

Name and abbreviation	Chemical structure	Detected in:
1′-COOH-S-2840A (two stereoisomers)		Urine (15% M, 10.5% F) Faeces Bile Plasma Liver Kidney
1′-CH_2OH-S-2840B (two stereoisomers)		Urine Faeces Bile (free and glucuronide) Plasma Liver Kidney Rat, dog and human microsomes
1′-COOH-S-2840B (two stereoisomers)		Urine Faeces Bile Plasma Liver Kidney
7′-OH-S-2399		Faeces Liver Kidney Rat, dog and human microsomes
3′-OH-S-2840		Plasma (male) Liver Kidney (male) Rat, dog and human microsomes
N-des-Me-S-2840		Faeces Plasma Liver Kidney Rat, dog and human microsomes

Isoflucypram

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Explanation

Isoflucypram (BCS-CN88460) is the International Organization for Standardization (ISO)-approved common name for *N*-(5-chloro-2-isopropylbenzyl)-*N*-cyclopropyl-3-(difluoromethyl)-5-fluoro-1-methyl-*1H*-pyrazole-4-carboxamide, with the Chemical Abstracts Service number 1255734-28-1. Isoflucypram is a novel broad-spectrum fungicide. The chemical class is *N*-cyclopropyl-*N*-benzyl-pyrazole-carboxamides and it is a succinate dehydrogenase inhibitor. Isoflucypram has not been

evaluated previously by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and was reviewed by the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR). All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with relevant national or international test guidelines, unless otherwise specified. No additional information from a literature search was identified that complemented the toxicological information submitted for the current assessment.

Evaluation for acceptable daily intake

1. Biochemical aspects

In total five studies were conducted to identify the biochemical aspects of isoflucypram (also known by code BCS-CN88460). In two absorption, distribution, metabolism and excretion (ADME) studies in female and male rats, phenyl- or pyrazole-radiolabelled isoflucypram was used (Fig. 1; Bongartz & Bartelsen, 2017a, b). Whole-body autoradiographic distribution was investigated in two studies using male and female rats (Bongartz & Bartelsen, 2017c; Fernyhough & Kendrick, 2017). In addition to these studies an in vitro comparative metabolism study was also conducted (Lagojda & Doebbe, 2017). A study of the metabolic fate of metabolite isoflucypram-carboxylic acid (BCS-CY26497, M12) was conducted using male rats; for details see section 2.6(c) (Bongartz & Bartelsen, 2017d).

Figure 1. Position of radiolabels of isoflucypram

[*Pyrazole-4*-^{14}C]isoflucypram

[*Phenyl-UL*-^{14}C]isoflucypram

* indicates position of ^{14}C label

1.1 Absorption, distribution and excretion

(a) Oral route

In the first ADME study (Bongartz & Bartelsen, 2017a), the toxicokinetic behaviour (absorption, kinetics and excretion) and metabolism of isoflucypram (purity 98.4%; Lot/batch no. NLL 8674-19-4) were investigated. Four male and four female Wistar Unilever HsdCpb rats in each group were administered by oral gavage [*pyrazole-4*-^{14}C]isoflucypram (Fig. 1; radiobiochemical purity >98%) as a single low or single high dose (2 or 200 mg/kg bw, respectively) or a single low dose after bile duct cannulation. For the single high dose, the radiolabelled test compound was mixed 1:99 with unlabelled isoflucypram (purity, 98.4%; Lot/batch no. NLL 8674-19-4). Four male rats received by gavage a 14-day pretreatment of unlabelled isoflucypram (purity, 98.4%; Lot/batch no. NLL 8674-19-4) and the radiolabelled isoflucypram at a low dose. The test compound was suspended in an aqueous solution of tragacanth at pH 4. The rats were sacrificed three days post dosing, but in the case of bile duct-cannulated rats, two days post dosing. The total radioactivity represented the radioactivity related to the test compound and its metabolites, and this was determined in plasma, urine, bile, faeces, organs, and tissue samples at sacrifice. Metabolites were investigated in urine, bile, and extracts of faeces.

In the second study (Bongartz & Bartelsen, 2017b), the test compound (radiochemical purity, greater than 98%) was labelled in the phenyl moiety of the molecule; [*phenyl-UL*-^{14}C]isoflucypram. The test compound was administered to four male and four female Wistar (Hsd/Cpb: WU) rats at the low dose of 2 mg/kg bw, by oral gavage as a suspension in an aqueous solution of tragacanth at pH 4. The measurement of radioactivity in plasma, urine, faeces, bile, and lyophilized tissue was carried out by liquid scintillation counting (LSC).

Absorption

In the first ADME study (Bongartz & Bartelsen, 2017a), between 100.3% and 104.5% of the administered radioactivity (AR) was recovered according to measurement of the total radioactivity in plasma samples, urine, and faeces, as well as in bile, organs and tissues, at sacrifice.

The absorption of [*pyrazole-4*-^{14}C]isoflucypram started immediately after administration as shown by the concentration profile of the radioactivity in the plasma of the low-dose rats (2 mg/kg bw). For all low dose tests, including male rats pretreated with unlabelled isoflucypram over 14 days, the maximum plasma concentration (C_{max}) was reached within one hour post administration, the time to maximum concentration T_{max}. In the high dose tests using 200 mg/kg bw of isoflucypram, the mean maximum plasma concentration was measured as two hours after dosing for male rats and at four hours after dosing for females. The time course of the mean plasma levels was comparable between male and female rats in all the tests. Radioactivity could be detected in all plasma samples until 72 hours after dosing, the latest time of plasma sampling, values ranging from approximately 0.2% to 3.1% of the maximum plasma concentration measured. Low dose tests with bile duct-cannulated male and female rats showed that about 74% of the dose recovered was detected in the bile of male animals, and 82% in the bile of females. Absorption rates were calculated by summation of the recovered radioactivity in urine, bile, and body without gastrointestinal tract (GIT), and amounted to 79.9% of administered dose (AD) for male rats and 84.1% AD for female rats, (Table 1).

In the second ADME study (Bongartz & Bartelsen, 2017b), the absorption of [*phenyl-UL*-^{14}C]isoflucypram started immediately after oral administration, as shown by the concentration profile of the radioactivity in the plasma at individual time points. For both tests the mean plasma C_{max} was reached at one hour after administration (its T_{max}) with a mean concentration of 0.255 µg/mL in male rats and 0.265 µg/mL in female rats. The time course of the mean plasma levels was comparable between male and female rats. Radioactivity (below 1.93% of the maximum plasma concentration measured) could be detected in all plasma samples until 72 hours after dosing, the latest time of plasma sampling (Table 1).

Table 1. Percentage of total radioactive dose recovered (mean values, oral administration) in rats treated with [pyrazol-l-^{14}C] isoflucypram or [phenol-UL-^{14}C]isoflucypram

	Radiolabel position, dose and sex								
	[pyrazol-l-^{14}C]isoflucypram							**[phenol-UL-^{14}C] isoflucypram**	
	2 mg/kg bw		**200 mg/kg bw**		**2 mg/kg bw pretreatment**	**2 mg/kg bw bile duct-cannulated**		**2 mg/kg bw**	
Sex	**M**	**F**	**M**	**F**	**M**	**M**	**F**	**M**	**F**
Urine	13.57	11.98	7.06	8.44	12.31	5.60	2.26	10.66	7.99
Bile	NSC	NSC	NSC	NSC	NSC	74.10	81.64	NSC	NSC
Faeces	86.03	87.83	92.77	91.39	87.36	20.00	14.94	88.98	91.75
Total excreted	99.61	99.81	99.83	99.83	99.67	99.71	98.84	99.64	99.74
Body excluding GIT	0.365	0.172	0.152	0.153	0.286	0.241	0.209	0.283	0.223
GIT	0.029	0.016	0.017	0.012	0.044	NC	0.952	0.076	0.034
Total in body	0.394	0.188	0.169	0.165	0.329	0.295	1.162	0.359	0.257
Norm. factor	0.964	0.997	0.993	0.993	0.971	0.959	0.957	0.978	0.954
Absorption rate (%)	-	-	-	-	-	79.9	84.1	-	-

M: Male; F: Female; NC: not calculated; NSC: no sample collected; Source: Bongartz & Bartelsen, 2017a,b

GIT: gastrointestinal tract:

Norm. factor: For all steps during the preparation of samples (extraction and clean-up), the values of individual fractions were normalized to 100% of recovered radioactivity.

Kinetics

In the first ADME study (Bongartz & Bartelsen, 2017a), the distribution of the test substance and its metabolites from the central compartment (blood plasma) into the different organs and tissues was investigated by measuring the concentration of the total radioactivity in plasma. After a single oral administration of the low dose [*pyrazole-4-*^{14}C]isoflucypram to male and female rats, C_{max} was observed at one hour post administration. In the high-dose tests, C_{max} was reached at two hours for male rats and at four hours for females. For all tests the plasma concentration declined to values below 3.1% of C_{max} within 72 hours of administration. This indicates no retention occurred of test compound-related residues in the bodies of the animals. The plasma concentrations in the low- as well as the high-dose tests were calculated using a two-compartment model. The weighting of $1/y^2$ was used due to the two-phase nature of the process; a fast elimination phase after reaching plasma C_{max} followed by a slower elimination phase after approximately 24 hours. Relevant pharmacokinetic parameters are shown in Table 2.

In the second ADME study (Bongartz & Bartelsen, 2017b), after a single oral administration of low dose [*phenyl-UL-*^{14}C]isoflucypram to male and female rats, the dose-normalized mean maximum plasma concentrations of radioactivity were measured one hour post administration, and amounted to 0.255 µg/mL for males and 0.265 µg/mL for females. For both tests the concentration of total radioactivity in plasma declined to values below 1.93% of T_{max} within 72 hours post administration. This indicates no retention occurred of compound-related residues in the bodies of the animals. The plasma concentration was calculated using a two-compartment model. The weighting of $1/y^2$ was used due to the two-phase nature of the process; a fast elimination phase after reaching plasma C_{max} followed by a slower elimination phase after approximately 24 hours. Relevant pharmacokinetic parameters are shown in Table 2.

Table 2. Pharmacokinetics following administration of [pyrazole-4-^{14}C]isoflucypram and [phenyl-UL-^{14}C]isoflucypram in rats (dose-normalized kinetic parameters)

	Radiolabel, dose level and sex						
	[*pyrazole-4-*^{14}C]isoflucypram					[*phenyl-UL-*^{14}C] isoflucypram	
	2 mg/kg bw		200 mg/kg bw		Pretreatment 2 mg/kg bw	2 mg/kg bw	
Parameter	**Male**	**Female**	**Male**	**Female**	**Male**	**Male**	**Female**
T_{max} measured (hours)	1	1	2	4	1	1	1
T_{max} calculated (hours)	0.92	1.27	3.67	4.49	0.48	0.72	0.83
C_{max} measured (µg/mL)	0.493	0.479	0.155	0.122	0.574	0.255	0.265
C_{max} calculated (µg/mL)	0.468	0.472	0.179	0.109	0.504	0.248	0.222
$t_{½}$ e1imination 1 (hours)	0.18	0.36	2.40	2.96	0.05	0.10	0.11
$t_{½}$ e1imination 2 (hours)	44.9	31.5	38.6	88.9	37.8	44.5	31.1
$AUC_{0-\infty}$ (g/g × hour)	3.73	3.63	2.13	2.07	3.22	2.43	2.75

$AUC_{0-\infty}$: Total area under the concentration–time curve Source: Bongartz & Bartelsen, 2017a, b

There were no sex specific differences in the calculated total area under the concentration–time curve values ($AUC_{0-\infty}$) for low- or high-dose male or female rats. However, rats dosed with 2 mg/kg bw showed an approximately 1.75-times higher exposure compared to rats dosed with 200 mg/kg bw, due to lower absorption at higher dose levels.

Plasma concentration measurement in toxicity studies

Metabolites and toxicity studies in which plasma concentrations were measured were:

- *Isoflucypram-desmethyl-carboxylic acid (BSC-CX99799, M11)* and *isoflucypram-cyclopropyl-pyrazole-carboxamide (BCS-CX99798, M58)* were measured in the one-year oral toxicity study in dogs (Kennel, 2017a), the long-term studies in mice (Blanck, 2017) and rats (Odin, 2018; Desmaris, 2017), the preliminary study of two-generation reproductive toxicity study in rats (Renaut, 2019), the main two-generation reproductive study in rats (Renaut, 2018), and the development toxicity study in rats (Kennel, 2017b).

- *Isoflucypram-propanol (BCS-CY24813, M01), isoflucypram2-rpopanol (BCS-DC20298, M02), isoflucypram-desmethyl-propanol (BCS-DC22055, M06), isoflucypram-desmethyl-carboxylic acid (BSC-CX99799, M11),* and *isoflucypram-carboxylic acid (BCS-CY26497, M12)*, were measured in the long-term studies in rats (Odin, 2018; Desmaris, 2017).

The parent, isoflucypram was also measured. Detailed information on their levels is shown in the text and the tables in corresponding studies: Table 12, the one-year oral dog study; Table 13, the long-term study in mice; Table 14, the long-term study in rats; Table 16, the preliminary study of reproductive toxicity study in rats; Table 17, the main two-generation reproductive toxicity study in rats; Table 18, the developmental toxicity study in rats.

In summary, plasma concentrations of isoflucypram-cyclopropyl-pyrazole-carboxamide (BCS-CX99798, M58) and isoflucypram-desmethyl-carboxylic acid (BSC-CX99799, M11) were much higher than isoflucypram in all species in the studies examined. The levels of other metabolites measured in rat plasma were similar to, or higher than, those of the parent compound.

Distribution

In both ADME studies, the amounts of radioactive residues measured in organs and tissues were moderate.

In the first ADME study (Bongartz & Bartelsen, 2017a) at the low dose, female rats showed lower organ concentrations of radioactivity than did male rats. At sacrifice, low levels of radioactivity (mean values 0.152%–0.365% AD) were found in the bodies excluding GIT. Small amounts of radioactivity were detected in the GITs (0.012% –0.044 % AD), except for the GITs of bile duct-cannulated rats. Thus the elimination of the test compound-related radioactivity was nearly complete at sacrifice. Lower organ concentrations were observed following pretreatment with isoflucypram than with administration of a single oral dose. Dose-normalized mean organ concentrations in males following administration of a high dose were lower than following administration of a low dose. The highest concentration of radioactivity was detected in the liver of animals from all low-dose and high-dose tests. Calculated from the radioactivity recovered, the equivalent concentrations ranged from 0.0338 to 3.5395 mg/kg. The concentration in blood cells was high compared to that in organs and tissues, excluding liver; it ranged from 0.0197 to 2.4061 mg/kg. The mean concentration in the other organs and tissues ranged from 0.0009 to 1.6321 mg/kg. From the elimination kinetics of the total radioactivity from plasma it can be concluded that small amounts of residual radioactivity in organs and tissues remained subject to further elimination

In the second ADME study (Bongartz & Bartelsen, 2017b), the equivalent concentration of radioactivity in organs and tissues of male and female rats was within the same order of magnitude. At sacrifice, low levels of radioactivity compared to the administered dose (mean values: 0.290% AD in males and 0.234% AD in females) were found in the bodies excluding GIT. Low levels of radioactivity were detected in the GITs (0.077% AD in males and 0.036% AD in females). Thus, the elimination of the test compound-related radioactivity was nearly complete at sacrifice. The highest concentration of radioactivity was detected in the liver with a mean equivalent concentrations of 0.0347 mg/kg in males and 0.0374 mg/kg in females. The concentration in blood cells was high compared to the concentrations in organs and tissues, excluding the liver. Mean equivalent concentrations were 0.0225 mg/kg in blood cells of male rats and 0.0295 mg/kg in blood cells of female rats. The mean equivalent concentration in other organs and tissues ranged from 0.0011 mg/kg to 0.0115 mg/kg. From the elimination kinetics of the total radioactivity from plasma it can be concluded that small amounts of residual radioactivity in organs and tissues remained subject to further elimination.

Whole-body autoradiography studies were conducted with [*pyrazole-4-*^{14}C]isoflucypram (Bongartz & Bartelsen, 2017c) and [*phenyl-UL-*^{14}C]isoflucypram (Fernyhough & Kendrick, 2017). In each study one Wistar rat/sex per time point received a single mean target dose of about 5 mg/kg bw of isoflucypram by oral gavage. Animals were cryosectioned at 1, 4, 8, 24, 48, 72, 120, 144 or 168 hours after administration. The amounts of radioactivity in excreta (urine and faeces) and exhaled carbon dioxide were additionally determined. The results in male and female rats demonstrated that the radioactivity was widely distributed among all tissues. Radioactivity was distributed throughout the animal immediately after dosing with a clear preference towards the liver and kidney, the organs

responsible for metabolism and excretion respectively. No relevant sex-related differences were observed concerning the maximum equivalent concentrations in blood, organs, and tissues. Absorbed radioactivity was quickly and efficiently eliminated from both genders within 72 hours following administration and long-term retention of residues in any of the organs or tissues can therefore be excluded.

In the study of [*pyrazole-4-*^{14}C]isoflucypram, 14 metabolites were found and their levels in plasma, liver and kidney measured as a pilot metabolism study using one male rat at each time point. Changes over time in the concentration of the parent and its four most abundant metabolites in plasma, liver and kidney are summarized in Table 3. Isoflucypram was present at low levels, or not detected, at all time points, suggesting it was rapidly metabolized. Metabolites isoflucypram-desmethyl-carboxylic acid (M11) and -carboxylic acid (M12) were found to be prominent metabolites earlier than isoflucypram-cyclopronayl-pyrazole-carboxamide (M58) or isoflucypram-desmethyl lactic acid (M09) in the plasma, liver and kidney. Metabolite M58 was the principal metabolite in plasma. The plasma levels of isoflucypram and its metabolites fell to a low level within eight hours of dosing. The results indicated that isoflucypram was rapidly metabolized to M11 or M12 first, and then to M58 or M09. The results also indicated that M58, M11, M12 and M09 were present at greater than 10% total radioactive residue (TRR) in plasma, liver and kidney. In this study it was concluded that M58 was the most prominent metabolite identified (Table 3). (Bongartz & Bartelsen, 2017c)

Table 3. Identification of metabolites in a rat pilot study with [pyrazole-4-^{14}C]BCS-isoflucypram; metabolites identified at 10% or more of total radioactive residue (all shown as %TRR)

		Time point			
Metabolite	**Tissue**	**1 hour**	**4 hours**	**8 hours**	**24 hours**
M58	Plasma	14.4[a]	33.6	46.7	NE
	Liver	2.0	4.2	3.3	NId
	Kidney	7.4	19.8	17.7	16.2
M11	Plasma	34.3	21.2	9.0	NE
	Liver	14.8	15.2	7.2	7.3
	Kidney	16.7	12.8	5.8	NId
M12	Plasma	10.7	3.3	NId	NE
	Liver	21.9	9.6	8.3	7.0
	Kidney	17.6	6.7	3.8	NId
M09	Plasma	5.4	15.6	3.0	NE
	Liver	5.4	24.0	6.2	3.3
	Kidney	3.6	9.8	NId	NId
Isoflucypram	Plasma	NId	NId	NId	NE
	Liver	5.6	2.4	2.7	NId
	Kidney	1.4	2.4	NId	NId

a NE: Not examined; NId: Not identified Source: Bongartz & Bartelsen, 2017c

Excretion

In the first ADME study, at the low and high dose, excretion was almost completed by 72 hours after administration. At this time more than 98% of the recovered dose had been excreted via urine and faeces. In all low- and all high-dose tests the main portion of radioactivity (more than 80%) had been excreted by at the latest 24 hours after administration.

In all tests the main excretion route was via faeces. The faecal excretion component of individual rats ranged from about 83% to 96% of the recovered radioactivity, with exception of the tests using bile duct-cannulated rats. In bile duct-cannulated rats approximately 20% of the recovered dose (mean value) was detected in faeces of males and approximately 15% in faeces the of females. Approximately 74% of the mean dose recovered was detected in bile samples from male bile duct-cannulated rats and approximately 82% in the corresponding case of females.

In single low-dose tests the mean urinary excretion rate was 13.6% of TRR for male rats and 12.0% of TRR for females. In high-dose tests, mean values of 7.1% and 8.4% of TRR were recorded in the urine of male and female rats, respectively. The lower urinary excretion rates after high doses hint that there was a lower absorption of isoflucypram at higher dose rates (Table 4) (Bongartz & Bartelsen, 2017a).

In the second ADME study, the excretion of isoflucypram and its metabolites in male and female rats was almost complete 72 hours after administration. By this time more than 99% of the recovered dose had been excreted via urine and faeces. The mean urinary excretion of total radioactivity after 72 hours was higher in male rats (10.66% of TRR) than in females (7.99% of TRR). In both tests the main portion of radioactivity (more than 80% of TRR) was excreted at the latest after 24 hours post dosing.

In both dose tests the excretion was predominantly faecal and amounted to (mean values) 88.98% of TRR for males and 91.75% of TRR for females. The urinary excretion rates (mean values) were 10.66% of TRR for males and 7.99% for females (Bongartz & Bartelsen, 2017b).

Studies of cannulated rats indicated that the major elimination route of isoflucypram was faeces via bile; details are described below in 1.2 Biotransformation.

Table 4. Cumulative excretion of percent of radioactivity in % of total radioactive dose recovered in rats treated with [pyrazole-4-^{14}C] isoflucypram

Matrix	2 mg/kg bw single dose		200 mg/kg bw single dose		2 mg/kg bw for 14 days	2 mg/kg bw for single dose with bile duct-cannulation	
Time (hours)	Male	Female	Male	Female	Male	Male	Female
Urine							
4	2.24	2.74	0.58	0.95	5.02	NC	0.58
8	7.58	6.33	1.68	2.73	8.52	3.21	1.37
12	10.34	-	-	-	-	-	-
24	13.25	11.57	6.75	8.17	11.98	5.27	2.02
48	13.51	11.92	7.03	8.41	12.25	5.60	2.26
72	13.57	11.98	7.06	8.44	12.31	-	-
Bile							
4	-	-	-	-	-	38.42	33.65
8	-	-	-	-	-	59.99	54.50
24	-	-	-	-	-	72.61	78.57
32	-	-	-	-	-	73.14	80.49
48	-	-	-	-	-	74.10	81.64
Faeces							
24	82.77	83.65	90.73	89.98	81.86	19.61	13.85
48	85.84	87.69	92.63	91.32	87.06	20.00	14.94
72	86.03	87.83	92.77	91.39	87.36	-	-
Sum	99.61	99.81	99.83	99.83	99.67	99.71	98.84
Norm. factor	0.964	0.997	0.993	0.993	0.971	0.959	0957

- no sample collected; NC: Not calculated Source: Bongartz & Bartelsen, 2017a

Norm. factor: For all steps during the preparation of samples (extraction and clean-up), the values of individual fractions were normalized to 100% of recovered radioactivity.

(b) Dermal route

No data were provided.

1.2 Biotransformation

Metabolism in rats was investigated using [*pyrazole-UL*-^{14}C]isoflucypram or [*phenyl-UL*-^{14}C]isoflucypram in the first and second ADME studies respectively. In both of these studies urine and faeces were sampled at various time points during the individual tests to investigate the metabolism of pyrazole-radiolabelled isoflucypram. Metabolism was also investigated in bile duct cannulated male rats treated with a low dose of pyrazole-labelled isoflucypram. Parent compound and metabolites were analysed and quantified in urine, bile and extracts of faeces by radio high-performance liquid chromatography (HPLC). In a whole-body autoradiography study (Bongartz & Bartelsen, 2017c) with [*pyrazole-UL*-^{14}C]isoflucypram, a pilot metabolism investigation was performed in male rats after a single oral low dose. Parent compound and metabolites were analyzed and quantified in urine, plasma and extracts of faeces, liver and kidney.

A large number of metabolites were identified in urine, faeces and bile, suggesting an extensive metabolism of isoflucypram in rats. The number of metabolites detected in males was larger than in females, however the overall rate of metabolism was comparable in males and females. The rate of identification of the parent compound and metabolites was high. Quantitative results for pyrazole- and phenyl-labelled isoflucypram and its metabolites excreted in urine, bile (pyrazole-labelled only) and faeces after oral treatment are summarized in Tables 5 and 6, and as these tables show, some label-specific metabolites were identified.

Since the excretion of isoflucypram indicated that its major elimination was via faeces through bile, the metabolites found in urine and faeces in uncannulated rats and/or the metabolites found in bile in bile duct-cannulated rats were considered for quantification of metabolites. The metabolites which were identified in bile at levels of approximately 10% and above of absorbed dose in male and/or female rats were:

- Isoflucypram-propanol-GlucA (isomer 1 and/or 2) (M19)
- Isoflucypram-desmethyl-GlucA (isomer 1 and 2) (M35)
- Isoflucypram-desmethyl-diOH-GlucA (group of isomers) (M33)

(Bongartz & Bartelsen, 2017a,b)

Table 5. The quantitation of parent and metabolites in rats treated with [pyrazole-4-^{14}C]isoflucypram

		Dose, regime and sex						
		2 mg/kg bw for single dose		**200 mg/kg bw for single dose**		**2 mg/kg bw for 14 days**	**2 mg/kg bw for single dose with bile duct cannulation**	
Isoflucypram metabolites: Report name	**Code name (metabolite number)**	**M**	**F**	**M**	**F**	**M**	**M**	**F**
		Percentage of administered dose						
Isoflucypram-propanol	BCS-CY24813 M01	0.95 f	4.09 f	0.81 f	2.79 f	2.44 f	0.20 f	0.05 f
Isoflucypram-propanol-GlucA (isomers 1 and 2)	M19	1.17 f	-	-	-	1.31 f	-	-
Isoflucypram-propanol-GlucA (isomer 1)	M19	-	-	-	-	-	6.98 b	**12.27 b**
Isoflucypram-propanol-GlucA (isomer 2)	M19	-	-	-	-	-	3.47 b	3.46 b
Isoflucypram-2-propanol	BCS-DC20298 M02	0.70 f	064 f	0.83 f	0.59 f	1.19 f	0.07 f	0.04 f

Isoflucypram metabolites: Report name	Code name (metabolite number)	Dose, regime and sex						
		2 mg/kg bw for single dose		200 mg/kg bw for single dose		2 mg/kg bw for 14 days	2 mg/kg bw for single dose with bile duct cannulation	
		M	F	M	F	M	M	F
		Percentage of administered dose						
Isoflucypram-carboxylic acid	BCS-CY26497 M12	12.61 f	13.77 u/f (0.80/ 12.98)	5.80 f	6.62 u/f (1.14/ 5.48)	14.34 f	2.68 b/f (2.48/ 0.20)	1.54 u/b/f (0.13/ 1/35/ 0.06)
Isoflucypram-lactic acid	M10	2.67 f	-	1.18 f	0.39 f	2.20 f	-	-
Isoflucypram-desmethyl	M13	1.44 f	11.43 f	2.74 f	5.31 f	2.98 f	0.38 f	0.46 f
Isoflucypram-desmethyl-hydroxyphenyl-2-propanol	M14	4.32 u/f (0.13/ 4.19)	6.74 u/f (1.29/ 5.45)	3.74 f	2.03 u/f (0.19/ 1.64)	4.18 u/f (0.22/ 3.36)	1.44 u/b/f (0.10/ 1.30/ 0.05)	1.69 u/b (0.27/ 1.42)
Isoflucypram-desmethyl-hydroxyphenyl 1,2-propandiol	M15	8.11 f	-	0.76 f	-	6.07 f	-	-
Isoflucypram-desmethyl-diOH	M08	1.57 f	-	0.47 f	-	0.55 f	0.06 f	-
Isoflucypram-desmethyl-hydroxymethyl-diOH	M17	2.86 f	-	-	-	1.56 f	0.36 b	0.66 b
Isoflucypram-desmethyl-GlucA (isomer 1)	M35	-	-	-	-	-	3.86 b	**14.09 b**
Isoflucypram-desmethyl-GlucA (isomer 2)	M35	-	-	-	-	-	1.64 b	7.26 b
Isoflucypram-desmethyl-OH-GlucA (isomer 1)	M32	-	-	-	-	-	1.90 b	0.20 b
Isoflucypram-desmethyl-OH-GlucA (isomer 2)	M32	10.24 f	0.23 u	2.22 f	0.76 f	10.47 f	2.09 b	3.66 u/b/f (0.11/ 3.45/ 0.10)
Isoflucypram-desmethyl-diOH-GlucA (isomer 1)	M33	1.12 f	-	-	-	-	**5.56 b**	-
Isoflucypram-desmethyl-diOH-GlucA (isomer 2 and 3)	M33	-	-	-	-	-	**0.85 b**	-
Isoflucypram-desmethyl-diOH-GlucA (isomer 3)	M33	0.52 f	-	-	-	0.31 f	**1.52 b**	**1.42 b**

Isoflucypram metabolites: Report name	Code name (metabolite number)	Dose, regime and sex						
		2 mg/kg bw for single dose		200 mg/kg bw for single dose		2 mg/kg bw for 14 days	2 mg/kg bw for single dose with bile duct cannulation	
		M	F	M	F	M	M	F
		Percentage of administered dose						
Isoflucypram-desmethyl-diOH-GlucA (isomer 4)	M33	-	-	-	-	-	**2.17 b**	**2.71 b**
Isoflucypram-desmethyl-diOH-GlucA (isomer 5)	M33	-	-	-	-	-	**1.46 b**	**2.65 b**
Isoflucypram-desmethyl-triOH-GlucA	M34	0.98 f	-	-	-	0.54 f	2.80 b	-
Isoflucypram-desmethyl-propanol	BCS-DC22055 M06	1.60 f	8.68 f	1.22 f	3.34 f	4.43 f	0.59 b/f (0.48/ 0.11)	1.23 b/f (1.15/ 0.08)
Isoflucypram-desmethyl-propanol-GlucA (isomer 2)	M31	-	-	-	-	-	0.77b	4.55b
Isoflucypram-desmethyl-carboxylic acid	BCS-CX99799 M11	12.44 f	18.84 u/f (4.37/ 14.47)	10.23 f	7.76 u/f (3.63/ 4.13)	13.76 f	1.51 b	2.94 u/b (0.87/ 2.07)
Isoflucypram-desmethyl-lactic acid	M09	1.98 f	-	-	-	1.52 f	3.50 b	4.36 b
Isoflucypram-desmethyl-hydroxymethyl-carboxylic acid	M16	3.01 f	11.38 u/f (0.80/ 10.58)	2.31 f	4.12 u/f (0.49/ 3.63)	6.15 f	0.20 f	0.21 u/f (0.14/ 0.07)
Isoflucypram-desmethyl-carboxylic acid-GlucA (isomer 1) and diOH-GlucA (isomer 1 and 2)		1.81u/f (0.41/ 4.19)	1.16 f	-	-	1.56 f	1.64 b	2.13 b
Isoflucypram-desmethyl-oxo-GlucA, carboxylic acid-GlucA and isoflucypram-desmethyl-carboxylic acid-GlucA (isomer 2)		-	-	-	-	-	4.58b	4.58 b
Isoflucypram-desmethyl-propanol-GlucA (isomer 1), olefine, oxo-GlucA, lactic acid and desmethyl-diOH-GlucA (isomer 6)		-	-	-	-	-	**13.24 b**	**11.94 b**
Isoflucypram-desmethyl-SA		0.28 f	-	-	-	-	0.19 b	0.08 b

Isoflucypram metabolites: Report name	Code name (metabolite number)	Dose, regime and sex						
		2 mg/kg bw for single dose		200 mg/kg bw for single dose		2 mg/kg bw for 14 days	2 mg/kg bw for single dose with bile duct cannulation	
		M	F	M	F	M	M	F
		Percentage of administered dose						
Isoflucypram-cyclopropyl-pyrazole-carboxamide	BCS-CX99798 M58	0.76 u	0.60 u	1.63 u	0.40 u	1.67 u	1.00 u/b (0.9/ 0.09)	0.13 u
Isoflucypram-cyclopropyl-pyrazole-carboxamideOH-GlucA	M61	0.72 u	0.08 u	0.18 u	0.07 u	0.43 u	0.28 u/b (0.13/ 0.5)	-
Isoflucypram-cyclopropyl-pyrazole-carboxamide GlucA (isomers)	M60	2.85 u	0.80 u	0.89 u	0.53 u	2.36 u	3.37 u/b (1.99/ 1.37)	0.25 u
Isoflucypram-cyclopropyl-oxy-pyrazole-carboxamide	M59	0.31 u	-	-	-	0.27 u	0.10 u	-
Isoflucypram-N-methyl-pyrazole-carboxylic acid	BCS-AB72918 BCS-CR73065 M50	0.67 u	0.37 u	0.30 u	0.37 u	0.50 u	0.26 u	0.13 u
Isoflucypram-desfluoro-N-methyl-pyrazole-carboxylic acid	M51	0.50 u	-	0.19 u	-	0.41 u	-	0.10 u
Isoflucypram-pyrazole-amide	M64	0.86 u	0.08 u	0.31 u	-	0.50 u	-	-
Isoflucypram-pyrazole-carboxylic acid	M63	1.59 u	0.18 u	0.16 u	0.16 u	1.02 u	0.74 u/b (0.38/ 0.36)	-
Isoflucypram-pyrazole-carboxylic acid-Ala	M65	4.92 u	1.69 u	3.10 u	1.18 u	4.77 u	1.70 u/b (1.50/ 0.20)	0.16 u
Isoflucypram-desfluoro-N-methyl-cyclopropylpyrazole-carboxamide-OH-Cys and desfluoro-N-methyl-cyclopropylpyrazole-carboxamide-OH-Cys-Gly		-	-	-	-	-	1.57 b	0.24 b
Isoflucypram-desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-OH-GSH	M54	-	-	-	-	-	0.94 b	0.04 b

Isoflucypram metabolites: Report name	Code name (metabolite number)	Dose, regime and sex						
		2 mg/kg bw for single dose		200 mg/kg bw for single dose		2 mg/kg bw for 14 days	2 mg/kg bw for single dose with bile duct cannulation	
		M	F	M	F	M	M	F
		Percentage of administered dose						
Isoflucypram (parent compound)	BCS-CN88460	4.11 f	4.86 f	57.26 f	59.07 f	073 f	18.24f	15.56 b/f 1.24/ 14.31)
Total identified		7.06	86.61	96.34	96.47	88.17	93.92	100.79
Total characterized		10.48	10.61	1.46	3.06	10.03	7.86	2.20

M: Male; F: Female; Source: Bongartz and Bartelsen, 2017a

Number expresses the parent/metabolites found as percentage of administered dose in urine (u), bile (b) or faeces (f);

The number in the parenthesis indicates percentage of the parent compound/metabolite in urine, bile or faeces;

Numbers in bold represent ≥ 10% of absorbed dose based on the absorption rate of isoflucypram as 79.9% and 84.1% in males and females, respectively (see Table 1).

Table 6. The quantitation of parent and metabolites in rats treated with [phenyl-CL-^{14}C]isoflucypram

Isoflucypram metabolites: Report name	Code number (metabolite number)	2 mg/kg bw for single dose	
		Male	Female
		% of dose administered	
Isoflucypram-benzylalcohol-dioxo-GlucA (isomer 1)	M75	0.82 u	0.15 u
Isoflucypram-benzylalcohol-dioxo-GlucA (isomer 2)	M75	1.99 u	0.54 u
Isoflucypram-desmethyl-triOH-GlucA	M34	0.84 f	-
Isoflucypram-benzylalcohol-dioxo (isomer 1)	M74	0.39 u	0.21u
Isoflucypram-desmethyl-hydroxymethyl-diOH	M17	3.31 f	–
Isoflucypram-benzylalcohol-dioxo (isomer 2)	M74	0.55 f	0.38 f
Isoflucypram-desmethyl-hydroxyphenyl-1,2-propandiol	M15	11.41 f	-
Isoflucypram-desmethyl-carboxylic acid-GlucA (isomer 1) and diOH-GlucA (isomer 1 and 2)	-	4.38 f	2.50 f
Isoflucypram-phenyl-formyl-olefine, benzylalcohol-GlucA and benzylalcohol-oxo-GlucA (isomer 1)	-	1.46 u	1.45 u
Isoflucypram-desmethyl-diOH (group of isomers)	M08	14.60 f	1.13 f
Isoflucypram-benzylalcohol-oxo-GlucA (isomer 2)	M73	1.64 u	0.78 u
Isoflucypram-desmethyl-lactic acid	M09	6.53 f	4.06 f
Isoflucypram-desmethyl-diOH (isomer)	M08	7.41 f	19.35 f
Isoflucypram-lactic acid	M10	3.58 f	0.28 f
Isoflucypram-propanol-GlucA (isomer 1 and 2)	M19	1.10 f	1.13 f
Isoflucypram-benzylalcohol-oxo-desdihydro (isomer 1)	M72	0.14 u	0.11 u
Isoflucypram-benzylalcohol-oxo desdihydro (isomer 2)	M72	0.11 u	0.28 u
Isoflucypram-desmethyl-SA	M13	0.41 f	1.40 f
Isoflucypram-desmethyl-carboxylic acid	BCS-CX99799 M11	7.48 f	12.40 u/f (1.31/11.09)
Isoflucypram-desmethyl-propanol	BCS-DC22055 M06	2.63 f	14.24 f

Isoflucypram metabolites: Report name	Code number (metabolite number)	2 mg/kg bw for single dose	
		Male	Female
		% of dose administered	
Isoflucypram-carboxylic acid	BCS-CY26497 M12	9.76 f	14.55 u/f (0.39/14.16)
Isoflucypram-propanol	BCS-CY24813 M01	4.04 f	8.92 f
Isoflucypram-benzylalcohol-oxo	-	1.13 u	0.45 u
Isoflucypram-2-propanol	BCS-DC20298M02	1.12 f	0.88 f
Isoflucypram-desmethyl	M13	-	2.59 f
Isoflucypram (parent compound)	BCS-CN88460	1.08 f	4.12 f
Total identified	-	87.89	91.86
Total characterized	-	9.36	7.29

Source: Bongartz and Bartelsen, 2017b

Number expresses the parent/metabolites found as percentage of administered dose in urine (u), bile (b) or faeces (f);

The number in the parenthesis indicates percentage of the parent compound/metabolite in urine, bile or faeces;

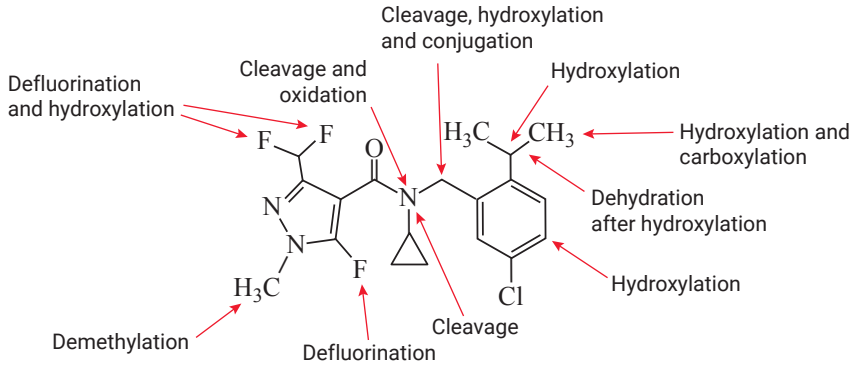
Based on the results from the first and second ADME studies, the main metabolic routes observed for rats treated with [*pyrazole-UL*-^{14}C]isoflucypram and [*phenyl-UL*-^{14}C]isoflucypram are as described below:

- Demethylation of the pyrazole moiety. This demethylation was one of the most prominent metabolic reactions in both sexes. Subsequent metabolism of desmethyl compounds was faster in male rats than females resulting in sex difference with respect to the percentage of administered dose for the resulting compounds.
- Hydroxylation at positions 1 and 2 of the propyl group in the phenyl ring, followed by defluorination of the difluoromethyl moiety leading to mono-, di- or trihydroxy compounds. Hydroxylation in other positions was also detected, but structural analysis could not discern its exact location.
- Further oxidation of the 1-propanol group leading to the formation of a carboxylic acid group, or in combination with a 2-propanol group, to a lactic acid moiety.
- Cleavage of the phenyl moiety, leading to cyclopropyl-pyrazole-carboxamide compounds.
- Cleavage of the phenyl moiety in combination with cleavage of the cyclopropyl ring, leading to a pyrazole-amide compound, followed by oxidation to carboxylic acid derivatives.
- Cleavage of the pyrazole moiety, leading to a number of benzyl alcohol compounds, which could subsequently undergo oxidation and dehydrogenation.
- Conjugation with glucuronic acid after hydroxylation, or via nitrogen, leading to one of several glucuronic acid conjugates.
- Conjugation with glutathione after defluorination of the cyclopropyl-pyrazole-carboxamide moiety, followed by degradation of the glutathione conjugate to glycine-cysteine or cysteine conjugates.
- Conjugation of the isoflucypram-pyrazole-carboxylic acid compound with alanine.
- Conjugation of the isoflucypram-desmethyl with sulfuric acid.
- Dehydration after hydroxylation of the propyl group, leading to isoflucypram-olefine.
- Oxidation in the pyrazole ring, leading to isoflucypram-cyclopropyl-oxypyrazole-carboxamide.

- Cleavage of the pyrazole moiety, leading to a number of benzyl alcohol compounds, followed by oxidation and dehydrogenation.
- Dehydrogenation of the propyl group after cleavage of the pyrazole moiety, leading to isoflucypram-phenyl-formyl-olefine.

Fig. 2 shows schematically the main positions in the molecule which are involved in the above metabolic reactions:

Figure 2. The position in the molecule, which are mainly involved in the metabolic reactions.



(Redrawn from Bongartz & Bartelsen, 2017a, b)

Based on the results of the two metabolic studies, proposed metabolic pathways for isoflucypram in rats are shown in Figures 3a–d (redrawn from Bongartz & Bartelsen, 2017a, b). Chemical structures of major metabolites in rats, including report names and code names, are listed in Appendix 1, Table 1.

Figure 3a. Predicted metabolic pathways of isoflucypram in rats

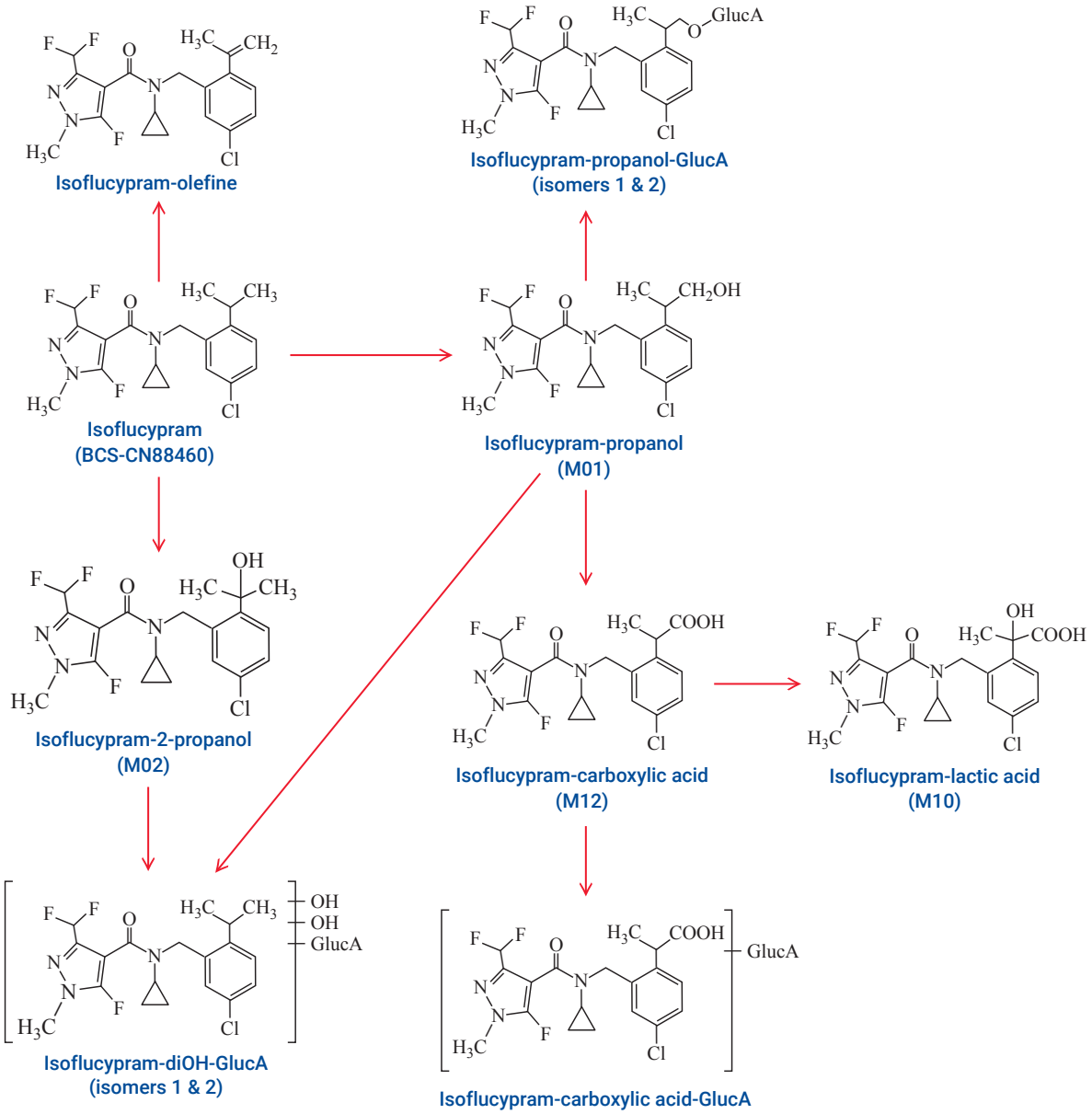


Figure 3b. Predicted metabolic pathways of isoflucypram in rats (Bongartz & Bartelsen, 2017a, b)

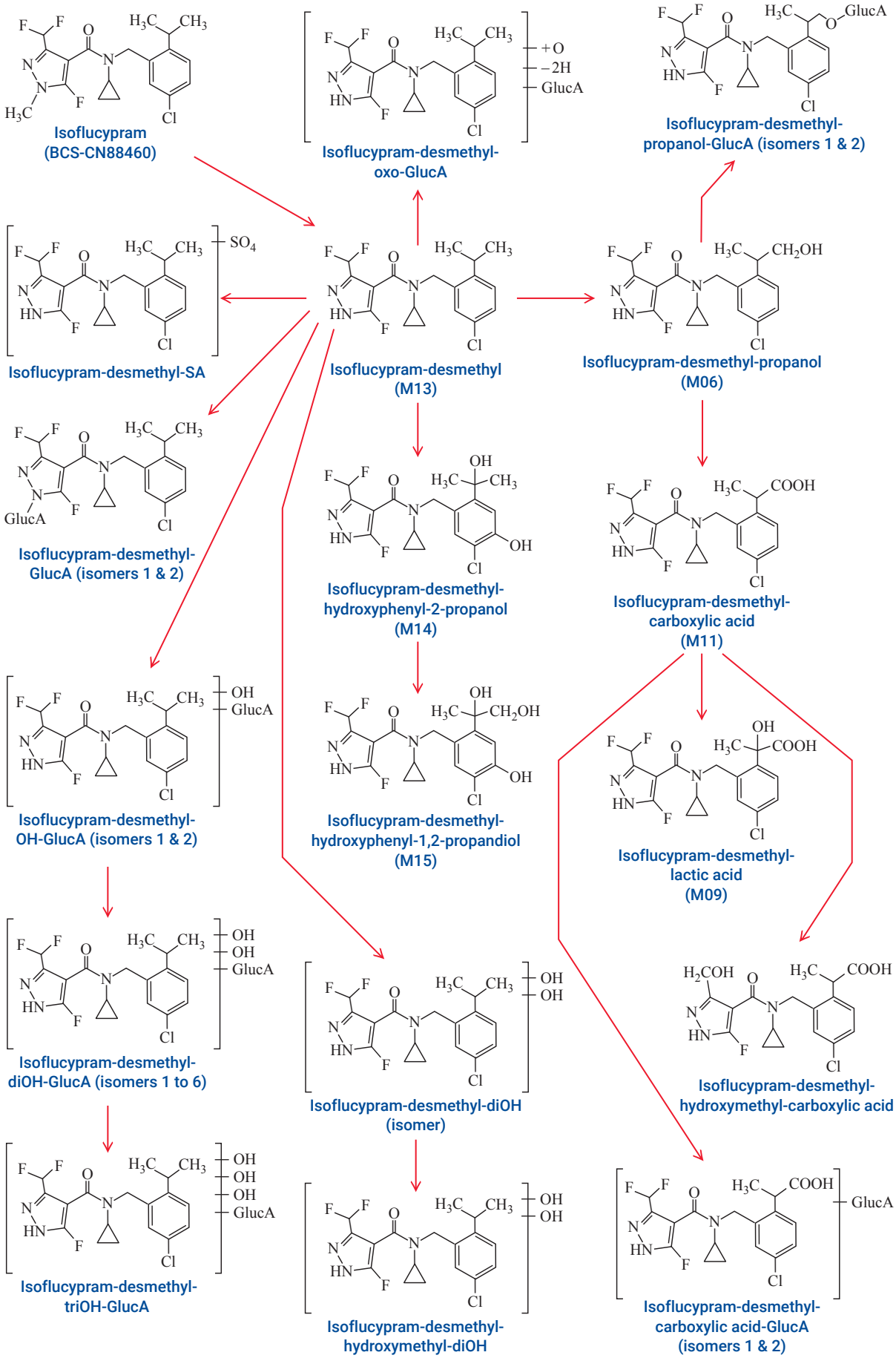


Figure 3c. Predicted metabolic pathways of isoflucypram in rats (Bongartz & Bartelsen, 2017a, b)

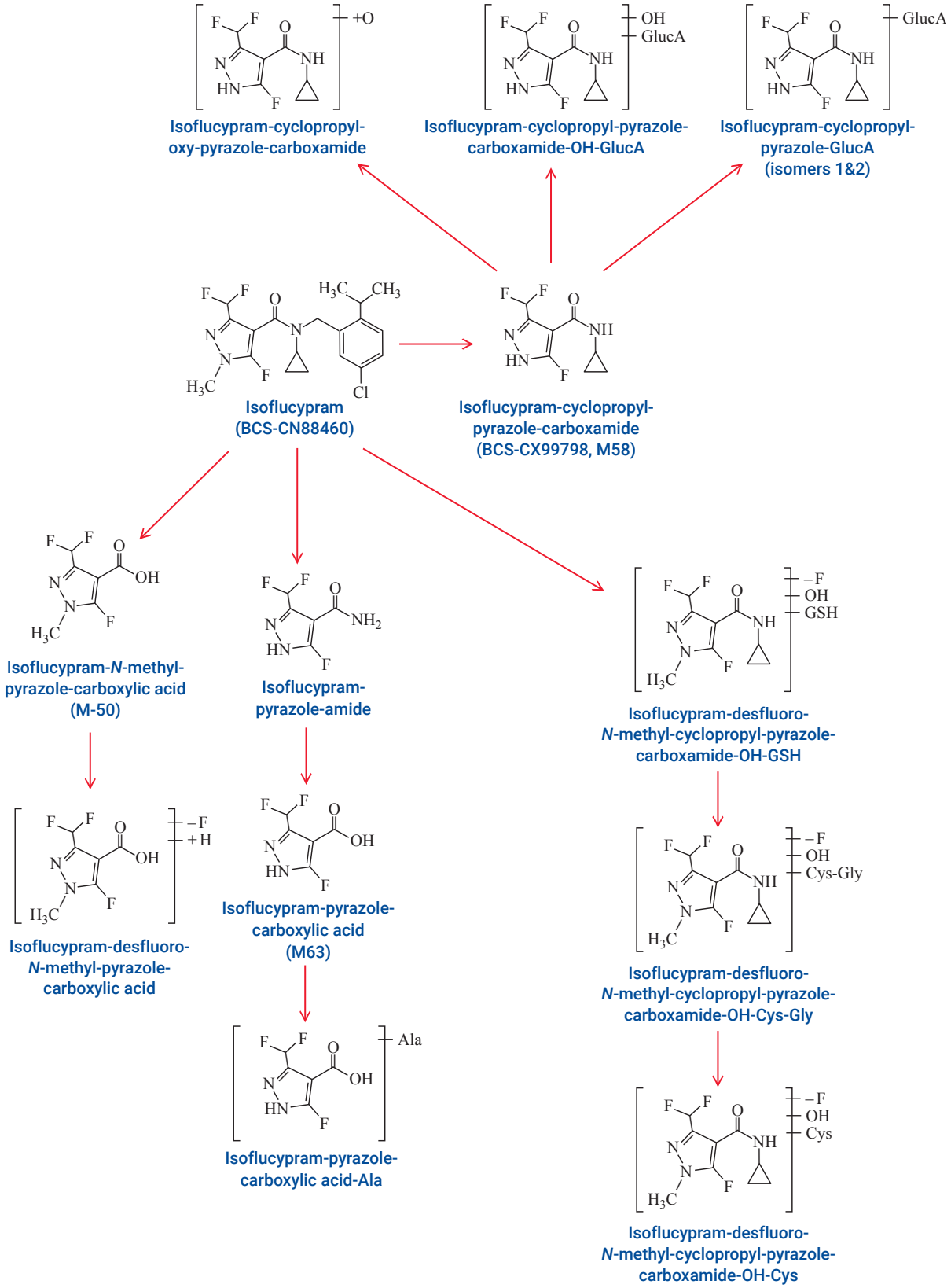
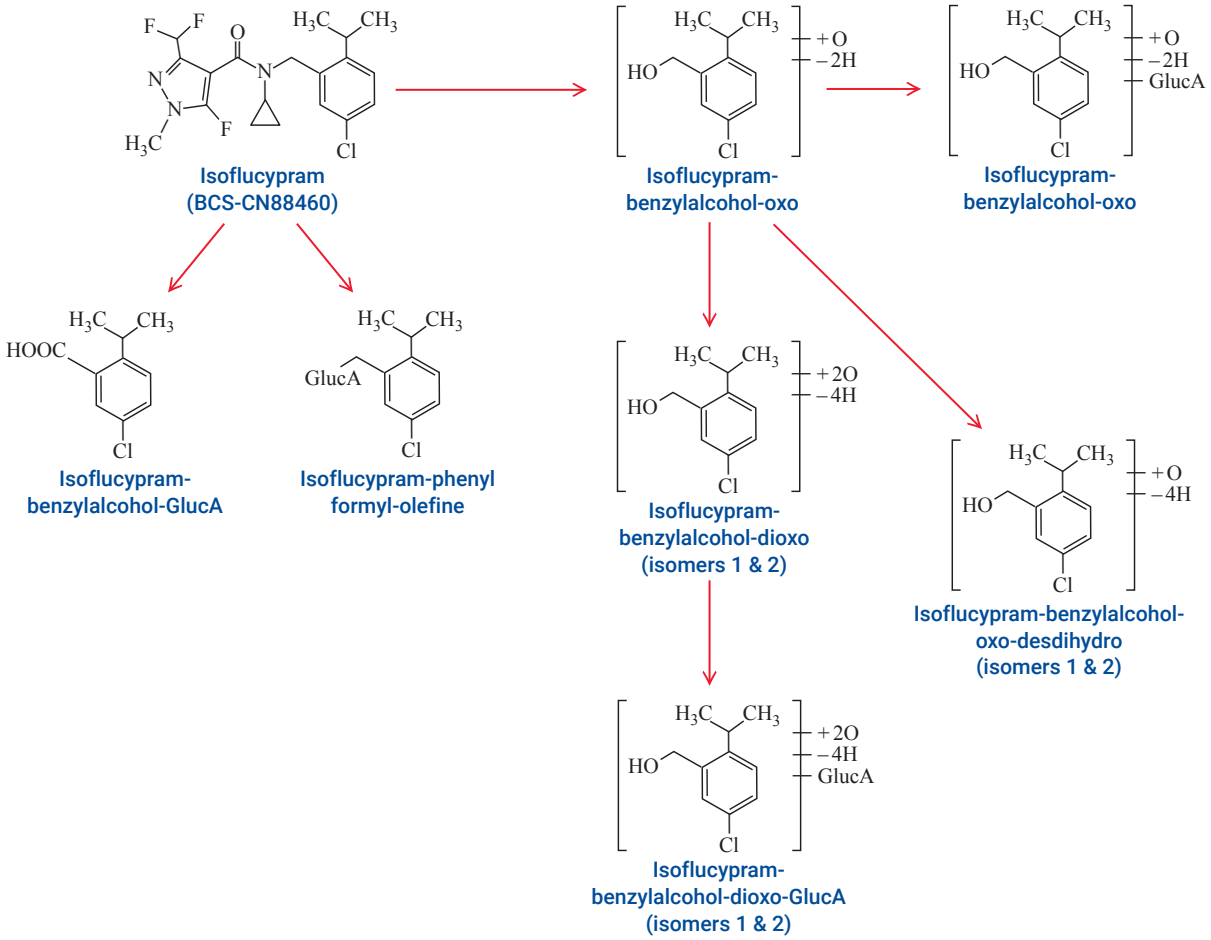


Figure 3d. Predicted metabolic pathways of isoflucypram in rats (Bongartz & Bartelsen, 2017a, b)



(Fig. 3a–d all redrawn from Bongartz & Bartelsen, 2017a, b)

In vitro studies

To determine the metabolite profiles in different species, [*pyrazole-UL*-^{14}C]isoflucypram was incubated with liver microsomes from humans, rats, mice, dogs and rabbits. The enzymatic activity of each of the liver microsomes was demonstrated using the positive control substance [^{14}C]testosterone.

The in-vitro metabolite profile of [*pyrazole-UL*-^{14}C]isoflucypram when incubated with liver microsomes was found to be moderately different in the various microsomal incubations and exhibited moderate to high transformation rates of the applied test compound during incubation. The highest metabolic transformation rate for human liver microsomes was observed after 60 minutes incubation at 13.2 µM, and metabolism in the period 0–60 minutes accounted for 83.7% of the total metabolic transformation achieved. With rat liver microsomes, [*pyrazole-UL*-^{14}C]isoflucypram had been metabolized to an extent between 62.7% and 98.0% after 60 minutes incubation, the lower metabolic transformation rates representing female rat liver microsomes. With mouse liver microsomes, [*pyrazole-UL*-^{14}C]isoflucypram was metabolized to an extent between 88.4% and 98.3% after 60 minutes incubation. With the male dog liver microsomes up to 95.6% of the [*pyrazole-UL*-^{14}C]isoflucypram was metabolized after 60 minutes incubation, and in rabbit liver microsomes up to 95.0%.

In the various liver microsome mixtures after incubation, metabolites M03, M04, M08, M09, M14, M16, M17, M18, M22 and M25 were usually the most abundant metabolites amounting to as much as 44.69% of the radioactivity. Except for isoflucypram, no attempt was made to identify metabolites during the study and comparison between samples was based on HPLC profiles. With human liver microsomes the most abundant metabolites (M04, M09, M16, M18 and M25) accounted for between 2.89% and 32.20% of the radioactivity. With rat liver microsomes the most abundant metabolites (M14, M18 and M25) accounted for between 1.02% and 44.69% of the radioactivity. Further abundant

metabolites were M03, M04, M08, M16, M17 and M22 ranging between 0.50% and 18.57% of the radioactivity. With mouse liver microsomes the most abundant metabolites (M04, M14, M16, M17, M18 and M25) accounted for between 1.20% and 30.88% of the radioactivity. With dog liver microsomes the most abundant metabolites (M04, M14, M16, M17, M18 and M25) accounted for between 0.93% and 21.28% of the radioactivity. With rabbit liver microsomes the most abundant metabolites (M4, M8, M14, M16 and M25) accounted for between 0.58% and 41.30% of the radioactivity. Other metabolites detected across all liver microsomes incubates accounted each for 8.62% or less of the radioactivity.

To summarize, a range of metabolites was detected in the liver microsomes mixtures after incubation. The metabolic pattern was comparable in human, mouse and rat liver microsomes systems and no unique human metabolite was detected (Lagojda & Doebbe, 2017).

1.3 Effects on enzymes and other biochemical parameters

No available data were provided.

2. Toxicological studies

2.1 Acute toxicity

Acute toxicity, eye- and skin-irritation and skin sensitization studies are summarized in Table 7.

Table 7. Summary of acute toxicity studies of isoflucypram

Species	Strain	Sex	Route	Purity Batch number	Result	Reference
Rat	RccHan: (WIST)	Female	Oral	94.2% 2013-006492	LD_{50} > 2000 mg/kg bw	Zelenák, 2014a
Rat	RccHan: (WIST)	Male and Female	Dermal	94.2% 2013-006492	LD_{50} > 2000 mg/kg bw	Zelenák, 2014b
Rat	Wistar Crl:WI	Male and Female	Inhalation	94.2% 2013-006492	Inhalation LC_{50} 2.518 mg/L (both sexes combined) 3.131 mg/L (males) 2.209 mg/L (females)	Mátyás, 2014
Rabbit	New Zealand White	Male	Skin irritation	94.2% 2013-006492	Negative	Zelenák, 2014c
Isolated chicken eyes	ROSS 308	Unknown	Eye irritation in vitro	94.2% 2013-006492	Neither severe irritant nor non-irritant (inconclusive)	Váliczkó, 2014
Rabbit	New Zealand White	Male	Eye irritation	94.2% 2013-006492	Mild irritant	Zelenák, 2014d
Mouse	CBA/J Rj	Female	LLNA	94.2% 2013-006492	Sensitizing EC3 = 29.0%	Hargitai, 2015

LLNA: Local lymph node assay; LD_{50}: Median lethal dose; LC_{50}: Median lethal concentration;
EC3: The amount of test substance needed to induce a three-fold increase in cell proliferation in a LLNA

(a) Lethal doses

Five female rats were sequentially administered a single dose of isoflucypram by oral gavage at a dose of 2000 mg/kg bw, followed by a 14-day observation period. All animals were observed individually after dosing at 30 minutes and at 1, 2, 3, 4, and 6 hours after dosing, then once each day for 14 days thereafter. No mortalities, clinical signs, effects on body weight, body weight gain or macroscopic findings were observed in any animal following oral gavage administration of isoflucypram. Oral median lethal dose (LD_{50}) was higher than 2000 mg/kg bw (Zelenák, 2014a).

Five male and five female rats were administered a single dose of isoflucypram dermally at a dose of 2000 mg/kg bw for 24 hours under a semi-occlusive bandage, followed by an observation

period of 14 days. There were no mortalities, systemic clinical signs, or treatment-related effects on body weight or body weight gain, nor were there any abnormalities noted at necropsy. Very slight erythema (score 1) was noted in five of 10 animals (two males and three females) on study day 1 only. All local signs observed were reversible by study day 2. Dermal LD_{50} was higher than 2000 mg/kg bw (Zelenák, 2014b).

Male and female rats (five/sex per concentration) were exposed for four hours to a liquid aerosol atmosphere of isoflucypram, diluted to 10–20% (w/w) with propan-2-one (acetone) then aerosolized, at concentrations of up to 2.87 mg/L. The mass median aerodynamic diameter (MMAD) in the various exposure groups ranged from 1.41 µM to 1.81 µM, with geometric standard deviations (GSDs) in the range 1.81–1.99. One male and one female died at 2.03 mg/L, while two males and all five females died at 2.87 mg/L, all during the exposure. Clinical signs included noisy or gasping respiration, decreased activity, ataxia (slight to severe), hunched back, slight sneezing, prostration, and coma. All surviving animals were symptom-free by study day 3 at the latest. Among surviving animals there were no findings of note at necropsy. Median lethal concentration (LC_{50}) in males and females was 3131 and 2209 mg/L, respectively. The LC_{50} for combined sexes was 2518 mg/L (Mátyás, 2014).

(b) Dermal irritation

Isoflucypram (0.5 g) was administered by topical semi-occlusive application to the intact shaved flank of three young adult, male rabbits for four hours. The treated skin surface was examined at 1, 24, 48, and 72 hours after patch removal. No clinical signs were observed on the skin of the treated animals at any time point after removal of the test item. No clinical signs of systemic toxicity were observed in the animals during the study and no mortalities occurred. The body weights of all rabbits were considered to be within the normal range of variability. Isoflucypram was not irritant to the skin of rabbits (Zelenák, 2014c).

(c) Ocular irritation

A total of three isolated chicken eyes were used for in vitro eye irritation screening. Isoflucypram (30 mg) was applied to the centre of the cornea of each eye so as to cover the entire surface of the cornea for ten seconds. After rinsing the eyes, some of the test material remained stuck to the corneal surface and fluorescein retention was observed in treated eyes. Based on the results, the test item cannot be regarded as a severe irritant or a non-irritant (Váliczkó; 2014).

A further in vivo study for eye irritancy was conducted in rabbits with isoflucypram, the irritant effects being evaluated by the Draize method. Test item (0.1 g) was placed into the conjunctival sac of the left eye of each of the three animals, with the untreated right eye serving as a control, and the eyes were then examined at 1, 24, 48, and 72 hours after application, with fluorescein staining performed 24 hours before application and 24, 48, and 72 hours after application of the test item. Conjunctival redness, chemosis, and discharge were noted in all three animals at the 24-hour examination. At 48 hours, a decreased amount of conjunctival redness with no chemosis or discharge was noted and all observations had returned to a pretreatment condition by the 72-hour examination. Isoflucypram applied to the mucosa of one eye in each of three rabbits caused conjunctival effects at one hour after application. These effects were fully reversible within 72 hours (Zelenák, 2014d). Isoflucypram was a mild irritant to rabbit eyes.

(d) Dermal sensitization

In an LLNA test five female mice per group received isoflucypram at 5%, 10%, 25%, or 50% on study days 1, 2, and 3. On study day 6 cell proliferation in the local lymph nodes was measured by incorporation of tritiated methyl thymidine. The observed stimulation index values were 1.2, 1.2, 2.5, and 5.6 at concentrations of 5%, 10%, 25%, and 50% respectively. No mortality or systemic toxicity was observed during the study. There were no visual signs of local irritancy at the site of application. A minimal amount of test item residue was observed on the ears of the animals in the 50% dose group on study days 1–6 and in the 25% dose group on study days 1–3. Alopecia was recorded in the 50% dose group on study days 3–6. No treatment-related effects were observed on body weight in any group. Isoflucypram was shown to have sensitization potential in the mouse local lymph node assay, with an EC3 value of 29.0% (Hargitai, 2015).

2.2 Short-term studies of toxicity

(a) Oral administration

Mouse

Study 1

A 28-day study was conducted using C57/6j mice. Five mice/sex per group were administered isoflucypram (purity 97.6%; Batch no. NLL 8224-6-4) in diet at concentrations of 0, 200, 800 or 2000 ppm (equal to 0, 32, 133 and 330 mg/kg bw per day for males, 0, 41, 149 and 374 mg/kg bw per day for females).

The treatment did not cause any mortalities or clinical signs during the study. At 2000 ppm body weight and food consumption were unaffected by the treatment in either sex except for a decrease in body weight gain (by 69% compared with controls) and a decreased food consumption (by 17% compared with controls) in females at first week time point. With regard to blood biochemistry measurements, higher levels of alanine transaminase (ALT) in both sexes and aspartate transaminase (AST) in males and slightly higher levels of alkaline phosphatase activity (ALP) in males were observed at 2000 ppm, and these changes were considered adverse effects on the liver. Total bilirubin was decreased at 200 ppm and above in females with statistically significance (1.0, 0.62, 0.46 and 0,52 µmol/L at 0, 200, 800 and 2000 ppm respectively) although the dose–response relationship was not clear. The decrease in bilirubin was a finding common to mice, rats and dogs when treated with isoflucypram. Based on studies with liver metabolism enzymes, the decrease in bilirubin was considered to be treatment-related but not toxicologically significant because a decrease in bilirubin (rather than an increase) indicates a change opposite to what would be expected with hepatotoxicity or haemolysis (for a postulated mode of action see Appendix 1 Fig. 1). On the basis of data on metabolic enzyme induction in the livers of mice, the decrease was considered a secondary effect to the marked induction of bilirubin-UDP-GT (uridine diphosphate glucuronosyltransferase) at 50 ppm and higher in mice (Rouquié, 2018a). At 2000 ppm absolute and relative liver weights were increased. Hepatocellular hypertrophy, minimal to slight hepatocellular necrosis (focal and single cells) were observed as treatment-related changes in most of the mice of both sexes at 2000 ppm. Focal or single cell hepatocellular necrosis was found one or two males at 800 ppm; the grading of these findings was minimal to slight. These findings were not considered treatment-related because they were not seen at higher concentrations (1000 ppm) in the longer, 13-week, feeding study in mice.

The no-observed-adverse effect level (NOAEL) for the four-week study in mice was 800 ppm (equal to 149 mg/kg bw per day) based on hepatotoxicity and decreased body weight gain and feed consumption at 2000 ppm (Repetto, 2012).

Study 2

Isoflucypram (purity 97.7%; Batch no. NLL8674-21-4) was administered for 90 days in diet to groups of ten C57BL/6J mice per sex at concentrations of 0, 100, 300 or 1000 ppm (equal to 0, 17.0, 51.0 and 168 mg/kg bw per day for males, 0, 19.5, 59.8, and 207 mg/kg bw per day for females). Bioanalytical measurements were not taken. On the day of necropsy a blood sample was collected from the retro-orbital venous plexus of each animal for selected clinical chemistry determinations. All animals were necropsied, selected organs weighed and designated tissues taken, fixed and examined microscopically.

Mortalities, treatment-related clinical signs, effects on body weight or food consumption were not observed in males or females by the treatment. At 1000 ppm a depressed body weight gain during the study (by ca 20% compared with controls) in males was considered to be treatment-related. Lower total bilirubin concentrations in males and females at 1000 ppm were treatment-related but not considered toxicologically significant due to the same reason as given in Study 1 above (four-week study; Repetto, 2012). At necropsy, absolute and relative liver weights were higher than controls in both sexes at 1000 ppm. At histopathological examination, the incidence of diffuse (mainly centrilobular) hepatocellular vacuolation was slightly increased in males and females at 1000 ppm. No treatment-related changes were found in either sex at 300 ppm or below. Major findings in the 13-week mouse study are summarized in Table 8.

The NOAEL in 13-week feeding study in mice was 300 ppm (equal to 51.0 mg/kg bw per day) based on a slight decrease in body weight gain, liver weight increase and hepatocellular vacuolation in the centrilobular area at 1000 ppm (equal to 168 mg/kg bw per day) (Blanck, 2013).

Table 8. The summary of the 13-week dietary toxicity study of isoflucypram in mice.

		Dietary concentration (ppm)							
		Males				Females			
Parameter	**Day**	**0**	**100**	**300**	**1000**	**0**	**100**	**300**	**1000**
		Weights and clinical chemistry							
Body weight (g)	92	26.44	25.69	26.42	25.25	22.40	21.92	21.70	22.10
Body weight gain (g)	1–92	5.83	5.02	5.84	4.72*	4.67	4.34	4.24	4.55
Total bilirubin (µmol/L)	92–94	1.23	0.88	0.88	0.71**	1.24	0.71**	0.92	0.84*
Total cholesterol (mmol/L)	92–94	1.892	2.001	1.809	1.570*	1.564	1.547	1.576	1.577
Liver weight (g)	92–94	0.912	0.918	0.989	1.018*	0.791	0.818	0.794	0.891*
Liver weight, (% of bw)	92–94	4.107	4.232	4.447*	4.840**	4.183	4.518*	4.410	4.886**
		Histopathology							
Hepatocellular vacuolation, mainly centrilobular, diffuse									
Minimal and slight		1	0	0	3 #	2	1	4	8 #

bw: Body weight Source: Blanck, 2013

Statistically significant: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; # Significant in the trend test: $p \leq 0.05$

Rat

Study 1

Isoflucypram (purity 98.6%; Batch no. NLL 8674-19-4) was administered in diet to groups of Wistar rats (five/sex per group) for at least 28 days at concentrations of 0, 300, 1000 or 3000 ppm (equal to 0, 22.8, 83.3 and 240 mg/kg per day for males, 0, 25.6, 86.5 and 285 mg/kg per day for females). A detailed physical examination was performed weekly throughout the study. Before necropsy a blood sample was collected from the retro-orbital venous plexus of each surviving animal for haematology and clinical chemistry determinations. All animals were necropsied, selected organs were weighed and examined for histopathological analysis. The remaining portions of the liver were homogenized for microsomal preparations to determine cytochrome P-450 and UDP-GT isoenzymes profiles. In addition, intra-abdominal fat from all animals was collected and frozen at approximately –20°C for subsequent analysis to investigate any potential for isoflucypram accumulation in this tissue.

Mortality, treatment-related clinical signs and food consumptions were unaffected by the treatment. At 3000 ppm, body weight was depressed by 10–15% in males throughout the study. Body weight gain in males was reduced in study weeks 1, 2, and 3. The overall cumulative body weight gain was reduced by 31% compared to controls. In females, body weight parameters were not affected. Blood biochemical analysis revealed higher total cholesterol in males (by 26%) compared with controls. Noteworthy haematological changes were a lower leucocyte counts (by 34%) and lower absolute lymphocyte counts (by 38%) found in males at 3000 ppm. The decrease in leucocyte count was considered a result of the lymphocyte decrease since a major portion of leukocytes in rodents are lymphocytes. The decrease was considered to be treatment-related. The Meeting noted a similar change was not observed in females at the same dose, and there was no corresponding morphological findings in the haematopoietic system, such as the spleen, lymphocytes, thymus or bone marrow.

Slight changes (within 10%) included higher mean erythrocyte counts, total protein and albumin levels in males at 3000 ppm, but these were not toxicologically significance. Lower total bilirubin concentrations in both sexes (by 64% in males and by 82% in females) at 3000 ppm were treatment-related changes, but were not considered adverse effects due to the following reason: a decrease in bilirubin (rather than an increase) indicates a change opposite to what would be expected for hepatotoxicity or haemolysis; also no indicators of hepatobiliary damage or haemolysis were seen. The liver enzyme induction analysis in this study showed that treatment with isoflucypram consistently induced a metabolic enzyme by binding to bilirubin and bilirubin-UDP-GT, in the liver (See Appendix Fig. 1). The cause of the decrease was considered to be excess elimination of serum bilirubin from blood, due to bilirubin-UDP-GT induction.

Liver weights were higher in males (by 13% for absolute, 31% for relative) and in females (by approximately 60%) at 3000 ppm. Thyroid weights were higher in males (by 21% for absolute, 41% for relative) and by 20–22% in females at 3000 ppm. Macroscopic observations showed enlarged and/or dark liver in some animals of both sexes, together with prominent lobulation in some females. Microscopically, panlobular hepatocellular hypertrophy and hepatocellular periportal microvacuoles were seen in both sexes at 3000 ppm. In the thyroid, minimal to slight follicular cell hypertrophy was noted in both sexes. In the kidney, increased tubular hyaline droplets, positive to α2μ-globulin antibody, were found in all males together with an increased incidence of bilateral basophilic tubules. The renal changes were not observed in females. In rats α2μ-globulin nephropathy is accepted as a male specific lesion and so this change was not considered to be relevant to humans.

Samples of fat and liver were taken at termination for analysis to examine any potential for isoflucypram accumulation and metabolic enzyme induction, respectively. Intra-abdominal fat showed that isoflucypram has no potential for accumulation in fat up to the highest dietary concentration tested, as only traces of isoflucypram (below the limit of validation of 50 μg/L) were detected in 4/5 males and 4/5 females. Hepatic enzyme induction measurements in males showed high induction of benzoxyresorufin-*O*-dealkylase (BROD) and pentoxyresorufin-*O*-deethylase (PROD) Phase I activities, as well as bilirubin- and 4-nitrophenol-UDP-GT Phase II activities. Slight increases were also noted in total cytochrome P450, ethoxyresorufin-*O*-deethylase (EROD), and T4-UDP-GT activities. In females, the greatest increases were noted for BROD activity and bilirubin-, 4-nitrophenol-, and T4-UDP-GT activities, while PROD activity was slightly increased. These metabolic enzyme inductions in the liver were accompanied by liver hypertrophy at 1000 ppm and above in both sexes. The induction of bilirubin-UDP-GT was seen at 300 ppm in females but only at 1000 ppm and above in males, indicating female rats were more sensitive to induction by isoflucypram than the males. A summary of the data for this study is shown in Table 9.

The NOAEL for four-week toxicity in rats was 1000 ppm (equal to 83.3 mg/kg bw per day) based on reduced body weight gain, increased cholesterol, liver hypertrophy with hepatocellular microvacuoles at 3000 ppm (equal to 240 mg/kg bw per day) (Totis, 2017).

Table 9. Summary of the four-week dietary toxicity study of isoflucypram in rats

	Dose (ppm)							
	Males				Females			
Parameter	**0**	**300**	**1000**	**3000**	**0**	**300**	**1000**	**3000**
Body weight (g)								
day 22	378.0	388.8	378.4	322.3**	209.2	210.5	215.6	206.1
day 29	402.7	418.4	402.1	356.8**	223.4	223.1	224.6	226.5
Body weight change (g)								
days 1–29	148.4	163.8	147.0	102.1**	47.8	46.3	49.7	49.0
Red blood cells (10^{12}/L)	8.962	8.648	8.992	9.576*	8.838	9.080	8.970	8.970
White blood cells (10^9/L)	12.81	13.84	10.89	8.48*	8.62	9.48	7.58	8.51
Lymphocytes (10^9/L)	10.71	11.06	8.86	6.68**	7.04	7.74	5.99	6.81
Cholesterol (mmol/L)	1.686	1.833	1.520	2.132*	1.886	2.000	2.365	2.396
Total protein (g/L)	63.0	64.0	63.6	66.8**	60.0	61.8	67.3**	63.2
Albumin (g/L)	39.2	40.5	40.2	42.4**	39.0	39.2	42.8	39.6
Bilirubin (μmol/L)	0.78	0.63	0.26*	0.28*	1.22	0.68*	0.30**	0.22**
Terminal body weight (g)	381.6	393.3	374.1	328.2**	208.4	208.2	207.9	210.0
Liver weight (g)	10.160	11.031	11.398	11.436	5.223	5.486	6.629**	8.562**
Liver weight (% rel. to body weight)	2.663	2.800	3.043*	3.483**	2.510	2.634	3.263**	4.085**
Thyroid weight (g)	0.0170	0.0182	0.0170	0.0205	0.0116	0.0130	0.0135	0.0142
Thyroid weight (% rel. to body weight)	0.0044	0.0047	0.00456	0.0062**	0.0056	0.0063	0.0065	0.0068

	Dose (ppm)							
	Males				Females			
Parameter	**0**	**300**	**1000**	**3000**	**0**	**300**	**1000**	**3000**
Macroscopic findings at necropsy (number affected of five rats/sex per group)								
Liver; enlarged	0	1	2	2	0	0	1	5
dark	0	0	0	3	0	0	3	2
prominent lobulation	0	0	0	0	0	0	0	3
Histopathology (number affected of 5 tissues examined per group)								
Hepatocellular: hypertrophy	0	0	0	3	0	0	1	4
microvacuolation	0	0	0	3	0	0	0	4
Thyroid follicular cell hypertrophy	0	1	1	2	0	0	1	3
Kidney basophilic tubules, bilateral	0	0	3	3	0	1	1	0
tubular hyaline droplets	0	3	5	5	0	1	0	0
Kidney α2μ-globulin immunohistochemical staining (number affected; mean severity score)								
Total	5	4	5	5	-	-	-	-
Mean severity	1.00	1.75	2.80	3.80	-	-	-	-
Liver enzyme biochemistry; mean of 5 rats/group (% of control)								
Total cytochrome P450[1]	100	101	124*	135**	100	86	89	92
EROD [a]	100	105	136*	157**	100	100	112	128
PROD [a]	100	85	161**	515**	100	116	140	143
BROD [a]	100	129	256**	734**	100	124	229**	524**
UDP-GT-4-nitrophenol [a]	100	122	214**	247**	100	107	198**	304**
UDP-GT-bilirubin	100	111	244**	317**	100	167*	250**	401**
UDP-GT-T4	100	102	147*	162**	100	135	222**	319**

[a] Liver enzyme positive control data obtained from Totis, 2007; Source: Totis, 2017

EROD: Ethoxyresorufin-*O*-deethylase; PROD: Pentoxyresorufin-*O*-deethylase;
BROD: benzoxyresorufin-O-dealkylase; UDP-GT: Uridine diphosphate glucuronosyltransferase;

Statistically significant: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Study 2

Isoflucypram (purity 97.7%; Batch no. NLL 8674-21-4) was administered for at least 90 days to 10 Wistar rats/sex per group at dietary concentrations of 0, 100, 300 or 1000 ppm (equal to 0, 6.34, 18.4, 63.5 mg/kg bw per day for males, 0, 7.92, 21.9, 80.9 mg/kg bw per day for females) . Ten animals per sex were added to the control and high-dose groups to assess the reversibility after one month. All surviving animals from the main study groups (dosing phase) were subjected to a neurotoxicity assessment (spontaneous motor activity, fore- and hindlimb grip strength, landing foot splay and rectal temperature parameters) during week 11 of the study. Urine samples were collected overnight from all animals on the week before scheduled necropsy. Before scheduled necropsy a blood sample was collected from the retro-orbital venous plexus of each animal for haematology and blood biochemistry determinations. All animals were necropsied, selected organs weighed and examined microscopically. The immunohistochemical detection of α2μ-globulin was performed in the kidney in all males from the control and treated groups.

No treatment-related change was observed in mortality, clinical sign, food consumption, haematology, urinalysis, or the results of ophthalmological and neurological examinations. At 1000 ppm body weight gains were reduced on several occasions during the study and the overall mean body weight gain and mean body weight in males were reduced by 12% and 7% compared to controls. In females,

body weight gains were reduced on day 8, thereafter, body weight gains were comparable to controls. The overall mean body weight gain in females was 8% lower than for controls, while mean body weight was similar to controls. These differences were quite small and only statistically significant over a few of the periods following commencement of treatment, however the consistent reductions in body weight gain in both sexes at 1000 ppm were considered to marginal but adverse effects caused by the treatment. In blood biochemistry at 1000 ppm, total bilirubin concentrations were low in both sexes, and total cholesterol concentration was slightly increased (by 19%) in females. In microscopic examination of the urinary system, cellular casts were found in the urine of five males at 1000 ppm while none were seen at 0 ppm. As a metabolic enzyme, bilirubin-UDP-GT had been induced in the liver at the same dose in an earlier four-week study (Totis, 2017). The decrease in serum bilirubin was considered not to be adverse.

In pathological examinations, relative liver weight was increased in males and females at 1000 ppm. Relative thyroid weight was also increased in males at the same dose. The histopathological changes corresponding with the increased organ weights were panlobular hepatocellular hypertrophy and follicular cell hypertrophy. No treatment-related finding in the thyroid reached statistically significance. Macroscopical examination revealed enlarged livers, which correlated with microscopic hepatocellular hypertrophy. The liver hypertrophy observed at 1000 ppm was considered to be an adaptive change. A higher severity of α2μ-globulin-positive droplets was observed in males at 1000 and 300 ppm when compared to controls. The α2μ-globulin nephropathy, including increased kidney weights and the cellular casts seen in urine (a male rat specific disease), was considered to be enhanced at 1000 ppm. No treatment-related changes were observed in other dose groups.

The changes seen in body weight, liver and thyroid during the treatment phase were almost completely reversed in both sexes during the recovery phase. The severity of α2μ-globulin nephropathy was also comparable to that of controls after the recovery period.

Results of the 90-day study in rats are summarized in Table 10.

In this study the NOAEL was 300 ppm (equal to 18.4 mg/kg bw per day) based on slightly depressed body weight gains at 1000 ppm (equal to 63.5 mg/kg bw per day) (Odin, 2017).

Table 10. Summary of the 90-day feeding study of isoflucypram in rats; main study phase

	Dietary dose (ppm)							
	Males				Females			
Parameter	**0**	**100**	**300**	**1000**	**0**	**100**	**300**	**1000**
	Main study phase							
Body weight, week 13 (g)	547.9	545.3	545.3	507.1	297.3	294.4	297.6	295.5
Body weight gain (g)	334.6	330.7	330.9	293.6	120.0	111.0	112.6	110.1
Total bilirubin, (μmol/L)	0.94	0.97	0.96	0.56*	1.85	1.52	1.16*	0.68**
Total cholesterol (mmol/L)	1.528	1.639	1.750	1.716	1.933	1.838	1.973	2.302*
Liver weight (g)	11.24	11.27	11.17	11.67	6.207	6.240	6.342	7.469**
Liver weight; rel.[a] (%)	2.162	2.175	2.163	2.397**	2.158	2.254	2.231	2.754***
Thyroid weight (g)	0.0188	0.0200	0.0179	0.0213	0.0153	0.0144	0.0159	0.0150
Thyroid weight; rel.[a] (%)	0.00363	0.00386	0.00347	0.00439*	0.00533	0.00520	0.00558	0.00553
	Main study phase – histology							
Liver (*N*)	10	10	10	10	10	10	10	10
Hepatocellular hypertrophy: periportal to panlobular								
minimal and slight	0	0	0	0	0	0	0	6
Kidney (*N*)	10	10	10	10	10	10	10	10
Hyaline droplets: proximal tubules								
minimal, slight and moderate	4	4	7	10	0	0	0	0

	Dietary dose (ppm)							
	Males				Females			
Parameter	0	100	300	1000	0	100	300	1000
Basophilic tubules: bilateral								
minimal and slight	4	0	1	6	0	0	0	0
Granular cast(s)								
minimal	0	0	0	2	0	0	0	0
Thyroid (*N*)	10	10	10	10	10	10	10	10
Follicular cell hypertrophy: diffuse								
minimal	1	0	1	5	0	0	0	5
Colloid alteration								
minimal	2	0	1	5	0	0	0	2
	Recovery phase							
Body weight (g) day 29	573.1	-	-	536.5	285.0	-	-	310.4*
Body weight gain (g)	21.1	-	-	37.7	−4.7	-	-	6.4
Terminal body weight (g)	554.8	-	-	509.6	283.3	-	-	292.4
Liver weight (g)	10.75	-	-	10.67	5.87	-	-	6.41
Liver weight; rel.[a] (%)	1.940	-	-	2.093*	2.066	-	-	2.195
Thyroid weight (g)	0.0210	-	-	0.0202	0.0156	-	-	0.0154
Thyroid weight; rel.[a] (%)	0.00381	-	-	0.00403	0.0051	-	-	0.00531
	Recovery phase – histopathology; *N* = 10, control and high dose only							
Kidney								
Basophilic tubules: bilateral								
minimal and slight	2	-	-	5	0	-	-	1
Granular cast(s)								
minimal	0	-	-	3	0	-	-	0
Thyroid;								
Colloid alteration								
minimal and slight	3			6	0			1

[a] Weight relative to body weight, expressed as percentage; N: Number of rats sampled; Source: Odin, 2017

Statistically significant: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Dog

Study 1

Isoflucypram (purity 98.0%; Batch number: NLL 8674-28-2) was administered for 28 days to groups of two beagle dogs per sex at dietary concentrations of 0, 300, 1000 or 3000 ppm (equal to 0, 12.7, 37.7 and 76.9 mg/kg bw per day for males, 0,11.3, 36.5, and 90.2 mg/kg bw per day for females). Similarly constituted groups of two males and two females at 0 and 3000 ppm isoflucypram for the 4-week treatment period subsequently received an untreated diet to check reversibility. All animals were subjected to a detailed necropsy. Selected organs were weighed and a range of tissues taken and processed for histopathological examination. Plasma concentrations of isoflucypram were measured at the end of the study.

Dietary administration of isoflucypram to male and female beagle dogs at 300, 1000 or 3000 ppm did not cause mortality nor did it induce any change seen at physical or ophthalmological examinations, haematology investigations or urinalysis. Plasma levels of isoflucypram showed a dose-related increase with a maximum concentration measured two or four hours after feed distribution. Metabolites were not measured.

At 3000 ppm increased salivation was observed on two occasions (days 15 and 22) for one male, and a wasted appearance was noted in one female on day 8. The salivation was a common change seen in the dog studies (see below). This clinical sign was considered to be a local effect but not a neurotoxic finding because this compound was a mild irritant to rabbit eyes and no neurological finding was otherwise observed due to this compound. At the end of the study, three animals lost weight (0.2−0.5 kg), and the fourth animal only gained 0.1 kg compared to a body weight gain of 0.6–1.2 kg in controls. Food consumption values were lower for almost all animals compared to controls or to their own pretest value. In blood biochemistry, increased ALP was observed in one male and two females; lower total cholesterol concentrations were observed in one male and one female. Terminal body weights of females were lower than for controls. Mean absolute and relative liver weights were higher in both sexes compared to controls; the livers were enlarged macroscopically in one male. Observed microscopically were centrilobular to panlobular hepatocellular hypertrophy in both sexes, and an increase in brown pigment accumulation in Kupffer cells in females at 3000 ppm. The brown pigment accumulation observed only in females was also found at 1800 ppm in the one-year study with this compound. Therefore, this change although minor was considered a treatment-related adverse effect. Liver effects in males at 3000 ppm were considered adaptive. No effect was seen at 1000 ppm or lower in either sex. Plasma concentrations of isoflucypram in both sexes reached a peak within two hours of feeding. The findings are summarized in Tables 11a and b.

The NOAEL for the four-week toxicity study in dogs was 1000 ppm (equal to 36.5 mg/kg bw per day) based on reduced body weight gain at 3000 ppm (equal to 90.2 mg/kg bw per day) (Garcin, 2014).

Table 11a. Summary of the 28-day feeding study of isoflucypram in dogs

		Dietary dose (ppm)							
		Males				Females			
Parameter		**0**	**300**	**1000**	**3000**	**0**	**300**	**1000**	**3000**
Terminal body weight (kg)	A	7.1	7.9	6.6	6.0	6.8	6.3	6.0	5.8
	B	7.2	6.9	8.7	7.7	6.1	6.1	6.5	5.1
Absolute liver weight (g)		254.3	263.3	282.9	320.5	191.2	228.7	257.9	244.7
Liver weight, as percentage of body weight		3.56	3.55	3.68	4.78	2.96	3.69	4.12	4.50
Alkaline phosphatase (IU/L)									
Pre-study day 20	A	110	107	131	227	129	141	133	100
	B	130	298	117	217	97	132	121	124
Study day 25	A	119	138	157	255	123	176	384	555
	B	117	280	171	372	115	171	157	361
Cholesterol (mmol/L)									
Pre-study day 20	A	2.79	2.99	2.74	3.20	2.91	3.45	2.69	2.92
	B	3.75	1.89	3.34	3.07	2.68	3.70	3.73	2.85
Study day 25	A	2.75	3.08	2.86	1.93	2.51	3.42	2.20	2.36
	B	3.34	2.14	3.05	3.13	2.66	4.43	3.80	1.90
Histopathology									
Hepatocellular hypertrophy, centrilobular to panlobular		0	0	0	2	0	0	0	1
Accumulation of brown pigment in Kupffer cells		0	0	0	0	0	0	0	2

A and B: Data for individual animals; Source: Garcin, 2014

Table 11b. Summary of the 28-day feeding study of isoflucypram in dogs; isoflucypram in plasma

Sex	Dietary concentration (ppm)	Animal ID	Time after food distribution			
			0 hours	1 hour	2 hours	4 hours
			Concentration of isoflucypram in plasma (mg/L)			
Male	0	1M0312 (A)	< LOQ	NS	NS	NS
		1M0313 (B)	< LOQ	NS	NS	NS
	300	2M0316 (A)	< LOQ	0.111	0.180	0.230
		2M0317 (B)	< LOQ	0.0555	0.0726	0.0563
	1000	3M0320 (A)	0.0164	0.529	0.919	0.965
		3M0321 (B)	0.0157	0.707	1.48	1.60
	3000	4M0324 (A)	0.0226	2.71	3.25	2.89
		4M0325 (B)	0.0187	2.52	3.36	3.36
Female	0	1F0314 (A)	< LOQ	NS	NS	NS
		1F0315 (B)	< LOQ	NS	NS	NS
	300	2F0318 (A)	< LOQ	0.127	0.188	0.240
		2F0319 (B)	< LOQ	0.0206	0.0336	0.0546
	1000	3F0322 (A)	< LOQ	0.180	0.589	0.631
		3F0323 (B)	< LOQ	NS	0.164	0.167
	3000	4F0326 (A)	< LOQ	1.37	1.87	1.57
		4F0327 (B)	< LOQ	0.907	1.80	1.44

A and B: Data for individual animals; < LOQ: Below limit of quantification (0.01 mg/L); NS: Not sampled

Source: Garcin, 2014

Study 2

Isoflucypram (purity 94.2%; Batch number: 2013-006492) was administered to four male and four female beagle dogs per group, all 8–9 months old. Animals received isoflucypram via dietary admixture at 0, 170, 500 or 1500 ppm (equal to 0, 5.5, 15.9 and 50.4 mg/kg bw per day for males, 0, 5.5, 16.2 and 54.0 mg/kg bw per day for females) for at least 13 weeks. A detailed physical examination was made prior to the start of treatment and monthly during the treatment period. Ophthalmological examinations were performed during the acclimatization phase and at the end of the study. Haematology, blood biochemistry analysis and urinalysis were performed once during the acclimatization phase, at week 7 and at the end of the study. A blood sample was also collected from selected animals for measurement of plasma concentration of isoflucypram (not metabolites) at the end of the study. All animals were subjected to a detailed necropsy. Selected organs were weighed and a range of tissues collected and processed for histopathological examination.

Up to 1500 ppm, the highest dose level tested, dietary administration of isoflucypram for at least 13 weeks induced no mortalities, no treatment-related changes in food consumption, nor any changes that were noted as a result of ophthalmological examination, haematology, urinalysis or gross observations. At the end of the study (week 12), the bioanalytical examination showed that plasma concentrations of isoflucypram before food distribution (that is, corresponding to 22.5 hours after the last feed distribution) were marginally above the limit of quantification in the high-dose group in both sexes and were below or close to the limit of quantification in the mid- and low-dose groups in both sexes. After feed distribution, the mean values in the treated groups showed a dose-related increase in plasma concentrations of isoflucypram, with similar results observed for the two sexes at both time points (four and seven hours after food distribution) and a peak concentration noted four hours after food distribution.

The only treatment-related clinical sign observed consisted of increased salivation at 1500 ppm in two females on five and seven occasions, respectively, all during the second half of the study. Similar salivation was also seen in the four- and 52-week dog studies. Macroscopical nor microscopical findings relating to salivation were not observed. Isoflucypram was a mild irritant to the eyes of rabbit

s. Therefore, the salivation was not considered adverse; it may have been a local effect. Mean body weight parameters were unaffected by treatment during the first half of the study, but towards the end of the study mean body weight at 1500 ppm was lower by 7% and 8% than for controls in males and females respectively, and overall mean body weight gain was lower by 39% than for controls in both sexes (statistically significant for overall mean body weight gain in males only). No effects on clinical signs or body weight were observed in the other treated groups.

In blood biochemistry, ALP activity was increased at 170 ppm (by ca 50% in males and 20% in females at week 12–13), 500 ppm (by ca 60% in males and 70% in females at week 12–13) and 1500 ppm (by ca 140% of the controls in males and 170% in females at week 12–13). The increases at 1500 ppm were statistically significance. The increases in ALP at 170 ppm and 500 ppm were not accompanied by other hepatobiliary damage, only liver hypertrophy-related changes. In addition, similar situations were seen to occur at similar doses in the one-year dog study of this compound. The adversity of increased ALP activity induced by pesticides in dogs has been previously reported (Yokoyama et al., 2019, 2021). In the absence of other findings related to hepatobiliary damage found at similar or lower doses to that causing an increase in ALP activity, the increase was not regarded as adverse, because increased ALP is not a sensitive marker for hepatobiliary damage in dogs. The ALP increase is reported to be a finding related to phenobarbital-type liver hypertrophy in dogs (Gaskill, Hoffmann & Cribb, 2004; Gaskill et al., 2005). The nature of the ALP increase in this study could be classed as an ALP increase without co-existing hepatobiliary damage. Taken together, the ALP increases observed up to 500 ppm were treatment-related but not adverse.

The observed decrease in total bilirubin is a common treatment-related change with this compound, seen in mice, rats and dogs. The decrease in bilirubin in the absence of accompanying related changes is considered to be treatment-related but not adverse in other species. Analyses of liver metabolic enzymes were conducted in mice and rats but not in dogs. Treatment-induced, CAR-mediated liver hypertrophy and bilirubin-UDP-GT induction, and/or increase in UGT1A1 expression in the liver has been shown in rodents. An available source reported on the induction of UDP-GT by phenobarbital treatment in the liver of beagle dogs (Makino et al., 2009). Integrating the available information described above, a plausible mode of action (MOA) for decreased bilirubin in dogs may consider it to be similar to the process in rodents. Therefore decreased bilirubin was not considered adverse in dogs.

At necropsy, mean terminal body weight at 1500 ppm was lowered by 6% in males and 9% in females (not statistically significant), and mean liver weight parameters were increased by 34–43% in males (statistically significant for liver relative to body weight and liver relative to brain weight ratios), when compared to controls. At microscopic examination, minimal centrilobular hepatocellular hypertrophy was observed in 3/4 males, together with cytoplasmic changes (eosinophilic intracytoplasmic vacuoles) in the liver for two of these males. This microscopic change is accepted as a typical feature of hepatocellular hypertrophy, with smooth-endoplasmic reticulum proliferation in hepatocyte (Greaves, 2012a). No other findings indicating hepatotoxicity were observed as a result of the treatment. Therefore, morphological findings were considered adaptive.

The major findings in this study are summarized in Table 12.

The NOAEL for 13-week toxicity in dogs was 500 ppm (equal to 15.9 mg/kg bw per day) based on reduced bodyweight gain at 1500 ppm equal to (50.4 mg/kg bw per day) (Kennel, 2015).

Table 12. Summary of the 13-week oral study of isoflucypram in dogs

		Dietary dose (ppm)							
		Males				Females			
Parameter	**Time**	**0**	**170**	**500**	**1500**	**0**	**170**	**500**	**1500**
Body weight (kg)	Day 92	9.00	8.48	8.73	8.40	8.35	8.03	8.13	7.70
Body weight gain, (kg)	Days 1–92	1.98	1.48	1.58	1.20**	1.73	1.50	1.40	1.05
Alkaline phosphatase (IU/L)	Prestudy	84.0	86.3	87.5	93.0	70.8	76.0	118.0	123.5
	Week 7	104.5	136.5	139.8	217.5*	103.3	122.3	176.3	291.3*
	Week 12–13	89.3	135.5	142.3	213.5*	103.5	128.0	171.8	284.0*

		Dietary dose (ppm)							
		Males				Females			
Parameter	**Time**	**0**	**170**	**500**	**1500**	**0**	**170**	**500**	**1500**
Total bilirubin (µmol/L)	Prestudy	0.65	0.88	0.53	0.33	0.68	0.93	0.73	1.03
	Week 7	0.78	0.90	0.40	0.45	0.73	0.33	0.68	0.48
	Week 12–13	0.75	0.70	0.33	0.30	0.73	0.33	0.40	0.33
Terminal body weight, (kg)	Termination	8.93	8.50	8.68	8.38	8.38	7.95	8.08	7.65
Liver weight (g)	Termination	252.2	269.4	284.0	338.5	279.4	278.3	291.1	308.1
Liver weight, relative [a] (%)	Termination	2.83	3.16	3.28	4.04**	3.34	3.48	3.60	4.03
Liver histology									
Hepatocellular hypertrophy, centrilobular;									
minimal + slight	Termination	0	0	0	3	0	0	0	0
Intracytoplasmic eosinophilic inclusion;									
Total	Termination	0	0	0	2	0	0	0	0
Plasma levels of isoflucypram (mean; mg/L)									
Prefeeding		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.040
4 hours after feeding		NS	0.053	0.184	1.528	NS	<0.048	0.147	1.280
7 hours after feeding		NS	0.036	0.124	1.030	NS	<0.044	0.119	0.825

[a] Weight relative to body weight, expressed as percentage; NS: No sampling Source: Kennel, 2015

< LOQ: Below limit of quantification (0.01 mg/L), the value of the LOQ was used for calculation of the group mean; the result was then expressed as "< mean value"

Statistically significant: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Study 3

Isoflucypram (purity 94.2%; Batch no. 2013-006492) was administered to groups of four male and four female beagle dogs (8–9 months old at study start) at dietary concentrations of 0, 150, 600 or 1800 ppm (equal to 0, 4.2, 18.8 and 60.2 mg/kg bw per day for males, 0, 4.2, 17.6 and 49.8 mg/kg bw per day for females) for at least 52 weeks. Each animal was checked for ill-health, moribundity and mortality twice a day or once daily at weekends. Observed clinical signs were recorded. A detailed clinical examination was performed approximately weekly and a detailed physical examination carried out monthly. Food consumption was recorded and body weight were measured weekly. Ophthalmology was performed pretest and the end of the study. Haematology, blood biochemistry and urinalysis were conducted at weeks 4, 7 and at the end of study. Blood samples were also collected from all surviving animals at four and seven hours after feeding at the end of study, from which to measure plasma concentrations of the parent compound and two metabolites, isoflucypram-cyclopropyl-pyrazole-carboxamide (BCS-CX99798, M58) and isoflucypram-desmethyl-carboxylic acid (BCS-CX99799, M11). All animals were necropsied, and selected organs were weighed, examined and prepared for histopathological analysis.

Doses of up to 1800 ppm of isoflucypram induced no treatment-related mortalities, and no treatment-related changes were noted at physical or ophthalmological examinations, from haematology or urinalysis measurements. One female at 150 ppm was sacrificed for humane reasons on day 176 due to a congenital anomaly (persistent fourth right aortic arch) that had significantly affected the clinical condition of this dog. At the end of the study (month 12), the plasma concentrations of isoflucypram and its metabolites were determined in the mid- and high-dose dogs of both sexes, and levels of the parent were found to be higher those of metabolites M58 and M11. The concentrations of the parent showed a dose-related increase, with a pattern of supranonlinearity at 1800 ppm. Metabolites M58 and M11 showed similar patterns, but with less steep concentration curves than for the corresponding dose of parent at 1800 ppm in both sexes. Peak concentrations were observed four or seven hours after food distribution.

The only treatment-related clinical signs at 1800 ppm consisted of increased salivation at in males, observed on 17 occasions during the study. There was no enhancement of this sign as the treatment period proceeded. Macroscopic and microscopic finding related to salivation were not observed. As described above, this common clinical sign to dogs was considered to be a local effect. The consistent lowest values of cumulative body weight gain in females at 1800 ppm throughout the study although no statistically significant changes were observed in body weight in both sexes. Feed consumption was decreased in females at 1800 ppm especially at Week 1. Feed consumption was unaffected in males. Taken together, the slight decrease in body weight gains in females at 1800 ppm was considered to be treatment related. Clinical chemistry determination revealed increased mean ALP in both sexes on one or more occasions. The ALP increase without accompanying hepatobiliary indicator was not considered adverse due to the same reason as that of the 90-day study. At necropsy, mean relative liver weights were increased in both sexes at 600 ppm and above at 15% higher than controls. A statistically significant increase was observed in males at 1800 ppm only, where the increase was 50% higher. At macroscopic observation, enlarged liver was noted in 3/4 males and 1/4 females. Histopathologically minimal to moderate centrilobular hepatocellular hypertrophy was observed in the liver in both sexes at 600 ppm and above, and eosinophilic intracytoplasmic inclusions of hepatocyte, an indicative change of smooth endoplasmic reticulum (sER) proliferation of hepatocyte in one male at 1800 ppm. Kupffer cell pigmentation were observed in two males and one female, and single cell necrosis in one female were also observed at 1800 ppm. Liver hypertrophy is basically adaptive to metabolism enzyme induction in the liver, however; excess liver hypertrophy was reported to have adverse potential (Hall, Elcombe & Taylor, 2012). The excess liver hypertrophy with the slight reaction in Kupffer cells and hepatocytes in both sexes at 1800 ppm was considered to be adverse.

The NOAEL in the one-year toxicity study in dogs was 600 ppm (equal to 17.6 mg/kg bw per day) based on severe liver hypertrophy with Kupffer cell pigmentation in males at 1800 ppm (equal to 49.8 mg/kg bw per day) (Kennel, 2017a).

Table 13. Summary of results in the one-year oral study of isoflucypram in dogs

		Dietary dose (ppm)							
		Males				**Females**			
Parameter	**Time**	**0**	**150**	**600**	**1800**	**0**	**150**	**600**	**1800**
Body weight (kg)	Day 1	7.08	7.05	6.93	6.98	7.40	8.10	7.10	**7.13**
	Day 9	7.23	7.25	7.05	7.00	7.45	8.17	7.23	**7.00**
	Day 93	8.60	8.55	8.05	8.23	8.75	9.73	8.13	**7.90**
	Day 184	9.15	8.90	8.45	8.60	9.30	10.30	8.73	**8.40**
	Day 361	9.65	9.10	8.60	8.83	9.95	11.10	9.08	**8.85**
Six-month cumulative body weight gain (kg)	13–26 weeks Mean	0.55	0.35	0.4	0.38	0.55	0.57	0.6	**0.5**
	SD	0.42	0.404	0.183	0.263	0.289	0.723	0.163	**0.408**
	26–52 weeks Mean	0.5	0.2	0.15	0.23	0.65	0.8	0.35	**0.45**
	SD	0.49	0.216	0.265	0.171	0.592	0.173	0.252	**0.28**
Food consumption (g)	Week 1 [% of control]	608 -	629 [103]	608 [100]	592 [99]	627 -	728 [98]	583 [93]	**468 [75]**
	Weeks 1–52 [% of control]	627 -	590 [98]	630 [104]	677 [112]	616 -	678 [110]	605 [98]	552 [90]
Alkaline phosphatase, mean ± SD (IU/L)	Prestudy	89.8 ± 12.45	98.0 ± 12.68	132.3 ± 2919	137.3 ± 49.65	116.0 ± 63.68	114.0 ± 57.31	96.3 ± 28.44	82.0 ± 15.68
	Month 4	69.8 ± 18.50	87.3 ± 15.90	156.5 ± 24.85*	266.0 ± 60.15**	110.3 ± 52.40	102.5 ± 33.28	132.5 ± 61.20	185.5 ± 39.95
	Month 6	63.0 ± 19.36	85.0 ± 18.92	127.3 ± 19.38	243.3 ± 79.28**	105.0 ± 51.63	83.0 ± 36.18	144.5 ± 43.75	176.3 ± 49.23
	Month 12	57.3 ± 0.386	82.5 ± 13.53	152.5 ± 41.22	268.8 ± 92.94**	108.0 ± 56.47	106.0 ± 34.04	180.0 ± 53.77	250.8 ± 79.71*

		Dietary dose (ppm)							
		Males				Females			
Parameter	**Time**	**0**	**150**	**600**	**1800**	**0**	**150**	**600**	**1800**
Total bilirubin (µmol/L)	Pre-study	0.35 ± 0.443	0.23 ± 0.171	0.08 ± 0.150	0.30 ± 0.216	0.23 ± 0.206	0.30 ± 0.316	0.18 ± 0.222	0.60 ± 0.445
	Month 4	0.98 ± 0.525	0.55 ± 0.370	0.45 ± 0.252	0.25 ± 0.129	1.10 ± 0.483	0.63 ± 0.206	0.65 ± 0.480	0.40 ± 0.141
	Month 6	0.80 ± 0.638	0.53 ± 0.222	0.35 ± 0.300	0.10 ± 0.082	1.48 ± 0.562	0.55 ± 0.443*	0.45 ± 0.238**	0.28 ± 0.263**
	Month 12	0.78 ± 0.386	1.13 ± 0.754	0.80 ± 0.589	0.30 ± 0.346	1.20 ± 0.702	0.53 ± 0.611	0.55 ± 0.412	0.53 ± 0.287
Body weight (kg)	Termination	9.68	9.10	8.53	8.78	10.03 ±	11.10	9.03	8.88
Liver weight (g)	Termination	241.8	215.5	266.2	346.2**	255.6	292.7	271.6	279.0
Liver weight, relative [a] (%)	Termination	2.53	2.38	3.12	3.96**	2.56	2.69	3.01	3.21
Histopathology									
N	Termination	4	4	4	4	4	3	4	4
Hepatocellular hypertrophy, centrilobular									
minimal	Termination	0	0	2	0	0	0	3	1
slight	Termination	0	0	0	2	0	0	0	3
moderate	Termination	0	0	0	2	0	0	0	0
total	Termination	0	0	2	4	0	0	3	4
Intracytoplasmic eosinophilic inclusion									
total	Termination	0	0	0	1	0	0	0	0
Kupffer cell pigmentation: focal									
total	Termination	0	0	0	2	0	0	0	1
Single cell necrosis: focal									
total	Termination	0	0	0	0	0	0	0	1
Plasma isoflucypram and metabolites, mean (mg/L)									
Isoflucypram (parent)	Prefeeding	< LOQ	< LOQ	< 0.013	< 0.048	< LOQ	< LOQ	< 0.016	0.145
	4 hours after feeding	NS	< LOQ	0.108	2.540	NS	< 0.02	0.225	1.365
	7 hour after feeding	NS	< 0.011	0.115	1.795	NS	< LOQ	< 0.231	0.603
M58 Isoflucypram-cyclopropyl-pyrazole-carboxamide (BCS-CX99798)	Prefeeding	< LOQ	< LOQ	0.031	< 0.093	< LOQ	< LOQ	0.037	0.087
	4 hours after feeding	NS	< LOQ	0.052	0.193	NS	< 0.01	0.077	0.140
	7 hour after feeding	-	< 0.012	0.074	0.225	NS	< 0.010	0.088	0.176
M11 Isoflucypram-desmethyl-carboxylic acid (BCS-CX99798)	Prefeeding	< LOQ	< LOQ	0.129	0.114	< LOQ	< 0.01	0.101	0.183
	4 hours after feeding	NS	< 0.011	0.225	0.553	NS	< 0.020	0.305	0.385
	7 hour after feeding	NS	< 0.018	0.225	0.433	NS	< 0.012	0.254	0.380

[a] Weight relative to body weight, expressed as percentage; Source: Kennel, 2017a

< LOQ: Below the limit of quantification (0.01 mg/L);
the value of the LOQ was used for calculation of the group mean; the result was then expressed as "< mean value";

NS: No sampling: *N*: Number examined;

Statistically significant: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

(b) Dermal application

No data available.

(c) Exposure by inhalation

No data available.

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

To investigate oncogenic potential, male and female C57BL/6J mice were fed a diet treated with isoflucypram (purity 94.2%; Batch no. 2013-006492) for 18 months. In addition, an interim sacrifice was performed after 52 weeks of treatment to assess chronic toxicity. Groups of 60 male and 60 female C57BL/6J mice were fed diet containing 0, 50, 250 or 1250 ppm of isoflucypram for 52 weeks. After 52 weeks 10 males and 10 females from each group allocated to the chronic phase of the study were necropsied at the scheduled interim sacrifice. The remaining 50 animals per sex of each group, allocated to the carcinogenicity phase of the study, continued treatment until the scheduled final sacrifice after at least 78 weeks of treatment. The mean intake of isoflucypram over 18 months was 0, 5.9, 29.0 and 147 mg/kg per day for males, 0, 7.8, 38.1 and 190 mg/kg per day for females. In addition to the routine examinations in the guideline, a blood sample was collected from selected animals for bioanalytical examination after three months, one year and at the end of the study. All animals were subjected to necropsy, with selected organs weighed at scheduled interim and final sacrifice. Designated tissues were fixed and examined microscopically.

Analysis for plasma concentration of isoflucypram produced values below or slightly above the LOQ throughout the study in all treated groups. A dose-related increase in the two major metabolites M58 (isoflucypram-cyclopropyl-pyrazole-carboxamide; BCS-CX99798) and M11 (isoflucypram-desmethyl-carboxylic acid; BCS-CX99799) was observed in their plasma concentrations with similar levels observed throughout study.

Treatment-related effects were not noted on feed consumption, at physical or haematological assessments throughout the study, nor was earlier development or increased incidence of tumours in either sex observed. At 1250 ppm there was a marginal increase in mortality amongst females. Mean body weight was lower than controls in both sexes. Overall, the cumulative body weight gain between weeks 1 and 78 was 11% lower in males and 13% lower in females than for controls.

At 1250 ppm terminal body weights appeared unaffected by the treatment in either sex at the one-year interim kill, and body weight gains were depressed by 6% in both sexes at the termination compared with controls. Absolute and relative liver weights were increased with statistical significance at 1250 ppm. At microscopic examination, an incidence of multinucleated hepatocytes in males at 12 months and 18 months was increased in the livers from the 1250 ppm group. Observed in female livers at 1250 ppm were a higher incidence and severity of diffuse bile duct hyperplasia, a higher incidence of hepatocellular necrotic foci, and a lower incidence and severity of diffuse hepatocellular vacuolation. In addition in the 1250 ppm groups, also observed were a slightly greater incidence and/or severity of amyloid deposition in the liver, submaxillary lymph node, kidney (females only), adrenal gland, and thyroid gland. The observed increase in kidney weight without accompanying histopathology was not considered treatment-related. The changes seen in this study are summarized in Table 14.

The NOAEL for long-term effects in mice was 250 ppm (equal to 29.0 mg/kg bw per day) based on reduced body weight and increased incidence of multinucleated hepatocytes at 1250 ppm (equal to 147 mg/kg bw per day). The treatment with isoflucypram did not induce carcinogenic effects in mice (Blanck, 2017).

Table 14. The summary of the carcinogenicity study of isoflucypram in mice.

		Dietary dose (ppm)							
		Males				Females			
Parameter	**Time**	**0**	**50**	**250**	**1250**	**0**	**50**	**250**	**1250**
N (initial)	Day 0	50	50	50	50	50	50	50	50
N killed for humane reasons	-	9	6	6	8	6	10	9	14
*N f*ound dead	-	0	0	0	1	0	2	1	1
Body weight (g)	Day 365	32.80	32.52	33.25	31.33***	26.98	27.01	26.86	25.43**
	Day 540	33.11	33.19	32.95	31.72**	27.92	27.87	27.74	26.52*
Body weight gain (g)	Days 1–92	7.28	6.90	7.14	6.54**	5.58	5.09**	4.83**	4.40**
	Days 1–540	12.51	12.49	12.35	11.14**	11.15	10.88	10.89	9.66**
N of mice examined	12 months	9	9	9	10	10	9	10	9
Liver weight rel. [a]		4.030	4.134	4.059	4.845***	4.611	4.741	4.842	5.270**
N of mice examined	18 months	41	44	44	41	44	38	40	35
Liver weight rel. [a]		4.129	4.375	4.157	4.822***	5.089	5.065	5.393	5.651***
Adrenal weight rel. [a]	18 months	0.014	0.014	0.016	0.018**	0.032	0.032	0.032	0.036**
Kidney weight rel. [a]	18 months	1.686	1.708	1.792**	1.947***	1.617	1.598	1.571	1.627
Histopathology of the liver									
Multinucleated hepatocytes (affected/observed)									
minimal, slight and moderate	12 months	1/10	0/10	0/10	8/10**	0/10	0/10	1/10	0/10
minimal, slight and moderate	18 months	3/50	1/50	4/50	43/50***	0/50	0/50	0/50	0/50
Bile duct hyperplasia: diffuse (affected/observed)									
minimal and slight	12 months	1/10	0/10	0/10	0/10	0/10	0/10	2/10	5/10**
minimal and slight	18 months	5/50	8/50	5/50	5/50	8/50	9/50	9/50	25/50***
Single cell necrosis: focal									
minimal and slight	18 months	4/50	0/50	2/50	10/50*	5/50	2/50	4/50	2/50
Hepatocellular necrotic focus (affected/observed)									
Minimal & slight	18-month	11/50	9/50	7/50	15/50	12/50	9/50	13/50	24/50*
Plasma isoflucypram and metabolites, mean (mg/L)									
Isoflucypram	3 months	NS	<LOQ	<LOQ	< 0.0102	NS	<LOQ	<LOQ	< 0.0184
Isoflucypram	12 months	NS	<LOQ	<LOQ	<LOQ	NS	<LOQ	<LOQ	< 0.0198
M58	12 months	NS	0.0294	0.125	0.486	NS	0.0414	0.312	0.986
M11	12 months	NS	0.0532	0.156	1.21	NS	0.0692	0.590	6.50
Isoflucypram	18 months	NS	<LOQ	<LOQ	< 0.0106	NS	<LOQ	<LOQ	< 0.0254
M58	18 months	NS	0.0334	0.134	0.584	NS	0.0656	0.356	0.844
M11	18 months	NS	0.0610	0.198	1.54	NS	0.0638	0.552	4.40

N: Number examined; NS: Not sampled Source: Blanck, 2017

[a] Weight relative to body weight, expressed as a percentage;

M58: Isoflucypram-cyclopropyl-pyrazole-carboxamide (BCS-CX99798)

M11: Isoflucypram-desmethyl-caroxylic acid (BCS-CX99799)

< LOQ: Below the limit of quantification (0.01 mg/L);
the value of the LOQ was used for calculation of the group mean; the result was then expressed as "< mean value";

Statistically significant: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Rat

Groups of 70 male and 70 female Wistar Rj:WI (IOPS HAN) rats were fed diets for two years containing isoflucypram (purity 94.2%; Batch no. 2013-006492) at levels of 0, 30, 150 or 450 ppm for males (equal to 0, 1.24, 6.27 and 18.6 mg/kg per day) and 0, 30, 150 or 800 ppm for females (equal to 0, 1.75, 8.54 and 46.6 mg/kg per day). Ten males and ten females from each group were necropsied after 52 weeks of treatment. The remaining 60 animals/sex per group continued treatment until sacrificed at the end of the study, at least 103 and 105 weeks for male and female rats respectively. Haematology, blood biochemistry and urinalysis were undertaken on selected animals during months 3–4, 6–7, 12, 18 and 24. Blood samples were withdrawn from anaesthetized rats by puncture of the retro-orbital venous plexus after overnight fasting. Plasma concentrations were measured for isoflucypram and metabolites, M58 (isoflucypram-cyclopropyl-pyrazole-carboxamide; BCS-CX99798) and M11 (isoflucypram-desmethyl-carboxylic acid; BCS-CX99799). Blood samples for such analyses were collected from the sublingual vein of the first five suitable animals from each treated group at 8:00 am without fasting, at the time points 3 or 4, 12 and 24 months. Four metabolites, M01 (isoflucypram-propanol, BCS-CY24813), M12 (BCS-CY26497), isoflucypram-2-propanol (BCS-DC20298) and M06 (isoflucypram-desmethyl-propanol; BCS-DC22055) and isoflucypram were measured using plasma collected at 24 months from two animals of each sex (two males in the control group) in a separate report (Desmaris, 2017). Selected organs were weighed, and designated tissues sampled and examined microscopically.

The major treatment-related effects and the plasma concentrations of isoflucypram, M58, M11 and other four metabolites are summarized in Table 15.

The plasma concentrations of isoflucypram were very low in both sexes at all time points. At all time points the mean plasma concentrations of M11 and M58 were higher than that of the parent and were found from the lowest dose, the M11 being the more abundant. Plasma concentrations of both M11 and M58 increased with a clear relationship to dose. The mean plasma concentration of M11 tended to increase with time, and its relationship to dose tended to show subproportionality at the high dose in both sexes. The mean plasma concentrations of M58 remained relatively stable over time. The subproportionality observed in M11 was not clearly reflected by levels of M58 (Odin, 2018). At 24 months plasma concentrations of M01, M12, M02 and M06 were low compare to M11 and M58, but similar to those of the parent compound in both sexes (Desmaris, 2017).

No treatment-related effects were observed on mortality, clinical signs, ophthalmology, urinalysis, haematology or macroscopic findings in males or females. Body weights in both sexes, body weight gains in males and food consumption in males were comparable between control and treated groups. In females at 800 ppm, body weight gains were slightly (up to approximately 10%) decreased compared to controls during the period 316–484 days. The decrease frequently showed statistically significance during this period but was not observed thereafter. The slight decrease was considered a treatment-related adverse effect due to consistent decrease over six months. Food consumption was slightly lower at week 1 (by approximately 4%) and week 78 (by approximately 10%) with statistical significance at 800 ppm.

With respect to blood biochemistry, total bilirubin concentrations were slightly lower in females at 800 ppm at all time points. The decrease in total bilirubin concentration was considered treatment-related but not an adverse effect. (See Appendix 2 Fig. 1).

Liver weights were slightly and significantly increased (by ca 10%) in females at 800 ppm at 12 and 24 months. The liver weights in males were not changed with statistical significance.

Corresponding histopathological changes to the increased liver weights in females were not found. At histopathological examination of the highest-dose groups the incidence and severity of colloid alteration and the incidence of minimum diffuse pigmentation in follicular cells was increased in the thyroid of both sexes and in males, respectively. Although liver hypertrophy or thyroid follicular hypertrophy was not clear in this study, the changes observed in the thyroid were considered to be associated with prolonged thyroid gland stimulation because the secondary effects on thyroid due to UDP-GT induction in the liver were found at the same concentration in rats in other MOA studies (See 2.6 Special studies). The histopathological changes seen in the thyroid due to the secondary effect of the liver were considered to be adverse.

Incidences of pheochromocytoma were higher in all treated groups than in controls. Incidences (four or five rats of 50 examined) were distributed among the groups, and no relationship between administered dose and blood concentrations of the parent or metabolites was discernible. The incidence in the concurrent control group was at the lowest bound in the historical control data (HCD) where its average was 3.35% (32 rats with tumours from 955 control rats) and its range was within 0–8.3% recorded for 16 studies conducted between 2000 and 2016 (Tinwell et al., 2022). There was no finding showing increased malignancy of this tumour. The incidence of pheochromocytoma was not increased in females. No MOA was postulated. Pheochromocytoma was known to be a common spontaneous tumour in aged male Wistar rats, and several xenobiotics were reported to induce this tumour in rats (Pelgrom & van Raaij, 2001). Available information indicated that the increases in all treated groups in males were considered to be unusual low incidence in the concurrent control group.

In the adrenal gland, the incidence of focal medullary hyperplasia was significantly increased in the males at the terminal kill. The severity of the finding was not affected. Focal hyperplasia or pheochromocytoma in the adrenal medulla is known common in males of several strain rats including Wistar (Brändli-Baiocco et al., 2018). The lesions were induced by xenobiotics which increase serum calcium levels (Greaves, 2012b). In the current study, however, there was no alteration of serum calcium levels in the rats and no proposed MOA for medullary hyperplasia due to this compound. It was therefore difficult to exclude the possibility that the statistically significant increase seen was a treatment-related.

Incidences were slightly increased of several histopathological findings in the female reproductive organs, including diffuse squamous metaplasia in the uterus and malignant Schwannoma. Their dose–response relationships were not clear. Neither other findings related to these changes, nor an increase in related tumour in other organs were seen. Therefore the slightly increased incidences observed were considered to be incidental.

In conclusion, there was no treatment-related increase in tumours in rats.

The study author considered the NOAEL for chronic toxicity was the highest dose because the slight decrease in body weight gain in females at 800 ppm, and the increases in histopathological findings in thyroid in both sexes at the highest doses were not adverse.

The NOAEL for chronic toxicity of isoflucypram in rats was 150 ppm (equal to 6.27 mg/kg bw per day) based on colloidal alteration and pigmentation in the follicular epithelium of the thyroid and increased incidence of hyperplasia in the adrenal medulla at 450 ppm (equal to 18.6 mg/kg bw per day). The NOAEL for carcinogenicity was 450 ppm (equal to 18.6 mg/kg bw per day), the highest dose tested (Odin, 2018; Tinwell et al., 2022).

The Meeting considered the dose selection of top dose in males was adequate to clarify chronic toxicity and carcinogenicity of isoflucypram for the following reasons:

- In this study, plasma concentration levels of major metabolites were kept at consistent levels at each dose. These levels demonstrated a clear dose–response relationship throughout the study.
- The results for males at the highest dose in this study were not influenced by α2μ-globulin nephropathy, the intensity of which was increased at 1000 ppm and above compared with the effect seen at 300 ppm in the short-term studies described above.
- No new finding indicating a chronic inflammatory reaction, leading to proliferating activity in any tissues/organs, was seen as a result of the treatment.

Table 15. The summary of carcinogenicity study of isoflucypram in rats.

	Dietary dose (ppm)							
	Males				Females			
Parameter	**0**	**30**	**150**	**450**	**0**	**30**	**150**	**800**
Body weight (g)								
day 316	670.4	673.7	667.7	664.7	352.7	354.1	358.6	337.1*
day 344	681.6	681.2	679.9	671.7	359.2	357.4	365.6	339.5**
day 540	700.8	722.8	714.2	708.6	417.5	416.1	439.1	394.3
day 708	665.4	695.8	670.6	699.0	446.1	470.2	456.2	435.4
day 729	-	-	-	-	454.5	468.4	464.9	442.7
Body weight gain (g)								
days 1–344	467.6	467.7	466.9	457.1	194.0	191.6	199.6	174.0**
days 1–708	456.3	479.8	460.5	488.8				
days 1–729	-	-	-	-	292.0	304.3	303.3	280.5
Total bilirubin (µmol/L)								
month 3–4	1.35	1.21	1.34	0.64**	1.75	1.66	1.64	0.66**
month 7	1.35	1.21	1.34	1.02*	2.04	1.94	1.87	1.00***
month 12	1.75	1.48	2.07	1.28*	2.87	2.87	2.80	1.29**
month 18	1.99	1.65	1.94	1.27	2.80	2.33	1.86	1.27***
month 24	2.03	1.63	1.97	1.42	2.05	1.95	1.80	1.01**
Total cholesterol (mmol/L)								
month 3–4	1.65	1.78	1.74	1.67	1.90	1.94	1.94	2.02
month 7	1.80	1.98	2.06	1.92	1.95	1.97	2.04	2.00
month 12	2.24	2.45	2.63	2.35	2.13	2.29	2.22	2.17
month 18	2.26	3.31	3.15	2.71	2.26	2.32	2.44	2.47
month 24	3.23	3.68	3.33	3.01	2.73	2.73	2.44	2.69
Liver weight								
month 12 (*N*)	8	10	9	10	10	8	10	10
Liver weight mean ± SD (g)	13.088 ± 2.1379	13.385 ± 1.6754	12.890 ± 1.9940	13.420 ± 1.3062	2.117 ± 0.1836	2.115 ± 0.1380	2.230 ± 0.1855	2.346* ± 0.1647
Liver weight, rel.[c] (%)	1.9	2.0	2.0	2.1	2.1	2.1	2.2	2.3*
month 24 (*N*)	17	22	23	26	26	24	20	29
Liver weight (g)	12.3	13.2	12.5	13.2	9.5	9.8	9.6	10.3
Liver weight, rel.[c] (%)	2.0	2.0	2.0	2.0	2.2	2.2	2.2	2.5**
Thyroid histopathology (affected individuals)								
N	59	59	59	59	60	60	60	59
Colloid alteration								
minimal	21	16	21	21	10	6	18	23**
slight and moderate	6	8	8	16	1	0	1	5
moderate	0	0	0	1	0	0	0	0
total	27	24	29	38	11	6	19	28***

	Dietary dose (ppm)							
	Males				Females			
Parameter	**0**	**30**	**150**	**450**	**0**	**30**	**150**	**800**
Pigmentation, follicular cells, diffuse								
minimal	2	3	6	11*	2	0	0	5
Adrenal histopathology; total affected [unscheduled kill/scheduled kill]								
N	60 [43/17][c]	60 [38/22]	60 [37/23]	60 [34/26]	60 [34/26]	60 [36/24]	60 [40/20]	60 [31/29]
Medullary hyperplasia, focal	13 [10/3]	11 [4/7]	13 [7/6]	23* [10//13]	4 [4/0]	7 [3/4]	12 [9/3]	5 [2/3]
Pheochromocytoma	0 [0/0]	5 [3/2]	4 [1/3]	4 [1/3]	1 [1/0]	0 [0/0]	1 [1/0]	0 [0/0]
Uterine histopathology (individuals affected)								
N					60	60	60	60
Endometrial stromal polyp					15	22	18	16
Endometrial adenocarcinoma					1	0	2	0
Malignant Schwannoma [#]					0	2	4	3
Reactive endometrial hyperplasia, diffuse					1	2	1	0
Squamous metaplasia, diffuse					0	1	7	7
Squamous metaplasia, focal					6	3	7	7
Vaginal histopathology								
N					60	59	60	59
Malignant Schwannoma					0	1	1	0
Mucification, diffuse					24	19	23	20
Epithelial atrophy, diffuse					24	21	24	22
Plasma concentrations of isoflucypram and metabolites (mg/mL) mean ± SD shown; mean values from Odin, 2018 (mg/L) or for individual animals from Desmaris, 2017,								
Time point				**Isoflucypram**				
Month 3–4	-	< LOQ	< 0.010	< 0.019	-	< LOQ	< 0.012	0.015 ± 0.0036
Month 12	-	< 0.01	< 0.015	< 0.015	-	< 0.01	0.014 ± 0.007	< 0.068 ± 0.063
Month 24	-	< 0.010	< 0.01	0.032 ± 0.012	-	< 0.01	< 0.017 ± 0.009	0.027 ± 0.010
Month 24[b]	< LOQ < LOQ	< LOQ 0.0012	0.011 0.020	0.030 0.036	- -	< LOQ < LOQ	0.021 0.014	0.017 0.042
				M11				
Month 3–4	-	0.068 ± 0.030	0.282 ± 0.160	0.526 ± 0.297	-	0.132 ± 0.056	0.339 0.181	0.610[a] ± 0.161
Month 12	-	0.074 ± 0.044	0.320 ± 0.129	0.523 ± 0.308	-	0.155 ± 0.064	0.423 ± 0.236	0.813 ± 0.232
Month 24	-	0.111 ± 0.046	0.540 ± 0.321	0.895 ± 0.888	-	0.121 ± 0.009	0.692 ± 0.543	1.470 ± 0.382
Month 24[b]	< LOQ < LOQ	0.10 0.039	0.93 0.49	0.82 0.93	- -	0.13 0.052	0.27 0.53	1.9 1.3
				M58				
Month 3–4	-	0.029 ± 0.009	0.162 ± 0.052	0.510 ± 0.192	-	0.014 ± 0.002	0.146 ± 0.038	1.113[a] ± 0.165

	Dietary dose (ppm)							
	Males				Females			
Parameter	**0**	**30**	**150**	**450**	**0**	**30**	**150**	**800**
Month 12	-	0.020 ± 0.004	0.123 ± 0.056	0.377 ± 0.094	-	0.015 ± 0.003	0.143 ± 0.065	1.278 ± 0.172
Month 24	-	< 0.015	0.069 ± 0.023	0.310 ± 0.096	-	< 0.012 ± 0.003	0.083 ± 0.045	1.230 ± 0.345
Month 24[b]	< LOQ < LOQ	0.023 < LOQ	0.082 0.039	0.44 0.25	-	< LOQ 0.013	0.042 0.090	2.2 1.3
M01	< LOQ < LOQ	< LOQ < LOQ	0.015 0.014	0.033 0.034	- -	< LOQ < LOQ	0.019 0.039	0.024 0.028
M12	< LOQ < LOQ	< LOQ < LOQ	0.027 0.069	0.072 0.084	- -	0.013 < LOQ	0.026 0.023	0.11 0.12
M02	< LOQ < LOQ	< LOQ < LOQ	0.034 0.061	0.24 0.19	- -	< LOQ 0.015	0.046 0.053	0.14 0.27
M06	< LOQ < LOQ	< LOQ < LOQ	0.11 0.046	0.20 0.080	- -	0.014 0.015	0.064 0.30	0.58 0.29

N: Number of individuals/organs examined; SD: Standard deviation; Sources: Odin, 2018; Desmaris, 2017

[a] Mean value calculated with one excluded value; [b] Individual plasma concentration; reported by Desmaris, 2017;

[c] Weight relative to body weight, expressed as percentage;

One tumour showed positive results by S100 protein immunohistochemically;

< LOQ: Below the limit of quantification (0.01 mg/L);
the value of the LOQ was used for calculation of the group mean; the result was then expressed as "< mean value";

Statistically significant: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

2.4 Genotoxicity

An adequate range of in vitro and in vivo genotoxicity studies was performed. The study results are summarized in Table 16.

Table 16. Summary of genotoxicity studies on isoflucypram

Type of study	Organism/cells	Dose range tested	Purity Batch	Result	Reference
		In vitro			
Gene mutation test; Ames	*Salmonella typhimurium* (TA100, TA98, TA1535, TA1537 and TA102)	±S9: 3–5000 µg/plate	94.2% No.2013-006492	±S9: negative	Sokolowski, 2014
Chromosome aberration assays	Human lymphocyte cells	Experiment 1: −S9: 7.8–23.9 µg/mL +S9: 13.6–41.8 µg/mL Experiment 2: −S9: 2.0–6.1 µg/mL +S9: 10–40.0 µg/mL	94.2% No.2013-006492	±S9: clastogenic	Bohnenberger, 2014
Gene mutation test; CHO	Chinese hamster lung V79/HPRT cells	Experiment 1: −S9: 4–32 µg/mL +S9: 8–128 µg/mL Experiment 2: −S9: 8–64 µg/mL +S9: 8–64 µg/mL	94.2% No.2013-006492	±S9: negative	Wollny, 2014
		In vivo			
Micronucleus (by gavage)	Mouse (NMRI) bone marrow	500–2000 mg/kg	94.2% No.2013-006492	Negative	Dony, 2014

(a) In vitro studies

An Ames bacterial mutagenicity and a V79/HPRT mammalian cell mutagenicity study were negative both with and without S9 activation (Sokolowski, 2014; Wollny, 2014). In the in vitro chromosome aberration study, there was a statistically significant increase in the incidence of chromosomal aberrations (both including and excluding gaps) in both the presence and the absence of S9 activation (Bohnenberger, 2014). There was no increase in the incidence of polyploid metaphases under any of the experimental conditions in that study.

(b) In vivo studies

In the in vivo mouse micronucleus assay, clinical signs of toxicity were observed after oral gavage administration (two administrations of 2000 mg/kg bw separated by 24 hours), but there was no effect of treatment on the ratio of polychromatic to normochromatic erythrocytes, nor was there any increase in the incidence of micronucleated polychromatic erythrocytes (Dony, 2014). Although direct evidence that bone marrow exposure to the compound was not assessed in this study, both male and female mice showed clinical signs. Ruffled fur, reduction in spontaneous activity, eyelid closure and hunchback were observed in a pre-experiment test where mice received 2000 mg/kg bw of isoflucypram dissolved in dimethyl sulfoxide (DMSO)/PEG 400 mixture, twice by gavage at 24 hours intervals. Serious clinical signs such as abdominal posture, reduction in spontaneous activity and tumbling was observed for one female immediately post dosing at both intervals. Toxic clinical signs including reduction of spontaneous activity and ruffled fur were also observed in the main experiment where mice received isoflucypram at 500 mg/kg bw twice, 1000 mg/kg bw twice, and 2000 mg/kg bw twice, the results showing a dose-related increase in severity of the effects.

The rat ADME studies covered in Section 1 above indicate that the compound does reach rodent bone marrow after oral gavage administration, and similar toxicological profiles were observed in mice and rats in toxicity studies. Isoflucypram and its metabolites in the chronic mouse study showed that significant amounts of the metabolites M58 and M11 were detected after dietary administration. All the information compiled indicate that isoflucypram and/or its metabolites are systemically available under the conditions of this study.

Isoflucypram was tested for genotoxicity in an adequate range of in vitro or in vivo systems. No evidence of genotoxicity was seen.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

A preliminary reproductive study was conducted to establish suitable treatment levels for a two-generation reproductive study. As an F0 generation, eight male and eight female Han Wistar rats received isoflucypram (purity 94.2%; Batch no. 2013-006492) in the diet at concentrations at 0, 100, 300 or 1000 ppm for four weeks before pairing, then throughout pairing and gestation. During the lactation phase the dietary concentrations for females were lowered by 50% to 50, 150 or 500 ppm. The aim was to maintain a constant achieved dose in dams during lactation periods, at which time increased feed intake, due to physiological demand on the dams, would otherwise lead to increased dosage of the test article. In the F1 generation, 12 males and 12 females from each group received treatment via the diet at 100, 300 or 1000 ppm from weaning until termination. Achieved doses in males were 0, 6.8, 19.7, and 65.2 mg/kg bw per day for pre-pairing F0 males, and 0, 12.2, 36.3 and 118.6 mg/kg bw per day for F1 maturation. Achieved doses in females were as follows: 0, 7.5, 23.7 and 76.7 mg/kg bw per day for F0 premating; 0, 7.8, 23.8 and 85.3 mg/kg bw per day for F0 gestation; 0, 7.8, 24.7, 77.2 mg/kg bw per day for F0 lactation; 0, 11.9, 36.2 and 125.0 mg/kg bw per day for F1 maturation. During the study, data was recorded on clinical condition, body weight, food consumption, estrous cycles before mating and scheduled termination, mating performance and fertility, gestation length, parturition and reproductive performance. Sperm analysis, organ weight, macroscopic and microscopic pathology investigations were undertaken on each adult generation. The clinical condition of offspring, litter size and survival, sex ratio, sexual maturation/puberty using several indicators of balano-preputial separation/anogenital distance were recorded and organ weight and macroscopic pathology investigations undertaken on each

generation. Plasma concentrations of isoflucypram and two major metabolites, M58 (isoflucypram-cyclopropyl-pyrazole-carboxamide; BCS-CX99798) and M11 (isoflucypram-desmethyl-caroxylic acid; BCS-CX99799) were measured in males at the end of treatment, in F0 females on day 17 after mating and on day 18 of lactation, and in F1 offspring on days 4 and 21.

Plasma concentrations of isoflucypram are shown in Table 17. In all generations, plasma concentrations of the isoflucypram were the lowest. Metabolite M11 levels were ten times higher than those of isoflucypram, and resembled the levels seen for M58. In the F0 generation, the plasma concentration levels of isoflucypram, M58 and M11 were comparable at each dose, including phase at which 50% dietary content was used during lactation in F0 females. In the F1 generation the concentrations of isoflucypram and both metabolites were found to be very low in offspring on postnatal day (PND) 4, but on day 18 of lactation (LD 18) concentrations in the offspring were higher than those in the F0 generation rats. The results indicated that rats of F0 and F1 generations were internally exposed to similar levels of isoflucypram and its metabolites, the M11 and M58 being prominent in the plasma in this study. In addition, plasma concentrations of both metabolites showed an almost linear relationship to dietary dose.

There were no treatment-related clinical signs and no increase in mortalities. Food consumption and body weight gain were unaffected by treatment up to 1000 ppm. Estrous cycles, precoital interval, mating performance and fertility were considered unaffected by treatment. Gestation length and gestation index were unaffected by treatment and there were no effects upon litter size, sex ratio or offspring survival to 21 days old. Body weight gain of offspring to 21 days old was unaffected by treatment with isoflucypram. For the selected F1 generation, mean body weights and body weight gains for males and females receiving isoflucypram were unaffected by treatment when compared with controls. Food consumption was unaffected throughout the maturation phase. Balano-preputial separation and vaginal opening were unaffected by treatment. There was no significant macroscopic finding at necropsy of the F0 or F1 adults, and necropsy of the F1 offspring did not reveal any treatment-related effects. Adjusted liver weights of F0 males and F1 males and females at 1000 ppm, and F1 males at 300 ppm were higher, with statistical significance, than those of controls. Treatment produced no effect on sexual maturation in either sex. For a summary of results see Tables 17a and b.

Table 17a. Preliminary two-generation reproductive study of isoflucypram in rats; plasma concentrations in F0 and F1 generations of isoflucypram and its metabolites (µg/L; each cell contains three results for individual animals examined)

	Dietary dose (ppm)					
Parameter	**100**	**300**	**1000**	**100/50[#]**	**300/150[#]**	**1000/500[#]**
Number of rats	**3**	**3**	**3**	**3**	**3**	**3**
	F0 generation					
	F0 males at the termination after mating			***F0 females on day 18 on lactation***		
Isoflucypram	ND/ND/ND	4.03/8.29/NR	18.3/9.48/19.2	8.69/8.41/23.2	21.5/38.2/13.8	29.0/11.1/16.6
M58	143/146/97.9	355/340/280	1100/951/867	77.0/44.1/58.6	221/223/207	1340/1490/910
M11	149/50.1/70.4	132/248/136	978/1140/443	59.1/61.6/48.1	245/203/371	1270/1330/778
	F0 females on day 17 after mating			***F1 pups on day 4 of age***		
Isoflucypram	7.41/16.0/3.09	6.09/9.99/12.3	21.9/31.5/13.4	ND/ND/ND	6.18/ND/ND	75.7/ND/12.3
M58	100/42.7/36.4	169/156/159	1090/1130/1370	11.1/12.8/12.8	32.3/31.7/26.0	83.3/42.3/79/8
M11	224/132/138	146/232/281	722/999/2490	ND/ND/ND	3.72/3.04/3.62	8.54/4.02/8.17
	F1 generation					
F1 generation				***F1 male pups on Day 21 of age***		
Isoflucypram				ND/ND/ND	ND/3.96/3.90	11.6/6.13/14.1
M58				222/148/86.8	487/336/370	1210/685/714
M11				144/102/57.4	498/478/318	1960/1070/956

F1 generation	*F1 female pups on Day 21 of age*		
Isoflucypram	ND/ND/ND	5.62/3.53/4.31	10.9/9.09/14.2
M58	111/126/78.0/	468/365/359	897/1400/1560
M11	103/103/76.3	525/397/373	1550/1100/991

[#] Dietary concentrations reduced to 50, 150 and 500 ppm from lactation period; Source: Renaut, 2019

ND: Not detectable; NR: no result due to lack of sufficient sample volume for repeat analysis

Table 17b. Preliminary two-generation reproductive study of isoflucypram in rats; timing of sexual maturation and body weights in the F1 generation

	Dietary dosage (ppm)							
	F1 males (a)				**F1 females (b)**			
	0	**100**	**300**	**1000**	**0**	**100/50[#]**	**300/150[#]**	**1000/500[#]**
Number of rats	12	12	12	12	12	12	12	12
Day of sexual maturity [a]:								
mean	49	48	47	50	35	34	35	35
standard deviation	4.3	21.8	1.9	3.8	3.5	1.9	2.9	2.5
Body weight [b] (g):								
mean	205	201	197	205	105	105	109	105
standard deviation	28.0	8.8	14.1	22.3	12.9	9.3	11.0	13.5

[#] Dietary concentrations reduced to 50, 150 and 500 ppm from lactation period; Source: Renaut, 2019

[a] Maturation markers in males and females were balanopreputial separation or vaginal open, respectively;

[b] Body weights at sexual maturation.

On the basis of the preliminary study, dietary administration of isoflucypram at concentrations up to 1000 ppm (500 ppm during lactation) caused no adverse effect on the parent animals, their reproductive performance or the development of their offspring. On the basis of these results a main reproductive toxicity study was carried out in which the highest dose was maintained at 100 mg/kg per day. (Renaut, 2019).

A main reproductive study was conducted to assess the influence of isoflucypram (purity 94.2 %; Batch no. 2013-006492) on reproductive performance when continuously administered via the diet through two successive generations of Han Wistar rats. For the F0 generation, three groups of 28 male and 28 female rats received isoflucypram orally, via the diet, at concentrations of 150, 450 or 1200 ppm. The treatment was for ten weeks before pairing, then throughout pairing and gestation. During the lactation phase dietary concentrations were lowered by 50% to 75, 225 and 600 ppm in order to ensure that the actual dose in relation to body weight remained relatively constant and to avoid overdosing the offspring as they transitioned toward eating a solid diet; the dosages used were based on the results of plasma concentration data from a preliminary study (Renaut, 2019). Administered doses were:

At premating:

- 0, 11.3, 34.1 and 94.4 mg/kg bw per day for F0 males;
- 0, 13.9, 41.6, 109 mg/kg bw per day for F1 males;
- 0, 13.0, 40.8, 104. mg/kg bw per day for F0 females;
- 0, 14.6, 44.5, 113 mg/kg bw per day for F1 females.

At gestation:

- 0, 11.7, 35.9, 94.7 mg/kg bw per day for F0 females;
- 0, 12.9, 38.4, 103 mg/kg bw per day for F1 females.

At lactation:

- 0, 11.5, 35.8, 94.5 mg/kg bw per day for F0 dams;
- 0, 11.3, 34.1, 92.9 mg/kg bw per day for F1 dams.

The F1 generation comprised 24 male and 24 female progeny from each group, and they continued to receive the relevant diet, as for the F0 generation, throughout the study until termination. During the study, data was recorded on clinical condition, body weight, food consumption, blood biochemistry, estrous cycles, mating performance and fertility, gestation length and parturition observations and reproductive performance. Sperm analysis, organ weight, macroscopic and microscopic pathology investigations were undertaken on each adult generation. The clinical condition of offspring, litter size and survival, sex ratio, sexual maturation (selected F1 generation only), body weight gain and anogenital distance were recorded; organ weights were recorded and macroscopic pathology investigations undertaken on each generation. Plasma concentrations of isoflucypram and two major metabolites, (M58 and M11) were measured in males at the end of treatment, females on LDs 17 and 18, and F1 offspring on day 21.

The results of individual measurements of plasma isoflucypram are shown in Table 18. In all generations plasma concentrations of the parent were the lowest. Metabolite M11 was present at levels around 10 times higher than isoflucypram, and resembled the levels M58. In the F0 generation, the plasma concentration levels of isoflucypram, M58 and M11 were comparable at each dose including the 50% reduction during lactation. In the F1and F2 generations the concentrations of isoflucypram and both metabolites were found to be very low in the offspring on LD 21. The results for plasma concentrations of M58 and M11 indicated that the rats of both sexes from F0 and F1 generations were internally exposed to similar levels of isoflucypram and its metabolites. In addition, plasma concentrations of both metabolites showed an almost linear relationship to ingestion of the test article. The results were similar to those of the preliminary study.

Table 18. Two-generation reproductive toxicity study of isoflucypram and two metabolites in rats; Individual plasma concentrations of isoflucypram, M58 and M11 in F0, F1 and F2 generations; (plasma concentrations in µg/L; each cell contains three results for individual animals examined)

	Dietary dose (ppm)					
Parameter	**150**	**450**	**1200**	**150/75[#]**	**450/225[#]**	**1200/600[#]**
N	**3**	**3**	**3**	**3**	**3**	**3**
			F0 generation			
	Males at the termination after mating			***Females on day 18 of lactation***		
Isoflucypram	5.41/5/22/3.09	9.19/8.91/8.53	28.0/24.6/18.9	15.1/13.6/17.1	18.4/18.3/21.4	21.0/22.1/29.7
M58	108/112/170	462/497/569	1560/1190/1280	89.3/93.8/97/97.3	573/490/559	1990/2650/1580
M11	102/90.9/92.7	217/348/279	557/714/399	111/64.1/82.9	340/400/334	804/2610/459
			F1 generation			
	Males at the termination after mating			***Females on day 18 of lactation***		
Isoflucypram	4.11/5.30/4.88	11.3/5.64/7.65	15.6/17.7/29.6	33.5/24.3/24.4	32.1/24.3/24.4	26.2/49.7/21.2
M58	207/185/129	446/434/473	1130/1879/1640	161/148/98.3	482/458/424	1360/1990/1400
M11	175/136/122	346/444/269	454/1600/631	142/335/364	728/568/493	1510/2270/1200
				Male pups, 21 days old		
Isoflucypram				ND/6.03/5.56	5.44/4.45/4/23	7.00/10.9/8.80
M58				285/168/267	881/903/450	1100/1670/994
M11				140/170/167	350/773/316	286/1610/1180
				Female pups, 21 days old		
Isoflucypram				ND/3.17/ND	7.40/7.60/7.48	11.7/10.7/10.5
M58				304/261/160	1480/747/371	2040/2370/907
M11				119/103/130	645/422/395	904/1670/1140

Parameter	Dietary dose (ppm)					
	150	450	1200	150/75[#]	450/225[#]	1200/600[#]
N	3	3	3	3	3	3
	F2 generation					
				Male pups, 21 days old		
Isoflucypram				ND/3.37/3.22	7.33/5.47/7.93	32.9/11.5/14.3
M58				312/482/216	632/359/568	1500/1720/2340
M11				180/179/162	525/415/358	2720/926/1400
				Female pups, 21 days old		
Isoflucypram				5.20/ND/4.51	5.27/6.04/5.10	14.0/11.7/14.4
M58				303/273/224	540/737/458	1310/1730/1370
M11				145/112/223	439/627/355	1060/1180/1920

[#] Dietary concentrations reduced to 75, 225 and 600 ppm from lactation period; Source: Renaut, 2018

ND: Not detectable (less than 3.00 µg/L); NR: No result due to lack of sufficient sample volume for repeat analysis;

N: Number sampled

The major findings from the reproductive toxicity study are summarized in Table 19. No adverse effects on clinical signs, survival, body weights or food consumption were observed in either parents or offspring of the F0 and F1 generations. In blood biochemical examination, statistically small but statistically significant increases in total cholesterol were observed in F1 males (ca 10%) at 450 and 1200 ppm but without a clear dose–response relationship. In females, increases in total cholesterol were clear (ca 30%–50%) in both generations at 1200/600 ppm in the F0 and F1 generations. Total cholesterol increases in females were considered to be adverse. The increase for males was not found at the same concentration as it was in the long-term study in rats; this small and inconsistent increase in total cholesterol in males was therefore not considered to be adverse. Several blood biochemical parameters were increased/decreased with statistical significance at the highest doses in males or females. The magnitude of these effects was however were very small (within ca 10% of controls) and no other findings were detected that corresponded with these changes. The changes in males were therefore not considered toxicological significant. Significant decreases in bile acid in F0 males at the two highest doses, which were not, however, observed in F1 males, were not considered to be toxicologically significant. In adults of the F0 and F1 generations, and young (three weeks old) rats of the F2 generation, liver weights were slightly increased in all treated groups in both sexes, and the absolute and relative thyroid weights were increased at 1200 ppm in F0 males, with statistical significance. The slight increase (c 10%) in liver weight in the F0 generation in the absence of hepatotoxic findings or thyroid effects was considered adaptive. Thyroid weights were increased in F0 adult males at the two highest doses. An increase in thyroid weight in combination with follicular cell hypertrophy and colloid alterations, was also found in males at 1000 ppm in the 90-day rat study, however, in this reproductive toxicity study, the dose–response relationship was not clear and the increases were not accompanied with any morphological change. It was considered to be important that the thyroid weight was not affected in the F1 generation where the treatment period employed covered from fertilization to adulthood. Taken together the increases seen in F0 males at the two highest doses were not considered adverse. There was no treatment-related histopathological findings in F0 and F1 generations.

Table 19. Summary of the two-generation reproduction study of isoflucypram in rats

	Dietary dose (ppm)							
	Males				Females[#]			
Parameter	**0**	**150**	**450**	**1200**	**0**	**150/75**	**450/225**	**1200/600**
Blood biochemistry	**(*n* = 10/sex per group)**							
Bilirubin µmol/L								
F0	1	1	1	1	1	1	1*	1*
F1	1	1	1	1	1	1	1	1
Bile acids µmol/L								
F0	41.4	34.9	26.2**	25.6**	39.4	46.4	27.8	24.6
F1	38.2	38.3	43.4	21.0	30.3	27.0	18.3	17.5
Cholesterol mmol/L								
F0	2.03	2.07	2.09	2.27	1.75	1.93	1.97	2.56**
F1	1.72	1.61	2.03**	1.97**	1.60	1.66	1.95	2.07*
Gestation index								
F0	-	-	-	-	100	100	96	100
F1	-	-	-	-	100	100	100	100
	F1 pups (*n* = 24/sex per group)							
Balano-preputial separation and vaginal opening								
Number of rats	24	24	24	24	24	24	24	24
Age (days ± SD)	46 ± 3.4	46 ± 3.1	47 ± 2.2	48 ± 4.0	33 ± 2.6	33 ± 2.3	33 ± 2.3	38 ± 2.9**
Body weight (g ± SD)	196 ± 23.9	192 ± 19.4	196 ± 18.6	202 ± 22.7	105 ± 13.3	103 ± 13.6	104 ± 14.6	124 ± 11.8**
Estrous cyclicity in adult F1 females in three-weeks before mating								
Regular estrous cycling; 4-day					23	23	21	23
4.5-day					1	1	1	1
5-day					0	0	0	0
acyclic[a]					0	0	2	0
Parental and offspring organ weights	**F0 generation**							
Liver weight (g) (M at week 17; F at PND 28)								
	14.45	14.45	15.01	15.94**	10.46	10.68	11.34**	12.33**
Liver weight relative to body weight (%) (M at week 17; F at PND 28)								
	3.30	3.25	3.42	3.70**	4.19	4.27	4.55**	5.04**
Thyroid weight (g) (M at week 17; F at PND 28)								
	0.017	0.018	0.020*	0.019*	0.017	0.016	0.016	0.017
Thyroid weight relative to body weight (%) (M at week 17; F at PND 28)								
	0.0038	0.0041	0.0045*	0.0045**	0.0067	0.0065	0.0063	0.0068
Thymus weight (g) (M at week 17; F at PND 28)								
	0.285	0.276	0.290	0.286	0.257	0.250	0.243	0.222*
Thymus weight relative to body weight (%) (M at week 17; F at PND 28)								
	0.0650	0.0623	0.0663	0.0666	0.1030	0.1002	0.0971	0.0906*

	Dietary dose (ppm)							
	Males				Females [#]			
Parameter	0	150	450	1200	0	150/75	450/225	1200/600
F1 generation								
Liver weight (g) (at PND 21)								
	2.174	2.272	2.408*	2.551**	2.031	2.210*	2.333**	2.571**
Liver weight relative to body weight (%) (PND 21)								
	4.513	4.671	4.874**	5.157**	4.417	4.703*	4.936**	5.293**
Liver weight (g) (M at week 17; F at PND 28)								
	16.14	15.43	16.18	17.58*	11.76	11.97	12.60*	13.02**
Liver weight relative to body weight (%) (M at week 17; F at PND 28)								
	3.44	3.27	3.51	3.87**	4.39	4.44	4.76**	4.97**
F2 generation								
Liver weight (g) (at PND 21)								
	2.245	2.410	2.454*	2.473*	2.181	2.401*	2.314*	2.382*
Liver weight relative to body weight (%) (PND 21)								
	4.498	4.787*	4.889*	5.127**	4.617	4.933*	4.873*	5.126**

M: Male; F: Female; SD: Standard deviation; PPD: Postpartum day; Source: Renaut, 2018

[#] Dietary concentrations were reduced during gestation and lactation to 75, 225, and 600 ppm in order to maintain a constant mg/kg bw/day dose;

[a] At least 10 days without estrus stage;

Statistically significant: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

There were no adverse effects on F1 males including the age of sexual maturation assessed by balano-preputial separation. In F1 females vaginal opening was significantly delayed at 1200/600 ppm compared with the mean age for controls. The delay did not reflect lower body weights at the current of time point. Subsequent estrous cyclicity, mating performance, fertility and reproductive performance (litter size, offspring survival and development), body/organ weights, macroscopic and microscopic changes in female reproductive tracts in F0 and F1 generations, ovarian follicle counts for F1 females were all unaffected. There were no effects of treatment on litter size, offspring survival, sex ratio or offspring body weight in either generation, nor anogenital distance of the F2 offspring.

The historical control data on the age of vaginal opening and body weight at the opening had been provided (Tinwell et al., 2022; see Table 20). The data on vaginal opening showed wide variability opening time. This degree of variability was reported not only in Wistar Han strain rats but also other strains (Goldman et al., 2000). When compared with these historical data, the vaginal opening age in this study was outside the previous variability. Therefore the delay to vaginal opening at 1200/600 ppm was considered to be treatment-related and its adversity was not excluded because the delay was in excess of the historical control range.

The study author considered adverse effects were not observed in parameters for parental, offspring or reproductive toxicity.

The study author proposed that the MOA for the delay in vaginal opening was low E2 level in the plasma due to excess E_2 metabolism in the liver when treated with isoflucypram. This was proposed because isoflucypram was reported to increase mRNA expression of Cyp3a23, an enzyme that metabolizes E_2 into 2-OH-estradiol in the liver (Rouquié, 2018b; Tinwell et al., 2022). Contrary to this, treatment with isoflucypram did not show any finding related to lower E_2 in either the short- or long-term toxicity studies (Totis, 2017; Odin, 2017; Odin, 2018) or in the special studies on endocrine disrupting properties (Totis, 2012; Kennel, 2011). Neither steroid nor gonadotropin hormones were measured, however, so it is difficult to conclude that the delayed vaginal opening was induced by lower E_2 level.

Table 20. Historical data for vaginal opening age and corresponding body weight in control groups from reproductive toxicity studies at the same test facility as the reproductive study[a]

Study number	F0 treatment commenced	Number of rats	Age (postnatal days)				Body weight (g) at opening			
			Min	Max	Mean	SD	Min	Max	Mean	SD
Feeding										
1	2013	12	30	35	31.8	1.80	74	115	93.4	9.52
2	2013	12	30	36	33.2	1.85	84	114	100.9	9.16
3	2013	12	30	35	32.7	1.72	79	114	95.0	10.92
4	2014	12	29	35	31.8	1.76	77	111	88.6	10.48
5	2014	24	28	37	33.5	2.72	78	127	105.0	13.10
6	2014	24	28	38	31.9	2.43	71	141	92.7	15.44
7	2016	24	29	43	33.8	3.07	77	135	103.8	15.26
8	2016	24	33	42	36.6	2.57	98	139	117.3	10.91
9	2017	24	29	37	33.7	2.28	82	127	106.9	11.55
10	2017	24	29	41	33.9	2.36	83	141	102.5	12.13
11	2019	24	31	39	34.1	1.98	73	121	102.9	10.41
Gavage										
1	2014	24	29	41	35.4	3.29	93	137	112.4	12.21
2	2015	12	29	38	34.3	2.63	87	126	110.0	12.30
3	2015	24	27	38	33.3	2.60	71	138	107.8	14.46
4	2018	12	28	37	33.9	2.81	80	122	104.6	11.11
5	2018	24	30	35	32.3	1.40	82	131	101.5	12.53
6	2018	12	27	47	35.4	5.00	71	153	106.8	24.08

SD: Standard deviation; Min: Earliest day of the opening; Max: Latest of the opening Source: Tinwell et al., 2022

[a] The Meeting noted that the data provided in this table was not fully matched to the criteria of historical control data for the guidance document by JMPR (JMPR, 2017)

The NOAEL for parental toxicity was 450/225 ppm (equal to 34.1 mg/kg bw per day; the lowest value in females) based on increased cholesterol and increased liver effects in females at 1200/600 ppm (equal to 92.9 mg/kg bw per day; the lowest adverse value in parents). The NOAEL for effects on offspring was 450/225 ppm (equal to 34.1 mg/kg bw) based on the delay to vaginal opening at 1200/600 ppm (equal to 92.9 mg/kg bw per day) The NOAEL for reproduction was 1200/600 mg/kg bw per day (equal to 92.9 mg/kg bw per day) the highest dose tested. (Renaut, 2018).

(b) Developmental toxicity

Rat

Isoflucypram (purity 94.2%; Batch no. 2013-006492) was administered to groups of 23 sperm-positive female Sprague Dawley rats by gavage from gestation day (GD) 6 to GD 20. The sperm-positive day was designated GD 0. Doses given were 0, 25, 125 or 625 mg/kg bw per day, with the test item suspended in aqueous 0.5% methylcellulose 400. At scheduled sacrifice on GD 21, a blood sample was collected from 10 pregnant rats for clinical chemistry determinations (not including thyroid hormone measurement) and from five pregnant rats for bioanalytical examination. Gravid uterine weight was recorded, and all dams were evaluated for the number of corpora lutea, and implantations (resorptions and dead or live fetuses). In addition, the liver and thyroid gland from all females were weighed and retained in 10% neutral buffered formalin. Histological examination was performed on the liver and thyroid gland from the first 10 pregnant dams in the control, mid-, and high-dose groups. Approximately half of the live fetuses from each litter underwent internal examination. The remaining fetuses were used for skeletal examination of both bone and cartilage.

In all groups, including the controls, at least 21 females were pregnant out of 23 mated females per group. Due to one accidental death at 25 mg/kg bw per day, there were in total 21, 21, 23, and 23 dams with viable fetuses at 0, 25, 125, and 625 mg/kg bw, respectively, by the end of the treatment period. Up to the highest dose level tested (625 mg/kg per day), there were no treatment-related maternal mortalities or clinical signs throughout the study. At necropsy, the bioanalytical examination showed that plasma concentrations of isoflucypram were below or marginally above the limit of quantification of 0.01 mg/L at the three dose levels tested, whilst there was essentially a dose-related increase in mean plasma concentrations of the metabolites M58 and M11.

There was no statistically significant change in body weight or body weight gain at any time during treatment. Food consumption was slightly decreased compared to controls (by 12%, statistically significant) between GDs 6 and 8. Thereafter, food consumption was similar to controls. At scheduled necropsy blood biochemistry revealed a lower bilirubin concentration at 125 mg/kg bw per day and above. These decreases were not considered adverse because of a MOA common to other rat studies. Decreased ALP at 625 mg/kg bw per day (by 34%) was not considered adverse as it was opposite to an increase that would be indicative of adversity. At the same dose, absolute liver weight increase (by 44%), enlarged liver at necropsy and minimal to slight hepatocellular centrilobular hypertrophy were observed in the liver of dams. In the thyroid, minimal follicular cell hypertrophy was found in 1/10 dams at this same dose.

At caesarean section of the 625 mg/kg bw per day group, the only treatment-related change was slightly lower fetal body weight (by 4%, 3% and 5% for combined sexes, males and females respectively). Fetal evaluation revealed no treatment-related malformations. At the external fetal examination, there were no treatment-related variations. The visceral fetal examination revealed an increased incidence of "renal pelvis" at the litter level at 625 mg/kg bw, compared to controls. The skeletal fetal examination revealed an incidence of incomplete ossification of where at least one bone of the zygomatic arch (unilateral/bilateral) was increased at 625 mg/kg bw at the litter level. Both variations were known to be common findings, however these incidences were above the range of their historical control data. On the other hand, both changes were isolated statistical increases and no consistent increase in other findings, such as malformations or variations, were observed. In addition, the critical period for inducing renal morphological abnormalities is considered to extend into the postnatal period, and minor manifestations of developmental toxicity such as incomplete ossification are considered to be reversible. In view of all the available information, the Meeting did not consider that both isolated increases were adverse for development. At caesarean section, there were no treatment-related effects at 125 mg/kg bw per day or lower.

The major changes observed in this study are summarized below in Table 21.

The maternal NOAEL for development was 125 mg/kg bw per day based on liver and thyroid effects, and reduced food consumption at 625 mg/kg bw per day. The embryo/fetal NOAEL for toxicity was 625 mg/kg bw per day, the highest dose tested. No teratogenicity due to the treatment was observed (Kennel, 2017b).

Table 21. Summary of the developmental toxicity study of isoflucypram in rats

Parameter		Isoflucypram dose (mg/kg bw per day)				HCD (%)
		0	25	125	625	
Number of rats examined		21	21	23	23	
Body weight (g)	on GD6	313.3	310.0	311.3	311.4	
	on GD8	317.9	314.7	316.7	314.0	
	on GD21	457.1	453.0	457.3	448.2	
Body weight gain (g)	GDs 6–8	4.6	4.7	5.4	2.6	
	GDs 6–21	143.8	143.0	146.0	136.8	
Food consumption (g)	GDs 1–6	25.06	25.68	26.73	25.25	
	GDs 6–8	26.38	26.12	26.6.3	23.11*	
	GDs 8–10	27.71	27.05	28.41	27.41	

Parameter	Isoflucypram dose (mg/kg bw per day) 0	25	125	625	HCD (%)
Organ weight/macroscopy:					
Number of rats examined:	21	21	23	23	
Liver weight (g)	14.1	14.0	15.5	20.3	
Thyroid weight (g)	0.0166	0.0154	0.0164	0.0170	
Liver, enlarged (affected rats)	0	0	0	14	
Biochemistry Number of rats examined	10	10	10	10	
Total bilirubin (µmol/L)	0.51	0.24	0.17**	0.08***	
Histopathology: Number of rats examined	10	0	10	10	
Hepatocellular hypertrophy, centrilobular, diffuse (affected rats) minimal to slight	0	NE	0	10	
Thyroid follicular cell hypertrophy, diffuse (affected rats) minimal	0	NE	0	1	
Plasma bioanalysis Number of rats examined	5	5	5	5	
Isoflucypram (mg/L)	< LOQ	< 0.012	0.015	0.017	
M58 (mg/L)	< LOQ	0.039	0.147	0.676	
M11 (mg/L)	< LOQ	0.339	0.273	0.607	
Maternal data					
Number of rats assigned	23	23	23	23	
Number of rats pregnant	21	22	23	23	
Number of rats non-pregnant	2	1	0	0	
Intercurrent death or sacrifice, total	0	1	0	0	
Intercurrent death or sacrifice, pregnant	0	1	0	0	
Pregnancy rate (%)	91	96	100	100	
Uterine data at scheduled sacrifice					
Total number corpora lutea	382	367	410	415	
corpora lutea per dam	18.2	17.5	17.8	18.0	
Total number implantations	328	320	352	369	
implantations per dam	15.6	15.2	15.3	16.0	
Total number litters	21	21	23	23	
Total number live fetuses	307	306	333	343	
live fetuses per dam	14.6	14.6	14.5	14.9	
Total number early resorptions	21	13	15	25	
early resorptions per dam	1.0	0.6	0.7	1.1	
Total number late resorptions	0	1	4	1	
late resorptions per dam	0	0	0.2	0	
Mean fetal weight, combined sexes (g)	5.59	5.63	5.63	5.37	
Mean fetal weight, males (g)	5.70	5.75	5.80	5.51	
Mean fetal weight, females (g)	5.47	5.48	5.49	5.21*	
Sex ratio (% males)	51.1	56.9	46.8	53.4	
Sex ratio (% males per litter)	51.0	56.3	47.4	53.9	
Pre-implantation loss per dam (%)	13.33	12.31	13.81	10.75	
Post-implantation loss per dam (%)	6.54	4.32	5.25	7.10	

Parameter		Isoflucypram dose (mg/kg bw per day)				HCD (%)
		0	25	125	625	
External examination of fetuses						
Number examined	Litters	21	21	23	23	-
	Fetuses	307	306	333	343	-
Tail, bent	Litters	0	1	2	1	-
	% of litters	0.0	4.8	8.7	4.3	0.0–4.3
	Fetuses	0	1	2	2	-
	% of fetuses	0.0	0.3	0.6	0.6	0.0–0.3
Visceral examination of fetuses						
Number examined	Litters	21	21	23	23	-
	Fetuses	148	148	159	167	-
Thymic remnant present (uni/bi)	Litters	3	4	6	8	
	% of litters	14.3	19.0	26.1	34.8	8.7–40.9
	Fetuses	3	6	7	12*	
	% of fetuses	2.0	4.1	4.4	7.2	2.5–10.3
Bladder distended	Litters	0	0	0	1	
	% of litters	0	0	0	4.3	0.0–0.0
	Fetuses	0	0	0	3	
	% of fetuses	0	0	0	1.8	0.0–0.0
Renal pelvis (uni/bi): dilated (grade less than severe)	Litters	0	1	2	6*	
	% of litters	0.0	4.8	8.7	26.1	0.0–21.7
	Fetuses	0	3	2	7*	
	% of fetuses	0.0	2.0	1.3	4.2	0.0–3.2
Skeletal examination of fetuses						
Number examined	Litters	21	21	23	23	
	Fetuses	159	158	174	176	
At least one bone of zygomatic arch (uni/bi) incomplete ossification	Litters	3	3	7	12*	
	% of litters	14.3	14.3	30.4	52.2	0.0–34.8
	Fetuses	4	6	10	15*	
	% of fetuses	2.5	3.8	5.7	8.5	0.0–8.8
Squamosal: incomplete ossification (uni/bi)	Litters	1	1	3	4	
	% of litters	4.8	4.8	13.0	17.4	0.0–9.1
	Fetuses	1	2	3	4	
	% of fetuses	0.6	1.3	1.7	2.3	0.0–1.2
Hyoid centrum: incomplete ossification	Litters	4	2	8	9	
	% of litters	19.0	9.5	34.8	39.1	0.0–26.1
	Fetuses	4	2	11	14*	
	% of fetuses	2.5	1.3	6.3	8.0	0.0–7.0
Femur: incomplete ossification (uni/bi)	Litters	2	4	5	5	
	% of litters	9.5	19.0	21.7	21.7	0.0–26.1
	Fetuses	2	4	8	15**	
	% of fetuses	1.3	2.5	4.6	8.5	0.0–6.4

HCD: Historical control data; uni/bi: Unilateral/bilateral; NE: Not examined Source: Kennel, 2017b

< LOQ: Below the level of quantification (0.01 mg/L);

Statistically significant: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Rabbit

Groups of 23 time-mated pregnant female New Zealand White rabbits were exposed to isoflucypram (purity 94.2%; Batch no. 2013-006492) by oral gavage from GD 6 to 28. The sperm-positive day was designated GD 0. The doses given were 0, 10, 70 or 500 mg/kg bw per day suspended in an aqueous solution of 0.5% methylcellulose 400. The volume of administration was 4 mL/kg based on the most recently recorded body weight. Clinical observations were recorded daily from GD 0, GD 1, or GD 2 until GD 29. Maternal body weights were recorded for all females on GDs 3, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 29. Food consumption was also measured for all the females during the intervals GDs 3–4, 4–5, 5–6, 6–8, 8–10, 10–12, 12–14, 14–16, 16–18, 18–20, 20–22, 22–24, 24–26, 26–28 and 28–29. At scheduled sacrifice, on GD 29, a macroscopic examination of the visceral organs was performed, gravid uterine weight was recorded, and the dams were evaluated for numbers of corpora lutea, and the number and status of implantations (resorptions, dead and live fetuses). In addition, the livers of all pregnant females were weighed at scheduled sacrifice. A portion of the liver from all females was retained in 10% neutral buffered formalin for possible histological examination. In the event, specimens were not examined and are retained in the archives. Live fetuses were removed from the uterus, counted, weighed, sexed and examined externally. The heads of the fetuses from approximately half of each litter were immersed in Davidson fixative then in Bouin's fluid and the internal structures examined after fixation. The bodies of all fetuses were dissected for soft tissues anomalies and sex determination. Fetuses were eviscerated, skinned and fixed in absolute ethanol before staining. A modification of the Staples and Schnell technique was applied and a subsequent skeletal examination performed. Pregnancy rate was 100% in all groups except the 10 mg/kg per day group, which included the sole non-pregnant female from the study. The dams plasma concentrations of isoflucypram, M11 and M58 were measured at caesarean section, which was 24 hours after the last dosing.

The major findings from this study are shown in Table 22. No treatment-related clinical signs were observed. At 500 mg/kg bw per day. Two does (animals 4F3239 and 4F3225) were sacrificed due to abortions on GD 23 and GD 26. Animal 4F 3225 decreased in body weight during GDs 6–8 and revealed a pale liver with marked multiple, diffuse white foci in the liver and slightly multifocal depression of spleen at necropsy. No macroscopic finding or body weight loss was observed with 4F3239. Similar body weight loss or macroscopic abnormalities as those found in 4F3225 were not observed in other pregnant rabbits at the same dose. There was no clear evidence as to whether the two abortions were related to treatment or not, however the possibility was not excluded since there were no abortions in other groups.

There was no statistically significant difference from controls in body weight at 500 mg/kg bw per day. At this dose body weight gains were slightly reduced during the period GD 6–14, and this was statistically significant in the period GD 6–8, although only amounting to ca 50 g compared to controls. At 500 mg/kg bw per day, the mean body weight gains up to GD 18 were depressed compared with controls, but differences were not statistically significant. At 500 mg/kg/day food consumption was reduced by approximately 20% during the first half of the treatment period. For GD 10–14 the reduction was statistically significant. Thereafter mean feed consumption was similar to the controls. The individualized data for body weight gain and food intake indicated that most animals displaying lower food intake during GD 6–18 showed the lower body weight gains. The Meeting considered that the slightly decreased body weight gains during the first half of the study were caused by lower food intake in the same period. Liver weight at 500 mg/kg bw per day was slightly increased (by 16%). As increased liver weight had been a common finding related to liver hypertrophy caused by the isoflucypram treatment in other species the increase was considered adaptive.

At caesarean section no treatment-related changes were noted in the Post-implantation data (mean number of early and late resorptions, percentage of Post-implantation loss) or fetal data (mean litter size, mean fetal body weight and percentage of males). No dead fetuses were observed. There were no treatment-related external, visceral or skeletal findings at the fetal examinations. There were no treatment-related effects on external, visceral or skeletal examinations.

No treatment-related effects were observed at other doses.

The plasma concentrations of the parent and two metabolites showed dose-related increases. The highest levels were of M11 followed by M58; the concentration of isoflucypram was the lowest.

The NOAEL for maternal toxicity in rabbits was 70 mg/kg bw per day based on a slight increase in abortions, slight decrease in body weight and reduced food consumption during the first half of the treatment period, at 500 mg/kg bw per day. The NOAEL for embryo/fetal toxicity in rabbits was 500 mg/kg bw per day, the highest dose tested (Leconte, 2017).

Table 22. Summary of developmental toxicity study of isoflucypram in rabbits

	Isoflucypram dose (mg/kg bw per day)				HCD
Parameter	**0**	**10**	**70**	**500**	
			Maternal data		
Animals assigned (*N*)	23	23	23	23	NA
Animals pregnant (*N*)	23	22	23	23	NA
Pregnancy rate (%)	100	96	100	100	NA
Animals not pregnant (*N*)	0	1	0	0	NA
Maternal wastage:					NA
Intercurrent death or sacrifice, total	0	0	0	2	NA
Intercurrent death or sacrifice, pregnant	0	0	0	2	NA
Premature delivery	0	0	0	0	NA
Intercurrent death or sacrifice, non-pregnant	0	0	0	0	NA
Abortions	0	0	0	2	NA
			Maternal weight and food data: mean ± standard deviation		
Body weight (kg)					
GD 6	3.493 ± 0.1899	3.475 ± 0.1825	3.477 ± 0.2170	3.533 ± 0.1965	
GD 8	3.521 ± 0.1939	3.506 ± 0.1851	3.484 ± 0.1961	3.515 ± 0.2036	
GD10	3.573 ± 0.1706	3.541 ± 0.1825	3.516 ± 0.1991	3.539 ± 0.2102	
GD18	3.707 ± 0.1901	3.672 ± 0.1846	3.633 ± 0.2348	3.540 ± 0.2238	
GD29	3.868 ± 0.1924	3.864 ± 0.1831	3.808 ± 0.2447	3.808 ± 02820	
Body weight gain (kg)					
GD3–6	0.011 ± 0.0707 [a]	0.054 ± 0.0716	0.064 ± 0.0763	0.048 ± 0.0573	
GD6–8	0.028 ± 0.0420	0.032 ± 0.0417	0.007 ± 0.0506	−0.019 ± 0.0544**	
GD8–10	0.051 ± 0.0522	0.035 ± 0.0310	0.031 ± 0.0329	0.024 ± 0.0402	
GD10–14	0.076 ± 0.0541	0.082 ± 0.0463	0.073 ± 0.0371	0.057 ± 0.0633	
GD14–18	0.058 ± 0.0419	0.049 ± 0.0421	0.044 ± 0.0730	0.044 ± 0.0564	
GD 18–22	0.044 ± 0.0571	0.065 ± 0.0438	0.047 ± 0.0490	0.048 ± 0.0466	
Food consumption (g)					
GD 6–8	149.3 ± 39.80 [a]	168.1 ± 18.40	160.3 ± 22.42	127.2 ± 41.81	
GD 8–10	162.7 ± 23.18	170.2 ± 26.42	159.7 ± 26.28	126.4 ± 45.01	
GD 10–14	143.7 ± 17.55	150.6 ± 25.36	141.0 ± 26.04	123.8 ± 32.19**	
GD 14–18	129.2 ± 32.49	145.2 ± 31.93	135.1 ± 43.84	120.5 ± 31.27	
GD 18–22	145.9 ± 27.87	149.2 ± 34.96	136.4 ± 45.38	135.8 ± 31.65	
GD 22–26	99.66 ± 25.62	114.2 ± 28.49	110.0 ± 32.07	103.2 ± 27.43	
GD 26–29	99.26 ± 30.33	108.6 ± 29.84	100.8 ± 31.27	100.86 ± 38.88	
Liver weight (g)	94.5	93.7	101.4	109.4**	

Parameter	Isoflucypram dose (mg/kg bw per day)				HCD
	0	10	70	500	
Plasma concentrations; mean ± standard deviation (µg/mL)					
Dams examined (*N*)	6	7	6	5	
Isoflucypram	<LOQ	<LOQ	<0.0127	0.0746±0.0419	
Metabolite M11	<LOQ	1.037±0.4383	5.27±1.551	20.2±6.14	
Metabolite M58	<LOQ	0.0147±0.0018	0.0715±0.0433	0.218±0.1188	
Uterine data at scheduled sacrifice					
Corpora lutea (*N*)	279	248	276	240	NA
Corpora lutea per dam	12.1	11.3	12.0	11.4	10.67–12.81
Total implantations (*N*)	243	199	226	204	NA
Implantations per dam	10.6	9.0	9.8	9.7	9.10–10.95
Total litters (*N*)	23	22	23	21	NA
Total live fetuses (*N*)	226	189	211	186	NA
Live fetuses per dam	9.8	8.6	9.2	8.9	8.23–9.90
Dead fetuses (*N*)	0	0	0	0	NA
Early resorptions (*N*)	10	6	8	6	NA
Early resorptions per dam	0.4	0.3	0.3	0.3	0.190–1.000
Late resorptions (*N*)	7	4	7	12	NA
Late resorptions per dam	0.3	0.2	0.3	0.6	0.000–0.318
Litters with total resorptions (*N*)	0	0	0	0	NA
Mean fetal weight (g)					
combined sexes	39.7	41.8	39.9	39.7	35.05–41.37
males	40.0	42.6	41.5	39.1	35.37–42.33
females	39.5	41.1	37.7	39.9	34.57–40.85
Sex ratio					
% males	45.6	47.1	49.3	44.1	NA
% males per litter	45.1	45.8	51.0	44.4	40.3–56.6
Pre-implantation loss per dam (%)	12.2	18.9	15.7	14.3	9.08–23.78
Post-implantation loss per dam (%)	6.1	4.7	8.6	8.8	5.47–19.25
Malformations					
Fetuses affected (*N*)	226	189	211	186	
Litters affected (*N*)	23	21	23	22	
Face malformation with split skeletal elements or cleft palate [a]	1 (1F3159)	0	1 (3F3206)	0	
Eye malformation [a]	0	0	0	1 (4F3231)	
Brain and/or renal malformation [a]	0	1 (2F3182)	1 (3F3196)	0	
Cardiac malformation(s) [a]	2 (1F3159; 1F3164)	1 (2F3176)	1 (3F3197)	0	

Parameter	Isoflucypram dose (mg/kg bw per day)				HCD
	0	10	70	500	
Vertebral or sternebral malformation [a]	1 (1F3165)	1 (2F3192)	1 (3F3201)	0	
Hindpaw malformation [a]	1 (1F3155)	0	0	0	
Total malformations (*N*): fetuses [litters]	5 [4]	3 [3]	4 [4]	1 [1]	
Total incidence (%): fetal [in litter]	2.2 [17]	1.6 [14]	1.9 [17]	0.5 [5]	

N: Number; NA: Not available; HCD: Historical control data Source: Leconte, 2017

[a] Number of fetuses with malformation given, with individual animal number in parenthesis;

<LOQ: Below the limit of quantification (0.01 µg/mL); for series with some individual values found <LOQ, the LOQ was used for mean calculations, and in such cases the result was expressed as "< of the mean value" and SD was not calculated.

Difference from control statistically significant: * $p \leq 0.05$; ** $p \leq 0.01$;

2.6 Special studies

(a) Neurotoxicity

Isoflucypram (purity 94.2%; Batch no. 2013-006492) was administered once by oral gavage to separate groups of Wistar rats (12/sex per group) at dose levels of 0, 200, 600 or 2000 mg/kg bw. An acute neurotoxicity assessment, including a functional observational battery (FOB) and spontaneous motor activity was performed on four occasions; during the prestudy phase, approximately six hours after dosing, and then seven and 14 days after dosing. All surviving animals were subjected to a complete necropsy. At least six animals per sex per group were subjected to neuropathological investigation with selected organs weighed and a range of organs fixed and examined microscopically. Up to and including the highest dose tested (2000 mg/kg bw), there was no mortality or any noteworthy treatment-related daily clinical signs. There were no treatment-related effects on body weight or brain weight parameters, and nothing notable at macroscopic or microscopic examinations. In addition, no treatment-related effects were observed in any of the neurotoxicology end-points, including neuropathological examination in either sex. Based on these results, a dose of 2000 mg/kg bw isoflucypram was the NOAEL for both sexes (Garcin, 2017).

(b) Immunotoxicity

No specific immunotoxicity study was conducted, however, no effect was apparent in the available toxicity studies that might suggest isoflucypram has potential for immunotoxicity.

(c) Endocrine disrupting properties

Several studies were conducted to investigate estrogenic, androgenic or steroidogenic modality of isoflucypram. No estrogenic, androgenic or steroidogenic effect was observed in isoflucypram. A summary of the results of these studies appears in Table 23).

Table 23. Summary of studies conducted with isoflucypram into estrogenic, androgenic and steroidogenic modality related endocrine end-points

Study/design	NOAEL	LOAEL	Effects and conclusion	Purity Batch no.	Reference
Hershberger male Crl:CD(SD) rat study: oral gavage dosing 0, 400, 800 mg/kg bw/day	800 mg/kg bw/day (highest dose tested)	> 800 mg/kg bw/day	Isoflucypram is neither androgenic nor anti-androgenic in the developing male rat	Purity: 98.6% Batch number: NLL 8674-19-4	Totis, 2012
Uterotrophic/ vaginal opening study in immature female rats: oral gavage dosing 0, 400, 800 mg/kg bw/day	Estrogenicity/ anti-estrogenicity: 800 mg/kg bw/day (highest dose tested) Systemic toxicity: <400 mg/kg bw/day	Estrogenicity/ anti-estrogenicity: > 800 mg/kg bw/ day	Isoflucypram is neither estrogenic nor anti-estrogenic in the developing female rat; no effect on vaginal opening and uterine weights in immature female rats. In addition, the maximum tolerated dose was exceeded at 800 mg/kg bw/day and animals were therefore not evaluated for vaginal opening at this dose level	Purity: 93.6% Batch number: LJGD563-1-2	Kennel, 2011

(d) Mode of action studies

A series of studies were performed to confirm that the MOA of specific effects seen in the liver and thyroid of rats following exposure to isoflucypram was through the activation of the constitutive androstane receptor (CAR) and/or pregnane X receptor (PXR). There are no guidelines for these studies.

Direct effect on thyroid

Two in vitro assays were conducted to explore the potential of isoflucypram to interfere directly with thyroid homeostasis.

In the first assay, the potential for isoflucypram (purity 94.2%; Batch no. 2013-006492) to inhibit thyroperoxidase (TPO) activity was assessed using pooled thyroid microsomes isolated from male Han Wistar rats. Activity of TPO was measured by oxidation of guaiacol to a coloured product. As a reference compound 6-propyl-2-thiouracil (6-PTU) was used, and this gave the expected inhibition of TPO, exhibiting a half-maximal inhibitory concentration (IC_{50}) of 1.673 µM (95% confidence intervals 1.157–2.322 µM). Isoflucypram was tested at 0, 0.01, 0.03, 0.01, 0.3, 1, 3, 10, 30, 60 and 100 µM, based on solubility evaluations conducted prior to the test. No inhibition of TPO activity was detected up to the highest concentration tested (100 µM) and it was therefore concluded that isoflucypram did not interfere with TPO activity in rat thyroid microsomes (Haines, 2020).

In a second assay the potential for isoflucypram (purity 94.2%; Batch no. 2013-006492) to inhibit sodium/iodide symporter (NIS) activity was assessed by measuring NIS-mediated iodide uptake in a rat thyroid-derived cell line, Fisher rat thyroid low serum 5% (FRTL-5). Activity of NIS was measured by exploiting the ability of iodide to catalyse the reduction of cerium(IV) which is yellow, to a colourless cerium(III) product. Sodium perchlorate was used as a reference NIS inhibitor and this exhibited an IC_{50} of 0.3394 µM (95% confidence intervals 0.2292–0.5351 µM), consistent with published reference values. Isoflucypram was not soluble in assay buffer at over 5 µM and was therefore tested at 0, 0.00032, 0.0016, 0.008, 0.04, 0.2, 1 and 5 µM in the assay. No significant inhibition of NIS activity was observed up to the highest concentration of isoflucypram tested (5 µM). Due to limited solubility in the specific assay buffer, the results for the test item isoflucypram were considered inconclusive. (McGinnis, 2020)

On the basis of the data generated from these two in vitro assays, isoflucypram was considered to have no potential to interfere directly with thyroid homeostasis and this provides further support to the assertion that the mild effects seen in vivo were secondary to liver effects.

MOA studies for liver and thyroid effect in rodents

A mode of action study for liver and thyroid effects was conducted using female mice. Isoflucypram (purity 94.2%; Batch no. 2013-006492) was administered for seven days via the diet at concentrations of 0, 50, 110, 250, 560 or 1250 ppm (equal to 0, 8.87, 18.6, 43.9, 94.1 and 224 mg/kg per day). An additional control and 1250 ppm group were placed on a control diet for one month after treatment had ceased to assess the reversibility of any effects. These concentrations were selected on the basis of results obtained in subchronic studies, and aimed to provide mechanistic information on events occurring at dietary concentrations used in the 18-month mouse study. As both the male and female mice had responded similarly in previous studies, females alone were selected. After necropsy on all animals, the liver was weighed. Liver and thyroid tissues were fixed for microscopic examination. Immunohistochemical staining for Ki67 and BrdU was performed to check for cell proliferation. A small piece of liver was flash frozen for gene expression analyses. At both sacrifice times livers of 10 animals per group were homogenized for microsomal preparations in order to determine cytochrome P-450 and UDP-GT isoenzyme profiles.

The major effects observed are summarized in Table 24. Administration of isoflucypram had no effect on clinical signs, body weight, body weight gain, or food consumption. Absolute and relative liver weights were increased in a dose-related manner. No histopathological findings or cell proliferation activity due to the treatment were found in the liver. There was no effect on thyroid follicular cell proliferation at any dose. Total cytochrome P450 content and the activities of PROD, BQ and bilirubin-UDP-GT, as well as associated changes in gene expression, increased in a dose-related manner. These results demonstrated that isoflucypram induced significant and reversible changes in the liver of the female mouse, effects which are associated with CAR/PXR activation (Rouquié, 2018a).

Table 24. Summary of the seven-day dietary study of liver and thyroid cell proliferation in response to isoflucypram administration to female mice

	Dietary dose (ppm)					
	0	**50**	**110**	**250**	**560**	**1250**
Day 8						
Body weight (g)	19.55	19.65	19.58	20.25	19.73	19.44
Liver weight (g)	0.964	1.013	1.018	1.105**	1.116**	1.216**
Liver weight as % of body weight	4.929	5.148	5.193	5.448**	5.653**	6.253**
Post reversibility phase						
Body weight (g)	21.90	ND	ND	ND	ND	21.23*
Liver weight (g)	1.120	ND	ND	ND	ND	1.090
Liver weight as % of body weight	5.108	ND	ND	ND	ND	5.135
Liver macroscopy; 15 mice examined						
Liver enlarged (mice affected);						
day 8	0	1	0	3	4	8
post reversibility phase	0	ND	ND	ND	ND	0
Total cytochrome P450 content, and activities of specific Phase I and Phase II enzymes						
Day 8						
Cytochrome P450 (nmol/mg protein)	0.30	0.28	0.36	0.35	0.47**	0.55**
PROD (pmol/min/mg protein)	11.18	16.16**	19.52**	32.80**	60.09**	128.89**
BQ (nmol/min/mg protein)	4.39	4.29	4.03	4.94	6.57**	8.66**
Bilrubin-UDP-GT (nmol/min/mg protein)	0.988	0.849	1.229*	1.283**	1.282**	1.425**

	Dietary dose (ppm)					
	0	**50**	**110**	**250**	**560**	**1250**
Post reversibility phase						
Cytochrome P450 (nmol/mg protein)	0.37	ND	ND	ND	ND	0.40
PROD (pmol/min/mg protein)	13.25	ND	ND	ND	ND	12.28
BQ (nmol/min/mg protein)	4.88	ND	ND	ND	ND	4.79
Bilrubin-UDP-GT (nmol/min/mg protein)	1.256	ND	ND	ND	ND	1.087
Gene transcription data from liver (relative quantities compared to controls)						
Day 8						
Cyp1a1	1.327	1.233	1.291	1.190	1.309	1.436
Cyp1a2	1.037	1.091	1.146	1.032	1.233*	1.372**
Cyp2b9	0.913	0.957	0.942	0.872	0.909	0.940
Cyp2b10	1.239	2.123**	3.350**	5.852**	11.292**	22.691**
Cyp3a11	0.877	1.171**	1.387**	1.866**	3.406**	6.261**
Ugt2b5	0.927	1.069	1.197**	1.165**	1.437**	1.411**
Ugt1a1	0.959	1.044	1.031	1.074	1.220**	1.533**
Post reversibility phase						
Cyp1a1	0.985	ND	ND	ND	ND	1.007
Cyp1a2	0.721	ND	ND	ND	ND	0.775
Cyp2b9	0.706	ND	ND	ND	ND	0.679
Cyp2b10	1.013	ND	ND	ND	ND	1.169
Cyp3a11	0.883	ND	ND	ND	ND	0.828
Ugt2b5	0.949	ND	ND	ND	ND	0.951
Ugt1a1	0.881	ND	ND	ND	ND	0.903

ND: No data; PROD: Pentoxyresorufin-*O*-deethylase; Source: Rouquié, 2018a
UDP-GT: Uridine diphosphate glucuronosyltransferase; BQ: Benzyloxyquinoline *O*-debenzylation
Difference from control statistically significant: * $p \leq 0.05$; ** $p \leq 0.01$;

A similar MOA study to the study in mice described above was conducted using rats. In a seven-day feeding study, isoflucypram (purity 94.2%; Batch no. 2013-006492) was administered to 12 female Wistar Han rats at dietary concentrations of 0, 30, 75, 150, 450 or 800 ppm (equal to 0, 2.4, 6.0, 12, 36 and 67 mg/kg bw per day). Additional control and 800 ppm groups were placed on a control diet for one month after treatment to assess the reversibility of any effects. These concentrations were selected on the basis of results obtained in other toxicity and MOA studies. Females were chosen due to more marked effects on liver and thyroid that were apparent in females in the short-term studies. At scheduled sacrifice, blood samples were taken for thyroid hormone analysis, specifically thyroxine (T4), triiodothyronine (T3) and thyroid-stimulating hormone (TSH). The liver, thyroid and pituitary from each animal was also collected and the liver weighed. Samples of both the liver and thyroid were fixed and examined microscopically. Additional slides were stained with Ki67 to check of for cell proliferation. The pituitary and small portions of the liver were frozen in liquid nitrogen and used for gene expression investigations. The remaining portions of the liver were homogenized for microsomal preparations in order to determine cytochrome P-450 and UDP-GT isoenzyme profiles.

The major effects of the treatment in this study are summarized in Table 25. There was no effect on mortality, clinical signs, body weight, body weight gain or food consumption in any group. At 800 ppm a slight increase in plasma TSH levels and Tshβ transcript levels were observed, which could be associated with an increase in follicular cell proliferation in the thyroid. Absolute and relative liver weight was slightly increased and an enlarged liver was observed in 6/12 animals at the high dose of 800 ppm. In the liver, there was a dose-related increase in the incidence of centrilobular, periportal, and total cell proliferation. There was also an increase in total proliferative index in the thyroid.

The increase in cell proliferation in both liver and thyroid was reversible after withdrawal of treatment. Total cytochrome P450, activity of BROD and PROD, activities of UDP-GT enzymes, as well as transcription levels of associated genes, were increased in a dose-related and reversible manner. These results showed that isoflucypram increased specific hepatic enzyme induction associated with activation of CAR/PXR and cell proliferation in the liver and thyroid in the female rat, indicating that isoflucypram acts via the CAR–PXR MOA in the rodent (Rouquié, 2018b).

Table 25. Summary of the seven-day dietary study of cell proliferation in the liver and thyroid of female rats treated with isoflucypram

	Dietary dose (ppm)					
Parameter	**0**	**30**	**75**	**150**	**450**	**800**
Liver weight, day 8						
Absolute weight (g)	8.881	8.582	8.706	8.763	9.008	9.649
Liver weight as % of body weight	3.380	3.315	3.325	3.418	3.495	3.700**
Liver weight, post reversibility phase						
Absolute weight (g)	8.317	ND	ND	ND	ND	8.925
Liver weight as % of body weight	2.878	ND	ND	ND	ND	3.047*
Liver macroscopy; 12 mice examined						
Liver enlarged (mice affected);						
day 8	0	1	0	3	2	6
post reversibility phase	0	ND	ND	ND	ND	1
Proliferative index in liver and thyroid						
Day 8						
Liver; centrilobular	20.16	20.54	25.41	30.01	30.03	37.19
periportal	34.28	25.50	37.32	38.13	48.00	67.79**
total	27.22	23.02	31.37	34.07	39.02	52.50**
Thyroid	23.65	22.53	31.23	25.24	37.74	49.97***
Post reversibility phase						
Liver; centrilobular	5.96	ND	ND	ND	ND	2.99
periportal	12.72	ND	ND	ND	ND	4.63***
total	9.34	ND	ND	ND	ND	3.81***
Thyroid	11.85	ND	ND	ND	ND	13.05
Thyroid hormones						
Day 8						
Triiodothyronine, T3 (ng/mL)	0.47	0.49	0.52	0.63**	0.58*	0.53
Thyroxine, T4 (µg/dL)	2.59	2.51	3.09	2.35	2.70	2.33
Thyroid-stimulating hormone, TSH (ng/mL)	0.820	1.092	1.228	0.978	1.081	1.523
Post reversibility phase						
Triiodothyronine, T3	0.50	ND	ND	ND	ND	0.63**
Thyroxine, T4	2.76	ND	ND	ND	ND	2.83
Thyroid-stimulating hormone, TSH	1.064	ND	ND	ND	ND	0.851
Total cytochrome P450 content, and activities of specific Phase I and Phase II enzymes						
Day 8						
Cytochrome P450 (nmol/mg protein)	0.882	0.815	0.839	0.917	0.893	1.048**
BROD (pmol/min/mg protein)	3.873	3.583	3.421	3.844	6.809	5.444
EROD (pmol/min/mg protein)	21.371	27.788	31.908**	25.095	23.578	26.288

Parameter	Dietary dose (ppm)					
	0	30	75	150	450	800
PROD (pmol/min/mg protein)	1.548	1.763	2.238	1.998	2.270	2.548
p-nitrophenol-UDP-GT (nmol/min/mg protein)	6.495	5.868	5.945	6.427	6.950	7.797**
Bilrubin-UDP-GT (nmol/min/mg protein)	0.554	0.391	0.457	0.538	0.976*	1.489***
T4-UDP-GT (pmol/min/mg protein)	0.492	0.500	0.751	0.710	0.821	1.136**
Post reversibility phase						
Cytochrome P450 (nmol/mg protein)	0.943	ND	ND	ND	ND	0.918
BROD (pmol/min/mg protein)	4.031	ND	ND	ND	ND	4.530
EROD (pmol/min/mg protein)	22.059	ND	ND	ND	ND	29.803*
PROD (pmol/min/mg protein)	5.146	ND	ND	ND	ND	4.992
p-nitrophenol-UDP-GT (nmol/min/mg protein)	6.047	ND	ND	ND	ND	5.940
Bilrubin-UDP-GT (nmol/min/mg protein)	0.5013	ND	ND	ND	ND	0.4239
T4-UDP-GT (pmol/min/mg protein)	0.930	ND	ND	ND	ND	0.850
Gene transcription data from liver and pituitary (relative quantities compared to controls)						
Day 8						
Cyp1a1	1.000	0.302	1.105	0.948	1.324	0.804
Cyp1a2	1.000	0.838	0.935	1.046	1.024	0.840
Cyp2b1	1.001	0.882	4.632**	6.932**	42.802**	71.253**
Cyp3a23	1.00	0.877	1.225	2.02**	16.730**	28.490**
Cyp4a1	1.001	1.045	1.090	0.998	0.943	0.773*
Ugt1a1	1.000	0.917	1.099	1.355**	2.908**	4.293**
Ugt1a6	0.999	0.943	0.933	1.117	1.330**	1.421**
Ugt2b1	0.999	1.348	1.790*	1.716*	2.558**	3.703**
TSHβ	0.969	1.225	1.216	1.088	1.387	1.480
Post reversibility phase						
Cyp1a1	0.999	ND	ND	ND	ND	1.322
Cyp1a2	1.001	ND	ND	ND	ND	1.162
Cyp2b1	1.000	ND	ND	ND	ND	1.384
Cyp3a23	1.00	ND	ND	ND	ND	1.150
Cyp4a1	1.000	ND	ND	ND	ND	1.018
Ugt1a1	1.000	ND	ND	ND	ND	0.933
Ugt1a6	1.000	ND	ND	ND	ND	0.955
Ugt2b1	1.000	ND	ND	ND	ND	0.993
TSHβ	0.999	ND	ND	ND	ND	1.015

PROD: Pentoxyresorufin-*O*-deethylase; EROD: Ethoxyresorufin-*O*-deethylase; Source: Rouquié, 2018b
UDP-GT: Uridine diphosphate glucuronosyltransferase; BROD: Benzoxyresorufin-*O*-dealkylase; ND: No data;
Difference from control statistically significant: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Two 28-day studies were conducted to investigate MOAs of liver and thyroidal effects in rats.

Study 1

A MOA study was conducted in groups of 10 Wistar Rj:WI (IOPS HAN) female rats, with isoflucypram (purity 94.2%; Batch no. 2013-006492) administered via the diet at concentrations of 0, 300 or 1000 ppm

(equal to 0, 26.4 and 84.8 mg/kg per day). These concentrations were chosen on the basis of concentrations used in the short-term studies. Females were chosen because a possibility that the effect on the liver and thyroid would be greater in females than males, suggested by the results of previous studies. At final sacrifice blood samples were collected for thyroid hormone measurements, after weighing the liver. A portion was collected from the liver and pituitary of each animal for gene transcript analyses. The remaining portions of the liver were homogenized for microsomal preparations to determine Phase I (cytochrome P 450) and II (UDP-GT) isoenzymes profiles.

The major effects seen in this study are shown in Table 26. Isoflucypram administration had no effect on clinical signs or mortality. Body weight was unaffected by treatment, but body weight gain and mean food consumption were slightly reduced at 1000 ppm over the course of the study. At necropsy, absolute and relative liver weights were increased, as were the activities of BROD, bilirubin-UDP-GT, and T4-UDP-GT. Circulating concentrations of T3 and T4 were not altered, but the concentration of TSH as well as the amount of TSHβ transcript in the pituitary was increased in a dose-related manner (Totis, 2013).

Table 26. Summary of the first 28-day dietary study of hepatotoxicity and thyroid hormone concentrations in female rats.

	Dietary dose (ppm)		
Parameter	**0**	**300**	**1000**
Thyroid hormone concentrations			
Triiodothyronine, T3 (ng/mL)	0.90	0.97	0.94
Thyroxine, T4 (µg/dL)	2.36	2.31	2.13
Thyroid-stimulating hormone, TSH (ng/mL)	0.381	0.586	1.018**
Liver weight			
Absolute weight (g)	8.234	8.951	9.794**
Liver weight as % of body weight	3.269	3.54*	3.865**
Hepatic cytochrome P450 isozymes			
BROD (pmol/min/mg protein)	4.34	5.49*	6.83**
PROD (pmol/min/mg protein)	3.45	3.68	2.74*
Bilirubin-UDP-GT (nmol/min/mg protein)	0.35	0.81*	2.08***
T4-UDP-GT (pmol/min/mg protein)	1.03	2.09*	4.37**
Gene transcription data from liver and pituitary (relative quantities compared to controls)			
Cyp1a1	1.88	2.00	10.50**
Cyp2b1	0.25	6.61	31.10**
Cyp3a3	0.77	20.87	98.27***
Cyp4a1	0.96	0.88	0.75*
Por	0.56	0.85**	1.35**
Ugt1a6	0.69	0.91	2.16***
Ugt2b1	0.65	1.15**	2.39**
Sult2a2	0.67	1.14	1.31*
Ephx1	0.72	1.53**	2.85**
Gstm4	0.48	1.83**	4.41**
TSHβ	1.09	1.26	1.87*

BROD: Benzoxyresorufin-*O*-dealkylase; PROD: Pentoxyresorufin-*O*-deethylase; Source: Totis, 2013
Statistically significant: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; UDP-GT: Uridine diphosphate glucuronosyltransferase

Study 2

Another 28-day dietary study was conducted in groups of 12 female Wistar Rj:WI (IOPS HAN) rats to determine the effect of isoflucypram (purity 94.2%; Batch no. 2013-006492) on cell proliferation

in the liver and thyroid after repeated administration at dietary concentrations of 0, 30, 75, 150, 450 or 800 ppm (equal to 2.4, 6.0, 12, 37 and 69 mg/kg per day). An additional control and 800 ppm group were placed on a control diet for one month after treatment to assess the reversibility of any treatment-related changes. At both sacrifice times blood samples were taken for hormone analysis (T4, T3 and TSH) and the liver, thyroid and pituitary from each animal was collected. The liver and thyroid were weighed and fixed for microscopic examination and Ki67 staining for cell proliferation determinations. The pituitary and small portions of the liver were fresh frozen for gene expression investigations. The remaining portions of the liver were homogenized for microsomal preparations in order to determine cytochrome P-450 and UDP-GT isoenzyme profiles.

The major effects of treatment are summarized in Table 27. Isoflucypram administration had no effect on clinical signs, mortality, body weight parameters or food consumption. The concentrations of circulating T3 and T4 were not changed at any dose compared with controls, however circulating TSH concentration increased in a dose-related manner. Absolute and relative liver weights were increased, as was the incidence of enlarged liver and hepatocellular hypertrophy. In the thyroid, the incidence of thyroid follicular cell hypertrophy was increased only at the top dose of 800 ppm. There was no statistically significant effect on proliferative index in the liver, but thyroid proliferation index was increased with statistical significance. Total cytochrome P450 content was not changed by treatment, however the activities of BROD, PROD *p*-nitrophenol-UDP-GT, T4-UDP-GT and bilirubin-UDP-GT, as well as their associated gene transcript levels, were increased in a dose-related manner. All observations were reversible following the recovery period (Rouquié, 2018c).

Table 27. Summary of the second 28-day dietary study of hepatotoxicity and thyroid hormone concentrations in female rats

	Dietary dose (ppm)					
Parameter	**0**	**30**	**75**	**150**	**450**	**800**
Thyroid hormone concentrations						
Day 29						
T3 (ng/mL)	0.41	0.39	0.40	0.44	0.41	0.37
T4 (µg/dL)	2.21	2.24	2.17	2.49	2.64	2.27
TSH (ng/mL)	0.720	0.734	0.857	0.882	0.942	1.421**
Day 57						
T3 (ng/mL)	0.44	ND	ND	ND	ND	0.41
T4 (µg/dL)	2.25	ND	ND	ND	ND	2.25
TSH (ng/mL)	0.612	ND	ND	ND	ND	0.862
Body and organ weight, day 29						
Terminal body weight (g)	274.1	284.7	278.4	281.8	277.7	272.1
Liver weight, absolute (g)	8.380	9.612**	8.777	9.049	9.125	9.839***
Liver weight as % of body weight	3.053	3.375**	3.154	3.212	3.280*	3.611**
Thyroid weight, absolute (g)	0.01608	0.01600	0.01558	0.01475	0.01692	0.01558
Thyroid weight as % of body weight	0.00589	0.00564	0.00562	0.00524	0.00610	0.00574
Body and organ weight, post reversibility phase						
Terminal body weight (g)	301.5	ND	ND	ND	ND	299.7
Liver weight, absolute (g)	8.349	ND	ND	ND	ND	8.764
Liver weight as % of body weight	2.768	ND	ND	ND	ND	2.924
Thyroid weight, absolute (g)	0.01602	ND	ND	ND	ND	0.01763
Thyroid weight as % of body weight	0.00533	ND	ND	ND	ND	0.00588
Macroscopy and microscopy, day 29; (number affected of *n* = 12 examined)						
Enlarged liver	1	6	0	1	1	10

	Dietary dose (ppm)					
Parameter	**0**	**30**	**75**	**150**	**450**	**800**
Hepatocellular hypertrophy	0	0	0	0	0	5
Thyroid follicular cell hypertrophy	0	0	0	0	0	6
Macroscopy and microscopy, post reversibility phase; (number affected of *n* = 12 examined)						
Enlarged liver	0	ND	ND	ND	ND	2
Hepatocellular hypertrophy	0	ND	ND	ND	ND	0
Thyroid follicular cell hypertrophy	0	ND	ND	ND	ND	0
Proliferative index in liver and thyroid, day 29						
Liver, total	11.471	17.776	12.495	13.467	15.118	16.794
Thyroid	13.137	15.527	18.896	17.008	21.263**	23.311**
Proliferative index in liver and thyroid, post reversibility phase						
Liver centrilobular	1.913	ND	ND	ND	ND	0.528*
periportal	6.811	ND	ND	ND	ND	2.943**
total	4.363	ND	ND	ND	ND	1.737**
Thyroid	8.605	ND	ND	ND	ND	7.109
Total cytochrome P450 content, and activities of specific Phase I and II enzymes						
Day 29						
Cytochrome P450 (nmol/mg protein)	0.994	1.106	0.896	0.893	0.965	1.025
BROD (pmol/min/mg protein)	2.716	4.173*	3.441	3.878	4.422*	6.737**
EROD (pmol/min/mg protein)	33.672	29.272	27.039	27.573	26.711	20.323**
PROD (pmol/min/mg protein)	4.149	4.227	4.060	4.790	4.738	4.784
p-nitrophenol-UDP-GT (nmol/min/mg protein)	6.730	7.793	6.687	6.447	8.105*	7.940
Bilrubin-UDP-GT (nmol/min/mg protein)	0.771	0.833	0.878	0.779	1.524**	1.941**
T4-UDP-GT (pmol/min/mg protein)	1.070	1.787**	2.102**	1.573*	2.242**	3.255**
Post reversibility phase						
Cytochrome P450 (nmol/mg protein)	0.913	ND	ND	ND	ND	0.945
BROD (pmol/min/mg protein)	2.120	ND	ND	ND	ND	3.426
EROD (pmol/min/mg protein)	32.868	ND	ND	ND	ND	28.959
PROD (pmol/min/mg protein)	2.729	ND	ND	ND	ND	2.415
p-nitrophenol-UDP-GT (nmol/min/mg protein)	6.273	ND	ND	ND	ND	6.335
Bilrubin-UDP-GT (nmol/min/mg protein)	0.526	ND	ND	ND	ND	0.473
T4-UDP-GT (pmol/min/mg protein)	1.095	ND	ND	ND	ND	1.008
Gene transcription data from liver and pituitary						
Day 29						
Cyp1a1	1.00	1.142	1.182	1.477	1.364	2.659**
Cyp1a2	0.998	0.907	1.126	0.936	0.985	1.036
Cyp2b1	0.999	2.183	3.866	10.853**	26.395**	102.785**
Cyp3a23	0.993	0.800	2.061*	2.908**	12.835**	29.575**
Cyp4a1	1.001	1.022	1.207	0.907	0.962	0.750
Ugt1a1	0.999	0.946	1.355*	1.435**	2.650*	4.068**
Ugt2b1	1.000	1.253	1.106	1.436	2.351**	3.318**

	Dietary dose (ppm)					
Parameter	**0**	**30**	**75**	**150**	**450**	**800**
TSHβ	0.999	1.062	0.961	1.039	1.028	1.188
Post reversibility phase						
Cyp1a1	0.999	ND	ND	ND	1.921	
Cyp1a2	1.000	ND	ND	ND	1.090	
Cyp2b1	1.001	ND	ND	ND	1.271	
Cyp3a23	0.994	ND	ND	ND	0.798	
Cyp4a1	1.000	ND	ND	ND	0.894	
Ugt1a1	1.000	ND	ND	ND	0.857*	
Ugt2b1	1.000	ND	ND	ND	1.518*	
TSHβ	1.007	ND	ND	ND	0.924	

PROD: Pentoxyresorufin-*O*-deethylase; EROD: Ethoxyresorufin-*O*-deethylase; Source: Rouquié, 2018c
UDP-GT: Uridine diphosphate glucuronosyltransferase; BROD: Benzoxyresorufin-*O*-dealkylase; ND: No data;
Difference from control statistically significant: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

In summary, isoflucypram induced CAR/PXR mediated liver hypertrophy, and bilirubin-UDP-GT, UDPGT1A1 and UDPGT2B1 in the liver indicating UDP-GT induction. TSH levels and cell proliferation activity in the thyroid increased by the treatment, and recovered after withdraw of the treatment in rats (Rouquié, 2018a, b, c; Totis 2013). The thyroidal effect was due to a secondary effect on continuous stimulation to the thyroid through UDP-GT induction in the liver. However, judgement whether this MoA of isoflucypram was relevant to humans or not was not established because information of thyroidal effect of isoflucypram on humans was inadequate.

The MoA studies also indicated that the treatment with isoflucypram was induced the phase II metabolism enzymes activity and increased expressions of their genes in the liver in rats. The major induced phase II enzymes were UDP-GT including bilirubin-UDP-GT and T4-UDP-GT. There was no direct information in liver enzyme induction in dogs, however available data integrated indicated that the lower bilirubin level, a common effect in mice, rats and dogs observed in the toxicity studies, was occur the same MoA, excess elimination of plasma bilirubin binding to these enzymes from the blood (See figure in Appendix 2).

(e) Studies on metabolite M12 (isoflucypram-carboxylic acid; BCS-CY26497)

A metabolism study and genotoxicity studies in vitro and in vivo were conducted for metabolite M12, and in silico analysis for genotoxicity was also carried out.

Metabolism of M12; pilot metabolism study in rats

The absorption, excretion, and metabolism of [*pyrazolyl-4-*^{14}C]isoflucypram-carbolyxic acid (radiochemical purity > 98% or above for both HPLC and TLC), one of the major metabolites of isoflucypram, were investigated in male Wistar rats. The rats received a single target dose of about 5 mg/kg bw of the test compound by oral gavage. The elimination of radioactivity from blood was followed quantitatively by collecting micro samples from one single rat at individual time points. In addition, the amount of radioactivity in excreta (urine and/or faeces) was determined over selected time periods for all rats. The amount of radioactivity was quantified by liquid scintillation counting.

Isoflucypram-carbolyxic acid was absorbed very quickly from the GI tract after a single oral administration. The highest plasma level was measured at 0.25 hours. Excretion of radioactivity was all most via faeces and only a minor part with urine. Excretion was nearly completed within 24 hours. metabolite M12 was the main compound in the faecal extract. A number of major downstream metabolites deriving from M12 were excreted predominantly via the faecal route, but some of these major metabolites were detected in urine, albeit at very low levels. Demethylation of the pyrazole moiety and hydroxylation in the propionic acid group were the most prominent metabolic reactions. Cleavage of the phenyl moiety and the cyclopropyl ring were also observed as important reactions. Further conjugation

with glucuronic acid after hydroxylation or via nitrogen and conjugation with alanine was found (Bongartz & Bartelsen, 2017d).

Studies on the genotoxicity of M12

Reverse mutation assay with Salmonella typhimurium

This study was performed to investigate the potential of metabolite M12 (purity 96.7%; Batch no. NLL 9728-6-4) to induce gene mutations according to the plate incorporation test (Experiment I) and the pre-incubation test (Experiment II) using the *Salmonella typhimurium* strains TA1535, TA1537, TA98, TA100, and TA102.The assay was performed in two independent experiments both with and without liver microsomal activation (S9). Each concentration, including the controls, was tested in triplicate. The test item was assayed at the following concentrations: pre-experiment and Experiment I: 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate; Experiment II: 33, 100, 333, 1000, 2500 and 5000 µg/plate.

No precipitation of the test item occurred in the overlay agar in the test tubes in either experiment. Precipitation of the test item in the overlay agar on the incubated agar plates was observed in Experiment I at 5000 µg/plate. In Experiment II no precipitation was observed on the incubated agar plates. The undissolved particles had no influence on the data recording. Plates incubated with the test item showed normal background growth up to 5000 µg/plate with and without S9 mix in all strains used. No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5) occurred in the test groups with or without metabolic activation. No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with M12 at any dose level, neither in the presence nor absence of metabolic activation. Nor was there any tendency towards higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance. Appropriate reference mutagens were used as positive controls and produced a distinct increase in induced revertant colonies.

Under the experimental conditions reported, the test item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. Therefore, M12 (isoflucypram-carbolyxic acid; BCS-CY26497) is considered to be non-mutagenic in this *Salmonella typhimurium* reverse mutation assay (Naumann, 2018a).

Chromosome aberration test in human lymphocytes in vitro

The test item, metabolite M12 (purity 96.7%; Batch no. NLL 9728-6-4) dissolved in dimethyl sulfoxide (DMSO), was assessed for its potential to induce structural chromosomal aberrations in human lymphocytes in vitro in two independent experiments. The highest applied concentration in this study (2068 µg/mL of test item) was chosen with regard to the purity (96.7%) of the test item and with respect to the current OECD Guideline 473. In Experiment I, in the absence and presence of S9 mix, no cytotoxicity was observed up to the highest applied and evaluated concentration, which showed precipitation. In Experiment II in the absence of S9 mix after continuous treatment, clear cytotoxicity was observed at the highest applied and evaluated concentration, which showed precipitation. In Experiment I (in the absence and presence of S9 mix) neither a statistically significant nor a biologically relevant increase in the number of cells carrying structural chromosomal aberrations was observed after treatment with the test item. In Experiment II, in the absence of S9 mix, statistically significant increases above the range of the historical control data (0.1–2.2% aberrant cells, excluding gaps) were observed after continuous treatment at all evaluated concentrations (2.7–8.3% aberrant cells, excluding gaps). The statistical analysis of dose dependency via the trend test showed a marginal, but not significant result with a p value of 0.083. No evidence of an increase in polyploid metaphases was noticed after treatment with M12 as compared to the control cultures. Appropriate mutagens were used as positive controls; they induced statistically significant increases in cells, with structural chromosome aberrations.

Under the experimental conditions reported, the test item induced structural chromosomal aberrations in human lymphocytes in vitro. Therefore, M12 (isoflucypram-carbolyxic acid; BCS-CY26497) is considered to be clastogenic in this chromosome aberration test, when tested up to cytotoxic and/or precipitating concentrations (Naumann, 2018b).

Cell mutation assay at the thymidine kinase locus ($tk^{+/-}$) in mouse L5178Y lymphoma cells

The study was performed to investigate the potential of M12 to induce mutations at the mouse lymphoma thymidine kinase locus, using the cell line L5178Y. The assay was performed in two independent experiments, using two parallel cultures each. The first experiment was performed with and without liver microsomal activation (S9) and a treatment period of four hours. The second experiment was solely performed in the absence of metabolic activation with a treatment period of 24 hours. The maximum test item concentration of the pre-experiment and the main experiments (2068 µg/mL) was chosen with respect to the current OECD guideline 490 regarding the purity of the test item. No substantial and reproducible dose-dependent increase in mutant colony numbers was observed in either main experiment. No relevant shift in the ratio of small versus large colonies was observed up to the maximal concentration of test item. Appropriate reference mutagens, used as positive controls, induced a distinct increase in mutant colonies and thus showed the sensitivity of the test system and the activity of the metabolic activation system.

Under the experimental conditions reported the test item did not induce gene mutations at the mouse lymphoma *tk* locus in the cell line L5178Y. Therefore, M12 (isoflucypram-carbolyxic acid) was considered not to be mutagenic in this mouse lymphoma thymidine kinase locus assay in L5178Y (Sokolowski, 2018)

Micronucleus assay in bone marrow cells of the mouse

This study was performed to investigate the potential of metabolite M12 (purity 98.8%; Batch no. NLL 9728-5-5) to induce micronuclei in polychromatic erythrocytes (PCEs) in the bone marrow of NMRI mice. The test item was dissolved in a DMSO/PEG 400 mixture, which was also used as the vehicle control. Both test item and vehicle control were administered twice, orally at 24 hour intervals. The volume administered was 10 mL/kg bw. Twenty-four hours after the second administration of the test item the bone marrow cells were collected for micronucleus analysis. Seven males per test group were evaluated for the occurrence of micronuclei. At least 4000 PCEs per animal were scored for micronuclei. To describe a cytotoxic effect due to the treatment with the test item, the ratio between polychromatic and normochromic erythrocytes was determined in the same sample and reported as the number of PCEs relative to total erythrocytes. The dose levels were 500, 1000 and 2000 mg/kg bw. A correction factor of 1.01 was applied, based on test item purity data provided by the sponsor. The highest dose (2000 mg/kg bw; the maximum guideline-recommended dose) was estimated to be suitable from the results of two pre-experiments.

At 2000 mg/kg bw, one animal showed serious clinical signs and was euthanized 5–6 hours after the second application due to animal welfare concerns. At 1000 mg/kg bw, one animal showed clinical signs such as closed eyes. No clinical sign was observed at 500 mg/kg bw. After treatment with the test item the number of PCEs was dose-dependently decreased as compared to the mean value of PCEs for the vehicle controls, thus indicating that M12 did exert a slight cytotoxic effect in the bone marrow. The clinical signs at 1000 mg/kg bw and above suggested that bioavailability of the test item could be relied on. The micronucleus frequency of one animal at 500 mg/kg bw (0.7% compared to a mean concurrent control value of 0.13%) was above the range for historical vehicle controls but was considered an irrelevant outlier since all other values in this group were well within the range of historical vehicle controls.

Under the experimental conditions reported, the test item did not induce micronuclei as determined by the micronucleus test with mouse bone marrow cells. Therefore M12 (isoflucypram-carboxylic acid; BCS-CY26497) was considered non-mutagenic in this micronucleus assay (Dony, 2019).

The summary of genotoxicity studies with M12 (BCS-CY26497) is shown in Table 28.

Table 28. Results of genotoxicity studies performed with M12 (isoflucypram-carboxylic acid BCS-CY26497)

Type of study	Organism/cells	Dose range tested	Purity (%)	Result	Reference
In vitro gene mutation test	Ames: *Salmonella typhimurium;* strains TA100, TA98, TA1535, TA1537, TA102	± S9: 3–5000 µg/plate	96.7	−/+S9: negative	Naumann, 2018a
In vitro chromosome aberration assays	Human lymphocytes cells	Experiment 1 −S9: 13.4–2068 µg/mL +S9: 126–2068 µg/mL Experiment 2 −S9: 126–2068 µg/mL	96.7	−S9: 22 hours clastogenic	Naumann, 2018b
In vitro gene mutation assay	Mouse lymphoma L5178Y cells	Experiment 1 −S9: 64.6–2068 µg/mL +S9: 6.38–2068 µg/mL Experiment 2 −S9: 16.2–2068 µg/mL	96.7	−/+S9: negative	Sokolowski, 2018
In vivo micronucleus test	Mouse (NMRI) bone marrow micronucleus (gavage)	500–2000 mg/kg bw	98.8	Negative	Dony, 2019

An adequate number of in vitro and in vivo tests for genotoxicity were carried out to investigate the genotoxicity of metabolite M12 (isoflucypram-carboxylic acid; BCS-CY26497) and it was concluded by the weight of evidence approach, that M12 showed no indication of genotoxicity.

(f) In silico prediction of mutagenicity for metabolites of isoflucypram

In silico prediction of mutagenicity were made for the following metabolites of isoflucypram:

- M07 (isoflucypram-desmethyl-1,2-propandiol),
- M36 (isoflucyprma-desmethyl-1,2propandiol-N-GlucA),
- M10 (isoflucypram-lactic acid),
- M49 (isoflucypram-N-methyl-cyclopropyl-pyrazole-carboxamide),
- M50 (isoflucypram-N-methyl-pyrazole-carboxylic acid; BSC-AB72918, BCS-CR73065,),
- M58 (isoflucypram-cyclopropyl-pyrazole-carboxamide; BCX99798),
- M66 (isoflucypram-cyclopropyl-pyrazole-carboxamide-Ala),
- M67 (isoflycypram-desfluoro-cyclopropyl-pyrazole-carboxamide-Ala),
- M07 (isoflucypram-desmethyl-1,2-propandiol),
- M06 (isoflucypram-desmethyl-propanol, BCS-DC22055), and
- M77 (Isoflucypram-desmethyl-propanol-aldehyde; BCS-DH85957)

The in silico analyses, including the potential for genotoxicity of the metabolites of isoflucypram and their conjugates are summarized in Tables 29 and 30.

In order to predict the potential for gene mutation and/or chromosomal aberration due to the metabolites an in silico assessment was conducted using following applications of Derek Nexus, Leadscope, Toxtree and TopKat. Versions of these application were as follows:

- Derek Nexus, using Nexus version 2.2.2, Derek Nexus version 6.0.1 and Knowledge Base 2018 1.1. All alerts were run and the reports for each substance contain all alerts that were triggered.
- Leadscope Model Applier 2.4, using the Genetox Suite, and the following models were applied:
  - Mutagenicity
    - Gene mutation in bacteria
      - Bact Mut v. 2
      - E. coli – Sal A-T Mut v. 2
      - Salmonella Mut v. 4
    - Gene mutation in mammalian cells
      - HPRT Mut v. 1
      - Mouse lymphoma Act v. 2
      - Mouse lymphoma Unact v. 2
  - Chromosome damage
    - In vitro
      - In vitro Chromosome aberration model CHL v. 2
      - In vitro Chromosome aberration model CHO v. 2
    - In vivo
      - In vivo Chrom Ab Comp (A7P) v. 1
      - In vivo Chrom Ab Other (A7R) v. 1
      - In vivo Chrom Ab Rat (A7Q) v. 1
      - In vivo Micronucleus Mouse v. 2
- Toxtree version 3.1.0, using only the models for Cramer class, Ames mutagenicity, and in vivo mouse micronucleus; (non-genotoxic/genotoxic carcinogenicity).

In addition, a read-across analysis for the assessment of structural similarities of the substances was done using the following OECD QSAR Toolbox (version 4) profiles for genotoxicity and functional groups:

- Mechanistic profilers
  - DNA binding by OASIS v. 1.6
  - DNA binding by OECD v. 2.3
  - Protein binding by OASIS v. 1.6
  - Protein binding by OECD v. 2.3
- End-point specific profilers
  - DNA alerts for Ames by OASIS v. 1.7
  - DNA alerts for CA and MNT by OASIS v. 1.2
  - In vitro mutagenicity (Ames test) alerts by ISS* v. 2.4
  - In vivo mutagenicity (Micronucleus) alerts by ISS*
  - Protein binding alerts for Chromosomal aberration by OASIS v. 1.5

* This profiler is based on the Mutagenicity/Carcinogenicity module of the software Toxtree. It works as a decision tree for estimating in vitro (Ames test) mutagenicity, based on a list of 30 structural alerts (SAs). The SAs for mutagenicity are molecular functional groups or substructures known to be linked to the mutagenic activity of chemicals. As one or more SAs embedded in a molecular structure are recognized, the system flags the potential mutagenicity of the chemical. The present list of SAs is a subset of the original Toxtree list, obtained by eliminating the SAs for nongenotoxic carcinogenicity.

The assessments were conducted as three separate analyses. In the first analysis M07, M36, M10, M49, M50, M58, M66 and M67 were investigated, in the second, M07 and M36, and in the third M06 and M77 were the subjects. The first two analyses were combined as one report (Hueser, 2020a), and the third reported separately (Hueser, 2020b). As analytical methods were the same throughout the results of all three are described in the same tables.

The metabolites investigated were: M10 (isoflucypram-lactic acid), M49 (isoflucypram-N-methyl-cyclopropyl-pyrazole-carboxamide), M50 (isoflucypram-N-methyl-pyrazole-carboxylicacid), M58 (isoflucypram-cyclopropyl-pyrazole-carboxamide), M66 (isoflucypram-cyclopropyl-pyrazole-carboxamide-alanine), M67 (isoflucypram-desfluoro-cyclopropyl-pyrazole-carboxamide-alanine) in the first analysis; M07 and M36 in the second analysis; M06 (isoflucypram-desmethyl-propanol; BCS-DC22055) and M77 (isoflucypram-desmethyl-propanol-aldehyde; BCS-DH85957), its processing metabolite, in the third analysis.

Table 29. Genotoxicity predictions of metabolites of isoflucypram applying alerts triggered in Derek, Nexus, Leadscope, TopKat and Tox tree

	Isoflucypram metabolite analyzed										
End-point	**Parent**	**M10**	**M49**	**M50**	**M58**	**M66**	**M67**	**M07**	**M36**	**M06**	**M77**
Prediction											
Derek: Ames mutagenicity	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
Toxtree											
Cramer class	III (high)	III (high)	III (high)	III (high)	III (high)	III (high)	III (high)	III (high)	III (high)	III (high)	III (high)
Ames mutagenicity	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	SA11
In vivo mouse micronucleus	SA34	SA34	NA	NA	NA	NA	NA	NA	NA	SA34	SA11 SA34
Non-genotoxic carcinogenicity	SA31A	SA31A	NA	NA	NA	NA	NA	NR	NR	NR	NR
Genotoxic carcinogenicity	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	SA11
Leadscope Clastogen. in vitro	-	-	-	-	-	-	-	-	-	-	-
ToxKat: Ames mutagenicity	NEG	NEG[b]	NEG[b]	NEG[b]	NEG[b]	NEG	NEG	NEG[b]	NEG[b]	NEG	NEG
Leadscope: clastogenicity in vitro											
Chrom Ab CHL v.2	NEG[a]	POS[a]	POS[a]	POS	POS[a]	NEG	NEG	POS[a]	POS	NEG[a]	NEG[a]
Chrom Ab CHO v.2	NEG[a]	NEG	NEG[a]	NEG[a]	NEG[a]	NEG	NEG	NEG[a]	NEG[a]	NEG[a]	NEG[a]
Leadscope: clastogenicity in vivo											
Chrom Ab Comp v.2 (A7P)	NEG[a]	NEG[a]	NEG[a]	POS	NEG[a]	NEG	NEG	NEG[a]	NEG[a]	NEG[a]	NEG[a]
Chrom Ab Other v.1 (A7R)	NEG[a]	NEG[a]	NEG[a]	POS[a]	NEG[a]	NEG	NEG	NEG[a]	NEG[a]	NEG[a]	NEG[a]
Chrom Ab Rat v.1 (A7Q)	NEG[a]	NEG[a]	NEG[a]	NEG[a]	NEG[a]	NEG	NEG	NEG[a]	NEG[a]	NEG[a]	NEG[a]
Mouse micronucleus v.2	NEG[a]	NEG[a]	POS[a]	NEG	POS[a]	NEG	NEG	NEG[a]	NEG	NEG[a]	NEG[a]
Leadscope: gene mutation											
HGPRT Mut v.1	NEG[a]	NEG[a]	NEG	NEG[a]	NEG	NEG	NEG	NEG[a]	NEG[a]	NEG[a]	NEG[a]
Mouse lymphoma Act v. 2	NEG[a]	NEG[a]	NEG[a]	NEG	NEG[a]	NEG	NEG	NEG[a]	NEG	NEG[a]	NEG[a]
Mouse lymphoma Unact v.2	NEG[a]	NEG[a]	POS[a]	NEG	POS[a]	POS	POS	NEG[a]	NEG	NEG[a]	NEG[a]

	Isoflucypram metabolite analyzed										
End-point	**Parent**	**M10**	**M49**	**M50**	**M58**	**M66**	**M67**	**M07**	**M36**	**M06**	**M77**
Bacterial Mut v.2	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG[a]	NEG	NEG[a]	NEG[a]
E coli – Sal 102 A-T Mut v.2	NEG[a]	NEG[a]	NEG	NEG	NEG	NEG	NEG	NEG[a]	NEG[a]	NEG[a]	NEG[a]
Salmonella Mut v.4	NEG[a]	NEG	NEG[a]	NEG	NEG[a]	NEG	NEG	NEG[a]	NEG	NEG[a]	NEG[a]

Experimental data

	Parent	**No experimental data for these metabolites (only M12)**
Test method	**Result**	
Ames	NEG	
In vitro chrom. aberration	Clastogenic	
In vitro micronucleus test	NEG	
HPRT	NEG	
In vivo micronucleus test	NEG	

NEG: Negative; POS: Positive; NA: Not available - : No alert; Source: Hueser, 2020a, b (for the prediction)

SA34 H-acceptor-path3-H-acceptor; SA11 simple aldehyde (Ames carcinogenicity and genotoxic carcinogenicity);

SA31A Halogenated benzene; HPRT: Hypoxanthine guanine phosphoribosyl transferase;

[a] The evaluated substance is outside of the applicability domain for that particular model;

[b] The substance was partially outside the OPS and or OPS limits model (OPS = optimum prediction space, corresponds to the applicability domain);

Table 30. Read-across by genotoxicity profiling of isoflucypram metabolites according to the OECD QSAR toolbox

Profilers		**Alert**	**Isoflucypram**	**M10**	**M49**	**M50**	**M58**	**M66**	**M67**	**M07**	**M36**	**M06**	**M77**
Protein binding	By OECD	1	**X**	X	X		X	X	X	X	X	X	X
		2		NR	NR	NR	NR	NR	NR	NR	NR		X
	By OASIS	3		NR	NR	NR	NR	NR	NR	NR	NR		X
DNA binding	By OECD	4	**X**	X						X	X	X	X
	By OASIS	5	**X**	X	X	X	X	X	X	X	X	X	
		6	**X**	X	X	X	X	X	X	X	X	X	
		7	**X**	X	X	X	X	X	X	X	X	X	
		8	**X**	X	X	X	X	X	X	X	X	X	
Mutagen alerts by ISS													
	In vitro Ames	9		NR	NR	NR	NR	NR	NR	NR	NR		X
	In vivo MNT	10	**X**	X	X	X	X	X	X	X	X	X	X
		11		NR	NR	NR	NR	NR	NR	NR	NR		X
Carcinogenicity alerts by ISS													
	Nongenotoxic	12	**X**	X						X	X	X	X
	Genotoxic	13		NR	NR	NR	NR	NR	NR	NR	NR		X

MNT: Micronucleus test; NR: Not reported; X: Alert; - : No alert; Source: Hueser, 2020a

Bold **X:** Indicates substances concluded as negative for in vitro Ames mutagenicity and in vivo micronucleus test and carcinogenicity based on experimental data.

Alert no. 1 Acylation; direct acylation involving an acetate leaving group

Alert no. 2 Schiff base-formers; direct acting Schiff base formers, monocarbonyls

Alert no. 3 AN2 Schiff base-formation >> Schiff base formation with carbonyl compounds >> Aldehydes

Alert no. 4	SN1 iminium ion formation, aliphatic tertiary amines
Alert no. 5	AN2 Schiff base formation by aldehydes formed after metabolic activation, geminal polyhaloalkane derivatives
Alert no. 6	6. Radical, Radical mechanism via ROS formation (indirect), Geminal Polyhaloalkane Derivatives
Alert no. 7	SN2 >> Acylation involving a leaving group after metabolic activation Geminal polyhaloalkane derivatives
Alert no. 8	SN2 Nucleophilic substitution at sp^3 carbon atom after thiol (glutathione) conjugation Geminal polyhaloalkane derivatives
Alert no. 9	Simple aldehyde
Alert no. 10	H-acceptor-path3-H-acceptor
Alert no. 11	Simple aldehyde
Alert no. 12	Halogenated benzene (non-genotox),Structural alert for non-genotoxic carcinogenicity
Alert no. 13	Simple aldehyde (alert for genotoxicity)

Due to the available in vivo experimental data for isoflucypram that indicated negative, and the high structural similarity with M10, the negative predictions were considered reliable and there was no concern over chromosome aberration with respect to M10. Most predictions for M49, M50 and M58 were not considered to be reliable (Diot, Heinemann & Shipp, 2018). While some of the metabolites have more functional groups, none of the additional functional groups triggered an alert in Derek or Toxtree for genotoxicity. The influence of structural features on genotoxicity predictions made with the Leadscope models revealed a low concern for genotoxic potential for these metabolites. The high structural similarity to the parent demonstrated with the OECD Toolbox profilers further supported an overall low potential for genotoxicity (Hueser, 2020a).

Metabolite M77 (isoflucypram-desmethyl-propanol-aldehype; BCS-DH85957) is a processing metabolite of the isoflucypram plant metabolite M06 (isoflucypram-desmethyl-propanol; BCS-DC22055). Metabolite M06 is also found in urine of female rat , representing 10% or more of absorbed dose, and in rat plasma as seen from the toxicity study in rats. Based on in silico prediction, structural similarity to isoflucypram and its metabolite M06, and from read across, there is no concern that M06 and M77 possess genotoxic potential (Hueser, 2020b).

Read-across analysis of genotoxicity prediction for metabolites M56, M57, M02, M52 and M54

Read-across analysis for genotoxicity prediction was conducted using OECD QSAR Toolbox (v. 4) for the following metabolites:

- M56 (an unconjugated metabolite in sulfhydryl group of isoflucypram-desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-mercapto-Glyc),
- M57 (isoflucypram-desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-mercapto-Glyc-MA),
- M02 (Isoflucypram-propanol),
- M52 (isoflucypram-desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-OH-Cys) As the chemical structure of M52 is similar to M54 (isoflucypram-desfluoro-N-methyl-cyclopropyl-pyrazole-carboxamide-OH-GSH), M52 was selected for the in silico analysis,
- Isoflucypram, the parent, was also analyzed for comparison.

The unconjugated metabolites M56 and M57 showed no alert, fewer alerts than the parent, or essentially the same alerts as isoflucypram with respect to the specific end-points. This result indicated that M56 and M57 is not likely to be mutagenic.

Metabolite M02 showed no alert or essentially the same alerts as isoflucypram for the specific end-points, therefore M2 was not predicted to be mutagenic.

Metabolite M52 showed no alert or essentially the same alerts as for the parent isoflucypram for most of the specific end-points. However, different types of alerts from isoflucypram were found with the end-point-specific profilers, with microbial metabolism simulator. Based on the in silico analysis and with no further information available, the possibility that M52 and M54 were predicted to have genotoxic potential could not be excluded.

Major rat metabolites

As indicated in Tables 4, 5 and 6 of the ADME studies (Bongartz & Bartelsen, 2017a, b, c) the metabolites identified at levels of around 10% and more of absorbed dose in bile, or 10% or more of TRR in plasma, liver or kidney, were considered as major rat metabolites. If the major metabolite was in a conjugated form, an unconjugated precursor was considered to be a major metabolite. Metabolites that fell into this category were:

- M01 (isoflucypram-propanol)
- M09 (isoflucypram-desmethyl-lactic acid)
- M11 (isoflucypram-desmethyl-carboxylic acid; BSC-CX99799)
- M12 (isoflucypram-carboxylic acid; BCS-CY26497)
- M13 (isoflucypram-desmethyl)
- M19 (isoflucypram-propanol-GlucA, isomer 1)
- M33 (isoflucypram-desmethyl-diOH-GlucA, group of isomers)
- M35 (isoflucypram-desmethl-GlucA)
- M58 (isoflucypram-cyclopropyl-pyrazole carboxamide)

Plasma concentrations of M11 and M58 were measured in the long-term studies in mice (Blanck, 2017), and in rats (Odin, 2018), in the rat reproductive study (Renaut, 2018, 2019), in developmental studies in rats (Kennel, 2017a, b), and in rabbits (Leconte, 2017), and in the one-year dog study (Desmaris, 2017). Throughout these studies, both metabolites were found at very high levels compared with the parent. The Meeting noted a possibility that high concentrations of both metabolites might contribute to the toxicity of isoflucypram. These findings supported the assertion that both M11 and M58 were major metabolites in experimental animals used in the toxicity studies on isoflucypram.

In the rat long-term study, plasma concentrations of M01, M02, M06 and M12 were measured after 24 months, at termination (Odin, 2018; Desmaris, 2017). Plasma levels of these metabolites were lower than those of M11 or M58 and similar to or slightly higher than the parent, which was very low in the plasma due to its rapid elimination. Based on the low plasma concentrations of these metabolites compared with M11 and M58, it was difficult to clarify whether they contribute to the toxicity of isoflucypram. Metabolites M01 and M12 were identified as major metabolites in the ADME study described above. Metabolites M02 and M06 could not be identified as major rat metabolites because of lack of any toxicological information other than in silico predictions (see above).

(g) Toxicity of impurities

The toxicity of the impurity BCS-CN45153 was investigated in a 28-day rat study, a 28-day mouse study, and a modified uterotrophic assay in the immature female rat (see Table 31).

Table 31. Studies conducted on the impurity BCS-CN45153

Study	NOAEL	LOAEL	Effects at LOAEL	Reference
28-day rat study 0, 200, 600, 1800 ppm Dietary study	600 ppm 50 mg/kg bw/day for males; 54 mg/kg bw/day for females	1800 ppm 152 mg/kg bw/day for males; 145 mg/kg bw/day for females	Decreased body weight/ body weight gain; increased liver weight; hyaline droplet accumulation in males; uterine squamous metaplasia	Totis, 2011
28-day mouse study 0, 400, 2000, 4500 ppm Dietary study	400 ppm 64 mg/kg bw/day for males; 73 mg/kg bw/day for females	2000 ppm 337 mg/kg bw/day for males; 377 mg/kg bw/day for females	Increased liver weight; centrilobular hepatocellular hypertrophy	Repetto, 2010
Uterotrophic/vaginal opening assay By oral gavage	150 mg/kg bw/day	450 mg/kg bw/day	Advancement of vaginal opening	Lasserre, 2011

A 28-day dietary toxicity study with BCS-CN45153 (purity 97.6% ; Batch no. NLL 8224-6-4) was conducted in C57BL/6J mice at concentrations of 0, 400, 2000 or 4500 ppm (equal to 0, 64, 337, and 779 mg/kg bw per day for males, 0, 73, 377, and 819 mg/kg bw per day for females). At 4500 ppm, body weight gain was reduced in both sexes. Food intake was not affected by the treatment. Absolute and relative liver weights were increased in both sexes. In biochemistry, total cholesterol was elevated compared with controls in females, and slightly increased ALP activity was seen in males. Microscopically observed hepatocellular hypertrophy was accompanied by single cell necrosis of hepatocytes in males and females. At 2000 ppm there was increased liver weights accompanied by an increase in the incidence of hepatocellular hypertrophy. These changes were not considered adverse but adaptive due to the absence of any direct indicators of hepatotoxicity. A decrease in bilirubin level was considered treatment-related but not adverse, since it is an increase in bilirubin only that is of toxicological significance. No effect was observed at 400 ppm. The results indicated the liver was a target organ of BCS-CN45153.

The NOAEL for short-term toxicity of BCS-CN45153 in mice was 2000 ppm (equal to 337 mg/kg bw per day) based on the depression in body weight gains, liver hypertrophy with single cell necrosis of hepatocytes, and increased cholesterol and ALP at 4500 ppm (equal to 779 mg/kg bw per day) (Repetto, 2010).

A 28-day feeding toxicity study was conducted in rats. BCS-CN45153 (purity, 98.4%; Batch no. NLL8224-10-2) was administered continuously via the diet to groups of Wistar rats (five/sex per group) for at least 28 days at concentrations of 0, 200, 600 or 1800 ppm (equal to 0, 17.3, 50.0 and 152 mg/kg per day in males, 0, 17.6, 54.0 and 145 mg/kg per day in females). All animals were observed for mortality and clinical signs daily, body weight and food consumption were measured weekly. A detailed physical examination was performed weekly throughout the study. Before necropsy a blood sample was collected from the retro-orbital venous plexus of each animal for haematology and clinical chemistry determinations. All animals were necropsied, selected organs weighed and a range of tissues taken, fixed and examined microscopically. The remaining portions of the liver were homogenized for microsomal preparations in order to determine cytochrome P-450 isoenzyme profiles (EROD, PROD, BROD and LAH, lauric acid hydroxylase).

No mortalities, treatment-related clinical signs, nor changes in haematology parameters were observed during the study. Treatment effects were observed at 1800 ppm only. There were no effects on body weight throughout the study, except a slight decrease (by ca 6%) on the first week, corresponding to a reduced body weight gain per day of 26% in males. In females, body weight was reduced (by 7%–12%) throughout the study. This effect was essentially attributable to a body weight loss of 1 g during the first week of the study compared to a gain of 22 g for the controls. Food consumption was not affected by the treatment in males, but in females was decreased (by 13%–35%) throughout the study. Lower mean total bilirubin concentrations observed (by 75%) in females were not considered an adverse effect.

Absolute and relative liver weights were higher in males (ca 20%, statistically significant) and in females (by ca 12%). Minimal centrilobular hypertrophy of hepatocytes was observed in one male only. The low level of liver hypertrophy observed appears to have promoted a small degree of metabolic enzyme induction in the liver: PROD (two-fold increase in males), BROD (three-fold increase in males, four-fold in females) and cytochrome P450 (1.3-fold increase in males and females). Absolute and relative kidney weights were increased (by ca 20%) corresponding an with increased severity of hyaline droplets in 2/5 males, a finding that was not found in females. Although the immunohistochemical staining for the α2μ-globulin antibody was not performed, a most plausible MOA for hyaline droplet formation was considered to be α2μ-globulin, a male rat-specific change. The same male-specific kidney change was observed in rats treated with isoflucypram. In the thyroid, minimal follicular cell hypertrophy was observed in 3/5 males. This change was considered to be a secondary change due to liver enzyme induction, although thyroid hormones (UDP-GT or TSH) were not measured in this study. In the uterus, minimal squamous metaplasia of the endometrial glands was noted in 2/5 females, but the estrous cycle was not modified when compared to the controls.

The NOAEL for BCS-CN45153 from this 28-day rat feeding study was 600 ppm (equal to 50 mg/kg bw per day) based on depressed body weight gain, liver and thyroidal effect in males at 1800 ppm (equal to 152 mg/kg bw per day) (Totis, 2011). The toxicological profiles in the short-term study of BCS-CN45153 were very similar to those of isoflucypram except for the morphological finding observed in the uterus at 1800 ppm.

In a modified immature rat uterotrophic assay, BCS-CN45153 (purity 98.4%; Batch no. NLL 8224-10-2) was administered by oral gavage at doses of either 150 or 450 mg/kg bw per day for up to 20 days. Vaginal opening was recorded from day 10 after the start of dosing. Uterine weight (wet and blotted) was recorded 24 hours after the end of each dosing period (three days and 20 days).

Increased salivation was seen in both treatment groups, while at 450 mg/kg bw per day there was also a decrease in motor activity and lack of grooming. Body weight gains at 150 mg/kg bw per day were lower than those at 450 mg/kg bw per day. Body weight gains at 450 mg/kg bw per day were comparable to those of controls. The treatment did not affect either wet and blotted uterus weights. As 4/6 rats had already shown vaginal opening on the first day of checking (PND 29), the precise day of vaginal opening at 450 mg/kg bw per day could not be determined (Table 32). The study author concluded that BCS-CN45153 interferes with pubertal development in the immature female rat (Lasserre, 2011).

Table 32. Vaginal opening time and body weight in a modified uterotrophic assay of immature rats treated with BCS-CN45153

		Dose, mean ± SD (mg/kg bw per day)		
Parameter		**0**	**150**	**450**
Number of rats		6	6	6
Cumulative body weight gain (g):	day 2	3.7 ± 0.8	3.3 ± 1.2	3.3 ± 1.5
	day 11	48.6 ± 4.4	42.2 ± 4.0*	43.8 ± 3.3
	day 15	73.4 ± 6.0	62.5 ± 5.1*	67.2 ± 6.1
	day 20	102.2 ± 5.7	93.2 ± 7.7	99.4 ± 7.8
Blotted uterine weight:	absolute (g)	0.2439 ± 0.0845	0.1645 ± 0.0494	0.2192 ± 0.564
	relative to body weight (%)	0.1698 ± 0.0531	0.1230 ± 0.0346	0.1609 ± 0.0506
Vaginal opening day:	day 10 (PND28)	0	0	4
	day 13 (PND31)	1	0	4
	day 15 (PND33)	4	2	4
	day 17 (PND35)	5	3	6
	day 19 (PND37)	5	4	6
	day 21 (PND39)	6	6	6
Mean age (day) of vaginal open and SD		33.5 ± 2.6	36.0 ± 2.8	≤30.3 ± 3.6
Mean body weight (g) at vaginal opening and SD		110.0 ± 15.9	11.48 ± 19.1	≤87.9 ± 24.5

SD: Standard deviation; PND: Postnatal day; Source: Lasserre, 2011

Difference from control statistically significant: * $p \leq 0.05$

The morphological change in squamous metaplasia in the uterine endometrium that was observed at 1800 ppm in the 28-day toxicity study, and the early occurrence of vaginal opening in the immature rats are common, recognised changes induced by estrogenic agents. On the other hand, uterotrophic effects, a sensitive indicator for detection of an estrogenic agent, was not found in the 20-day treatment, a longer treatment than a conventional uterotrophic assay with immature rats. No further information was available. The possibility that BCS-CN45153 interferes with pubertal development in the immature female rat was not excluded because the MOA for the occurrence of early vaginal opening was unclear. The Meeting noted rats no effect of the vaginal opening was observed at 150 mg/kg bw group indicating the occurrence showed a dose–response relationship.

(h) Literature review

There was no published literature available on the toxicological evaluation of isoflucypram.

3. Observations in humans

The sponsor provided the information on medical surveillance of manufacturing plant personnel and monitoring studies. There was no specific report related to isoflucypram (Steffens, 2017).

Comments

Biochemical aspects

Isoflucypram was rapidly absorbed in rats when administered orally. Maximum plasma concentration (C_{max}) of radioactivity was reached at one hour post administration after a low single oral dose of 2 mg/kg body weight (bw), and at 2–4 hours with a high single oral dose of 200 mg/kg bw. The dose-normalized plasma C_{max} after administration of the high dose was 3–4 fold lower than that after the low dose indicating reduced absorption at the high dose. Absorption in rats at the low dose was around 80%. Radiolabelled isoflucypram was distributed throughout the rats immediately after dosing, with the highest levels in liver and kidney. No relevant sex-related differences in distribution were observed (Bongartz & Bartelsen, 2017a, b, c; Fernyhough & Kendrick, 2017). Isoflucypram was rapidly eliminated in both sexes (Fernyhough & Kendrick, 2017). Excretion was mainly via faeces (greater than 80%), following excretion in bile (70% or more) (Bongartz & Bartelsen, 2017a, b).

A number of metabolites were identified in samples from male and female rats. No relevant qualitative differences in the metabolite profile between sexes were observed in rats, although some desmethyl metabolites were excreted faster in males than in females (Bongartz & Bartelsen, 2017a, b). For the common metabolites the metabolite pattern was similar in all studies (Bongartz & Bartelsen, 2017a, b, c; Fernyhough & Kendrick, 2017). The most important metabolic reactions of [*pyrazole-4-*^{14}C]isoflucypram were demethylation, hydroxylation, carboxylation and conjugation. Conjugates of isoflucypram-propanol (M01) and isoflucypram-desmethyl (M13) were identified at greater than 10% of absorbed dose. The metabolites identified at greater than 10% total radioactive residue (TRR) in plasma, liver and kidney were isoflucypram-cyclopronyl-pyrazole-carboxamide (M58), isoflucypram-desmethyl-lactic acid (M09), isoflucypram-desmethyl-carboxylic acid (M11) and isoflucypram-carbolyxic acid (M12) (Bongartz & Bartelsen, 2017a, b). Measurements in toxicity studies confirmed that M58 and M11 occurred at high concentrations in plasma.

A comparative metabolism study was performed in vitro using liver microsomes from humans, dogs, rabbits, rats and mice. The metabolite patterns were comparable for human, mouse and rat liver microsome systems, but somewhat different with the dog and rabbit preparations. No unique human metabolite was detected (Lagojda & Doebbe, 2017).

Toxicological data

In rats, isoflucypram had an acute oral median lethal dose (LD_{50}) of greater than 2000 mg/kg bw, an acute dermal LD_{50} of greater than 2000 mg/kg bw and an acute inhalation median lethal concentration (LC_{50}) of 2.518 mg/L (Zelenák, 2014c, d; Váliczkó, 2014). Isoflucypram was non-irritating to the skin and mildly irritating to the eyes in rabbits. It was not skin sensitizing in a mouse local lymph node assay (LLNA) (Hargitai, 2015).

The effects of isoflucypram in short- and long-term studies in mice, rats and dogs included decreased body weight gain, liver hypertrophy and changes related to thyroid hormones.

In a 90-day study, mice received isoflucypram in their diet at concentrations of 0, 100, 300 or 1000 ppm (equal to 0, 17.0, 51.0 and 168 mg/kg bw per day for males, 0, 19.5, 59.8, and 207 mg/kg bw per day for females). The no-observed-adverse-effect level (NOAEL) was 300 ppm (equal to 51.0 mg/kg bw per day) based on a slight decrease in body weight gain, liver weight increase and hepatocellular vacuolation in the centrilobular area at 1000 ppm (equal to 168 mg/kg bw per day) (Blanck, 2013).

In a 90-day study, rats received isoflucypram at dietary concentrations of 0, 100, 300 or 1000 ppm (equal to 0, 6.34, 18.4, 63.5 mg/kg bw per day for males, 0, 7.92, 21.9, 80.9 mg/kg bw per day for females). The NOAEL was 300 ppm (equal to 18.4 mg/kg bw per day) based on a reduction in body weight gains at 1000 ppm (equal to 63.5 mg/kg bw per day) (Odin, 2017).

In a 13-week study, dogs received isoflucypram at dietary concentrations of 0, 170, 500 or 1500 ppm (equal to 0, 5.5, 15.9 and 50.4 mg/kg bw per day for males, 0, 5.5, 16.2 and 54.0 mg/kg bw per day for females). The NOAEL was 500 ppm (equal to 15.9 mg/kg bw per day) based on reduced body weight gain at 1500 ppm (equal to 50.4 mg/kg bw per day) (Kennel, 2015).

In a 52-week study, dogs received isoflucypram at dietary concentrations of 0, 150, 600 or

1800 ppm (equal to 0, 4.2, 18.8 and 60.2 mg/kg bw per day for males, 0, 4.2, 17.6 and 49.8 mg/kg bw per day for females). The NOAEL was 600 ppm (equal to 17.6 mg/kg bw per day) based on reduced body weight gain and liver effects with Kupffer cell pigmentation at 1800 ppm (equal to 49.8 mg/kg bw per day) (Kennel, 2017a).

The overall NOAEL for the 13-week and 52-week toxicity studies in dogs was 600 ppm (equal to 17.6 mg/kg bw per day). The lowest-observed-adverse-effect level (LOAEL) was 1800 ppm (equal to 49.8 mg/kg bw per day).

In an 18-month chronic toxicity and carcinogenicity study, mice received isoflucypram at dietary concentrations of 0, 50, 250 or 1250 ppm (equal to 0, 5.9, 29.0 and 147 mg/kg bw per day for males, 0, 7.8, 38.1 and 190 mg/kg bw per day for females). The NOAEL for chronic toxicity was 250 ppm (equal to 29.0 mg/kg bw per day) based on reduced body weight gain and increased incidence of multinucleated hepatocytes at 1250 ppm (equal to 147 mg/kg bw per day). The NOAEL for carcinogenicity in mice was 1250 ppm (equal to 147 mg/kg bw per day), the highest dose tested (Blanck, 2017).

In a two-year chronic toxicity and carcinogenicity study, rats received isoflucypram at dietary concentrations of 0, 30, 150 or 450 ppm for males (equal to 0, 1.24, 6.27 and 18.6 mg/kg bw per day), and 0, 30, 150 and 800 ppm for females (equal to 0, 1.75, 8.54 and 46.6 mg/kg bw per day). The NOAEL for chronic toxicity was 150 ppm (equal to 6.27 mg/kg bw per day) based on colloid alteration and pigmentation in the follicular epithelium of the thyroid, and increased incidence of hyperplasia in the adrenal medulla at 450 ppm (equal to 18.6 mg/kg bw per day). The NOAEL for carcinogenicity was 450 ppm equal to (18.6 mg/kg bw per day), the highest dose tested (Odin, 2018).

The Meeting concluded that isoflucypram is not carcinogenic in mice or rats.

Isoflucypram was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found (Dony, 2014; Bohnenberger, 2014; Sokolowski, 2014; Wollny, 2014).

The Meeting concluded that isoflucypram is unlikely to be genotoxic.

In view of the lack of genotoxicity and the absence of carcinogenicity in mice and rats, the Meeting concluded that isoflucypram is unlikely to pose a carcinogenic risk to humans.

In a two-generation reproductive study, rats received isoflucypram at dietary concentrations of 0, 150, 450 or 1200 ppm for ten weeks before pairing, throughout pairing and gestation, and at 0, 75, 225 or 600 ppm during the lactation phase. This was to maintain actual intakes equal to 0, 11.3, 34.1 and 92.9 mg/kg bw per day (on the basis of the lowest intake for each group). The NOAEL for parental toxicity was 225 ppm (equal to 34.1 mg/kg bw per day) based on increased cholesterol and increased liver effects at 600 ppm (equal to 92.9 mg/kg bw per day). The NOAEL for effects on offspring was 225 ppm (equal to 34.1 mg/kg bw per day) based on the delay of vaginal opening at 600 ppm (equal to 92.9 mg/kg bw per day). The NOAEL for reproductive toxicity was 600 ppm (equal to 92.9 mg/kg bw per day), the highest dose tested (Renaut, 2018).

In a developmental toxicity study, pregnant rats received isoflucypram by gavage at doses of 0, 25, 125 or 625 mg/kg bw per day from gestation day (GD) 6 to GD 20. The NOAEL for maternal toxicity was 125 mg/kg bw per day based on reduced feed consumption at 625 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 625 mg/kg bw per day, the highest dose tested (Kennel, 2017b).

In a developmental toxicity study, pregnant rabbits received isoflucypram by oral gavage at doses of 0, 10, 70 or 500 mg/kg bw per day from GD 6 to GD 28. The NOAEL for maternal toxicity was 70 mg/kg bw per day based on a slight increase in abortion, slight decrease in body weight and reduced food consumption during the early period of treatment at 500 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 500 mg/kg bw per day, the highest dose tested (Leconte, 2017).

The Meeting concluded that isoflucypram is not teratogenic.

In an acute neurotoxicity study, rats received isoflucypram by oral gavage as a single dose of 0, 200, 600 or 2000 mg/kg bw. The NOAEL was 2000 mg/kg bw, the highest dose tested (Garcin, 2017).

No subchronic neurotoxicity study was conducted. No findings indicative of neurotoxicity were observed in repeat-dose studies in mice, rats or dogs.

The Meeting concluded that isoflucypram is not neurotoxic.

No specific data were submitted regarding immunotoxicity. In routine toxicity studies there was no evidence of effects on the immune system.

The Meeting concluded that isoflucypram is unlikely to be immunotoxic.

Isoflucypram was neither androgenic nor anti-androgenic in a Hershberger assay in rats (Totis, 2012), and neither estrogenic nor anti-estrogenic in a uterotrophic assay using immature rats (Kennel, 2011).

Isoflucypram did not inhibit thyroperoxidase (TPO) activity using rat thyroidal microsomes or sodium/iodide symporter (NIS) activity in a cell line. Isoflucypram had no direct effects on the thyroid (McGinnis, 2020).

Thyroid effects through induction of liver metabolism enzymes were investigated using mice and rats. Isoflucypram induced CAR/PXR-mediated liver hypertrophy, and uridine diphosphate glucuronosyltransferase (UDP-GT), including bilirubin-UDP-GT activity and the expression of UDP-GT1A1 in the liver. Isoflucypram increased thyroid-stimulating hormone (TSH) levels and cell proliferation activity in the thyroid. These effects reversed after withdrawal of the treatment in rats (Rouquié, 2018a, b, c; Totis, 2013). The mode of action (MOA) of the thyroid effect was considered to involve changes in the thyroid gland secondary to the excess elimination of thyroid hormones from the blood circulation due to the induction of UDP-GT in the liver.

Toxicological data on metabolites and/or degradates

The table below shows an overall summary of the toxicological characterization of the metabolites requested for residue definition.

Summary overview of toxicological characterization of plant/livestock metabolites

Compound, codes	Rat ADME Toxicity covered by toxicological properties of parent compound (content in rat biofluids > 10% absorbed dose or 10% TRR)	Genotoxicity assessment (data, QSAR, read-across)	General toxicity	Health-based guidance values (HBGVs)
Isoflucypram (BCS-CN88460)	Parent	Not genotoxic (data)	Full dataset	ADI: 0.06 mg/kg bw/day
M01 Isoflucypram-propanol	Yes Conjugate > 10% in bile	Not genotoxic as covered by parent	Covered by parent	Parent ADI
M02 Isoflucypram-2-propanol	No	Not genotoxic in QSAR analysis	No data	TTC Cramer class III value: 0.0015 mg/kg bw/day
M06 Isoflucypram-desmethyl-propanol	No	Not genotoxic (QSAR and RA)	No data	TTC Cramer class III value: 0.0015 mg/kg bw/day
M11 Isoflucypram-desmethyl-carboxylic acid	Yes > 10% TRR in plasma, liver and kidney in ADME study	Not genotoxic as covered by parent	Covered by parent	Parent ADI
M12 Isoflucypram-carboxylic acid	Yes > 10% TRR in plasma, liver and kidney in ADME study	Not genotoxic as covered by parent	Covered by parent	Parent ADI

Compound, codes	Rat ADME Toxicity covered by toxicological properties of parent compound (content in rat biofluids > 10% absorbed dose or 10% TRR)	Genotoxicity assessment (data, QSAR, read-across)	General toxicity	Health-based guidance values (HBGVs)
M52 Isoflucypram-desfluoro-*N*-methyl-cyclopropyl-pyrazole-carboxamide-OH-Cys	No	Genotoxic alert (QSAR)	No data	Genotoxic TTC value: 0.0025 µg/kg bw/day
M54 Isoflucypram-desfluoro-*N*-methyl-cyclopropyl-pyrazole-carboxamide-OH-GSH	No Structurally similar to M52	Genotoxic alert (QSAR) by read-across from M52	No data	Genotoxic TTC value: 0.0025 µg/kg bw/day
M49 Isoflucypram-*N*-methyl-cyclopropyl-pyrazole-carboxamide	Not found in rats	Not genotoxic (QSAR and RA)	No data	TTC Cramer class III value: 0.0015 mg/kg bw/day
M56 Isoflucypram-desfluoro-*N*-methyl-cyclopropyl-pyrazole-carboxamide-mercapto-Glyc	Not found in rats	Not genotoxic (QSAR and RA)	No data	TTC Cramer class III value: 0.0015 mg/kg bw/day
M57 Isoflucypram-desfluoro-*N*-methyl-cyclopropyl-pyrazole-carboxamide-mercapto-Glyc-MA	Not found in rats	Not genotoxic (QSAR and RA)	No data	TTC Cramer class III value: 0.0015 mg/kg bw/day
M62 Isoflucypram-cyclopropyl-pyrazole-carboxamide-Glyc (isomers 1 and 2)	Not found in rats It is M58-Glyc M58 is a rat major metabolite	Not genotoxic as covered by parent	Covered by parent	Parent ADI
M66 Isoflucypram-cyclopropyl-pyrazole-carboxamide-Ala	Not found in rats It is M58-Ala M58 is a rat major metabolite M58-Ala is considered to convert into M58 upon consumption	Not genotoxic as covered by parent Not genotoxic (QSAR and RA)	Covered by parent	Parent ADI
M67 Isoflucypram-desfluoro-cyclopropyl-pyrazole-carboxamide-Ala	Not found in rats It is desfluorinated M58-Ala M58 is a major metabolite in toxicity studies in mice, rats, rabbits and dogs M58 is found at > 10%TRR in plasma, liver and kidney in ADME study in rats	Not genotoxic as covered by parent	Covered by parent	Parent ADI

Compound, codes	Rat ADME Toxicity covered by toxicological properties of parent compound (content in rat biofluids > 10% absorbed dose or 10% TRR)	Genotoxicity assessment (data, QSAR, read-across)	General toxicity	Health-based guidance values (HBGVs)
M68 Isoflucypram-cyclopropyl-pyrazole-carboxamide-acetic acid	Not found in rats It is M58-acetic acid M58 is a major metabolite in toxicity studies in mice, rats, rabbits and dogs M58 is found at > 10%TRR in plasma, liver and kidney in ADME study in rats	Not genotoxic as covered by parent	Covered by parent	Parent ADI
M69 Isoflucypram-cyclopropyl-pyrazole-carboxamide-OH-lactic acid (isomer 1 and 2)	M58-lactic acid M58 is a major metabolite in toxicity studies in mice, rats, rabbits and dogs M58 is found at > 10%TRR in plasma, liver and kidney in ADME study in rats	Not genotoxic as covered by parent	Covered by parent	Parent ADI

ADI: Acceptable daily intake; RA: read across; TRR: Total radioactive residue;
ADME: Absorption, distribution, metabolism and excretion; QSAR: Quantitative structure–activity relationship

BCS-CN45153, an impurity of isoflucypram

Two 28-day toxicity studies and a rat uterotrophic assay were submitted but did not raise a concern relative to the parent (Lasserre, 2011; Repetto, 2010; Totis, 2011).

Microbiological data

There was no information available in the public domain and no experimental data were submitted which addressed the possible impact of isoflucypram residues on the human intestinal microbiome.

Human data

The sponsor provided information on medical surveillance manufacturing plant personnel and monitoring studies. There was no specific report related to isoflucypram (Steffens, 2017).

The Meeting concluded that the existing database on isoflucypram was adequate to characterize the potential to the general population, including fetuses, infants and children.

Toxicological evaluation

The Meeting established an ADI of 0–0.06 mg/kg bw for isoflucypram based on a NOAEL of 6.27 mg/kg bw per day in the long-term study in rats and applying a safety factor of 100.

The Meeting concluded that it was not necessary to establish an ARfD for isoflucypram in view of its low acute oral toxicity and the absence of any other toxicological effects, including developmental toxicity, that would be likely to be elicited by a single dose.

Levels relevant to risk assessment of isoflucypram

Species	Study	Effect	NOAEL	LOAEL
Mouse	78-week study of toxicity and carcinogenicity [a,]	Toxicity	250 ppm, equal to 29.0 mg/kg bw/day	1250 ppm, equal to 147 mg/kg bw/day
		Carcinogenicity	1250 ppm, equal to 147 mg/kg bw/day [c]	-
Rat	Acute neurotoxicity study [b]	Neurotoxicity	2000 mg/kg bw [c]	-
	90-day toxicity study [a]	Toxicity	300 ppm, equal to 18.4 mg/kg bw/day	1000 ppm, equal to 63.5 mg/kg bw/day
	Two-year studies of toxicity and carcinogenicity [a]	Toxicity	150 ppm, equal to 6.27 mg/kg bw/day	450 ppm, equal to 18.6 mg/kg bw/day
		Carcinogenicity	450 ppm, equal to 18.6 mg/kg bw/day [c]	-
	Two-generation study of reproductive toxicity [a]	Reproductive toxicity	1200/600 ppm, equal to 92.9 mg/kg bw/day [c]	-
		Parental toxicity	450/225 ppm, equal to 34.1 mg/kg bw/day	1200/600 ppm, equal to 92.9 mg/kg bw/day
		Offspring toxicity	450/225 ppm, equal to 34.1 mg/kg bw/day	1200/600 ppm, equal to 92.9 mg/kg bw/day
	Developmental toxicity study [b]	Maternal toxicity	125 mg/kg bw/day	625 mg/kg bw/day
		Embryo/fetal toxicity	625 mg/kg bw/day [c]	-
Rabbit	Developmental toxicity study [b]	Maternal toxicity	70 mg/kg bw/day	500 mg/kg bw/day
		Embryo/fetal toxicity	500 mg/kg bw/day [c]	-
Dog	13-week and one-year studies of toxicity [a, d]	Toxicity	600 ppm, equal to 17.6 mg/kg bw/day	1800 ppm, equal to 49.8 mg/kg bw/day

[a] Dietary administration

[b] Gavage administration

[c] Highest dose tested

[d] Two or more studies combined

Acceptable daily intake(ADI) for isoflucypram *

0–0.06 mg/kg bw

Acute reference dose (ARfD) for isoflucypram,

Not necessary

* Applies to isoflucypram, M01, M11, M12, M62, M66, M67, M68, M69

Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure.

Critical end-points for setting guidance values for exposure to isoflucypram

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid; approximately 80%
Dermal absorption	No data
Distribution	Wide; highest levels in liver and kidney
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	Rapid; nearly complete via urine, bile and faeces within 24 h
Metabolism in animals	Extensive demethylation, conjugation and carboxylation
Toxicologically significant compounds in animals and plants	Isoflucypram, M01, M02, M06, M11, M12, M49, M52, M54, M56, M57, M62, M66, M67, M68, M69
Acute toxicity	
Rat, LD_{50}, oral	> 2000 mg/kg bw
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	2.5 mg/L
Rabbit, dermal irritation	Irritating
Rabbit, ocular irritation	Mildly irritating
Mouse, dermal sensitization	Sensitizing (LLNA)
Short-term studies of toxicity	
Target/critical effect	Body weight
Lowest relevant oral NOAEL	17.6 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	No data.
Lowest relevant inhalation NOAEC	No data.
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Body weight, liver multinucleated cells, hyperplasia adrenals
Lowest relevant NOAEL	6.27 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic
Genotoxicity	Unlikely to be genotoxic
Reproductive toxicity	
Target/critical effect	Liver, increased cholesterol, increased liver weight, delayed vaginal opening
Lowest relevant parental NOAEL	34.1 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	34.1 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	92.9 mg/kg bw per day (rat)
Developmental toxicity	
Target/critical effect	Body weight, food consumption, abortion
Lowest relevant maternal NOAEL	70 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	500 mg/kg bw per day, highest dose tested (rabbit)

Neurotoxicity	
Acute neurotoxicity NOAEL	> 2000 mg/kg bw, highest dose tested (rat)
Subchronic neurotoxicity NOAEL	No specific data but no evidence from routine studies
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity	No evidence from routine studies
Studies on toxicologically relevant metabolites	
Isoflucypram-carboxylic acid (M12 ; BCS-CY26497)	Unlikely to be genotoxic (Ames, chromosome aberration, micronucleus in vivo)
M02, M06, M07, M10, M36, M49, M50, M56, M57, M66, M67	Unlikely to be genotoxic (QSAR)
M52, M54	Genotoxic alert (QSAR)
Microbiological data	No information available
Human data	No clinical cases or poisoning incidents had been recorded

Summary

	Value	Study	Safety factor
ADI	0–0.06 mg/kg bw [a]	Two-year study of toxicity and carcinogenicity (rat)	100
ARfD	Not necessary		

[a] Applies to isoflucypram, M01, M11, M12, , M62, M66, M67, M68 and M69

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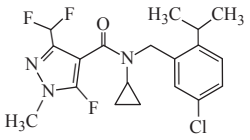
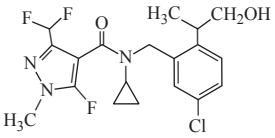
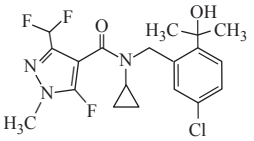
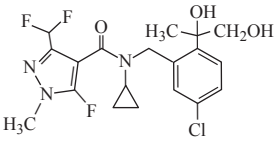
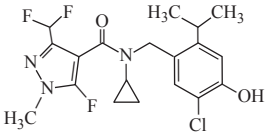
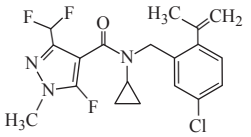
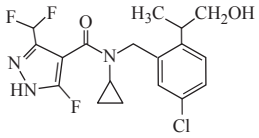
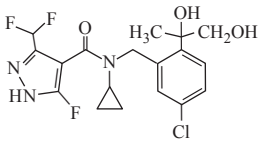
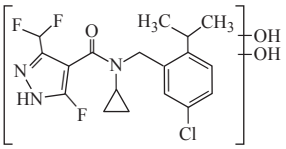
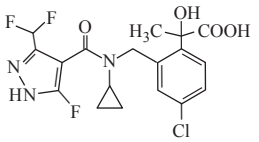
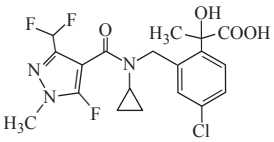
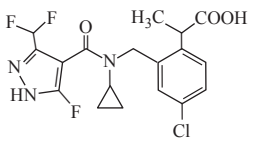
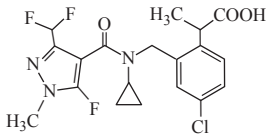
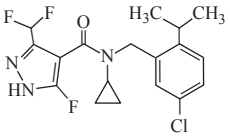
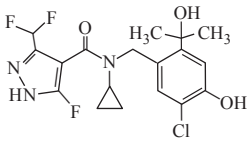
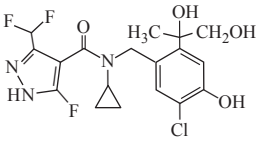
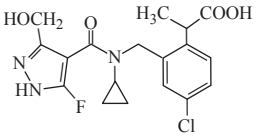
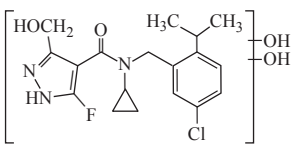
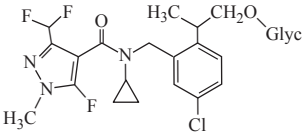
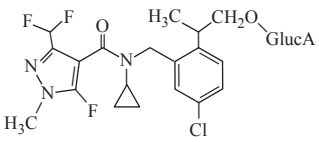
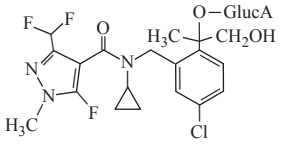
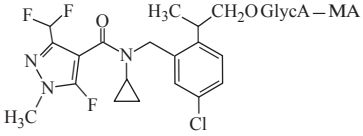
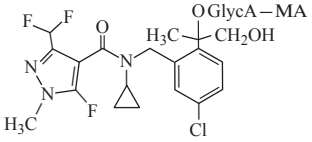
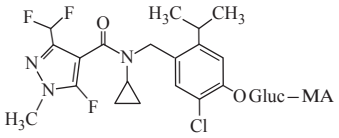
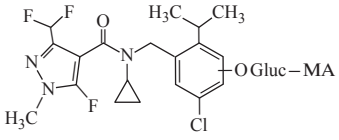
Appendix 1
List of parent compound and metabolites in animals and plants

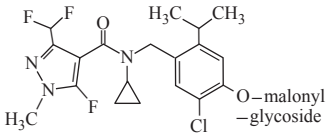
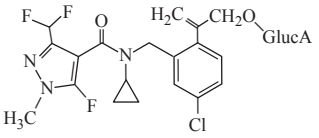
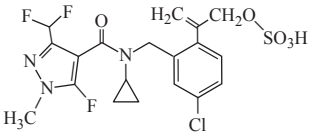
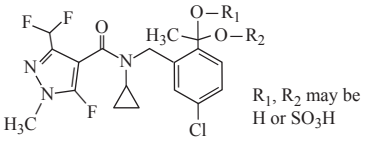
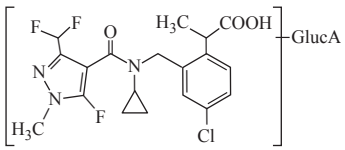
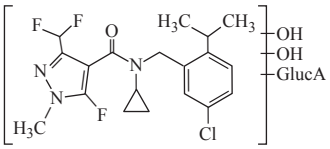
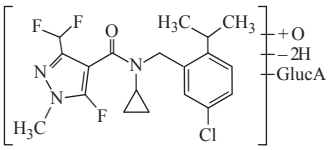
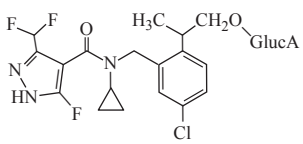
Table 1. The list of parent compound and metabolites in rats and plants

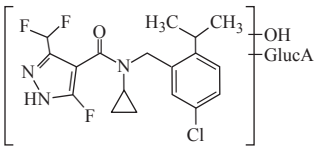
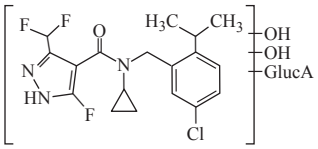
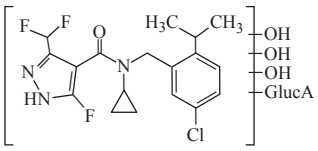
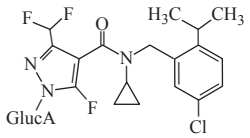
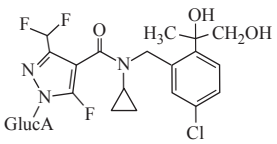
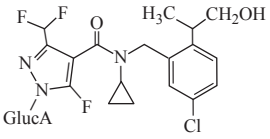
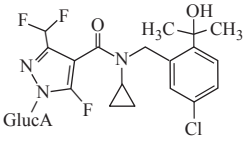
Number Name Identity	Structure	Identified in:
Isoflucypram CAS: 1255734-28-1 *N*-(5-chloro-2-isopropylbenzyl)-*N*-cyclopropyl-3-(difluoromethyl)-5-fluoro-1-methyl-*1H*-pyrazole-4-carboxamide [IUPAC]		Animal: rat (faeces, liver, kidney); hen (eggs, muscle leg, fat); goat (milk, muscle, fat, liver, kidney, faeces); sunfish (edible parts, viscera) Plant: soybean (forage, hay, straw, seed); wheat (hay, straw, grain); CRC (wheat forage, Swiss chard, turnip leaves); oilseed rape (intermediate harvest, mature plants, seeds); tomatoes; potato (tubers, leaves)
M01 Isoflucypram-propanol BCS-CY24813 *N*-[5-chloro-2-(1-hydroxypropan-2-yl) benzyl]-*N*-cyclopropyl-3-(difluoromethyl)-5-fluoro-1-methyl-*1H*-pyrazole-4-carboxamide [IUPAC]		Animal: rat (faeces); hen (eggs, muscle, fat, liver, excreta); goat (muscle, fat, liver, kidney, faeces, urine); sunfish (edible parts, viscera) Plant: wheat (hay, straw)
M02 Isoflucypram-2-propanol BCS-DC20298 *N*-[5-chloro-2-(2-hydroxypropan-2-yl) benzyl]-*N*-cyclopropyl-3-(difluoromethyl)-5-fluoro-1-methyl-*1H*-pyrazole-4-carboxamide [IUPAC]		Animal: rat (faeces); goat (milk, muscle, fat, liver, kidney, faeces, urine)
M03 Isoflucypram-1,2-propandiol *N*-[5-chloro-2-(1,2-dihydroxypropan-2-yl) benzyl]-*N*-cyclopropyl-3-(difluoromethyl)-5-fluoro-1-methyl-*1H*-pyrazole-4-carboxamide [IUPAC]		Animal: hen (excreta)
M04 Isoflucypram-hydroxyphenyl *N*-(5-chloro-4-hydroxy-2-isopropylbenzyl)-*N*-cyclopropyl-3-(difluoromethyl)-5-fluoro-1-methyl-*1H*-pyrazole-4-carboxamide [IUPAC]		Animal: goat (urine)

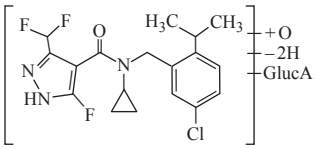
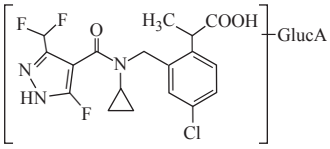
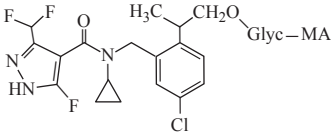
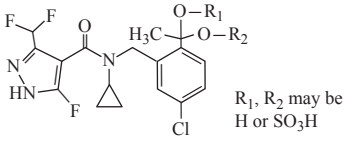
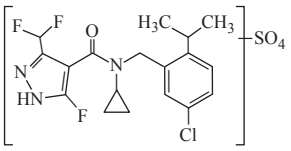
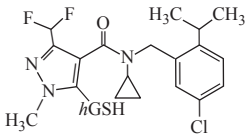
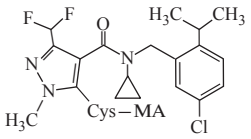
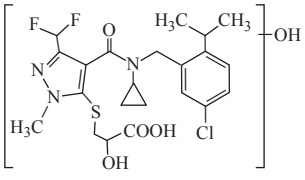
Number Name Identity	Structure	Identified in:
M05 Isoflucypram-olefine *N*-[5-chloro-2-(prop-1-en-2-yl)benzyl]-*N*-cyclopropyl-3-(difluoromethyl)-5-fluoro-1-methyl-*1H*-pyrazole-4-carboxamide [IUPAC]		Animal: rat (faeces, bile)
M06 Isoflucypram-desmethyl-propanol BCS-DC22055 *N*-[5-chloro-2-(1-hydroxypropan-2-yl) benzyl]-*N*-cyclopropyl-3-(difluoromethyl)-5-fluoro-*1H*-pyrazole-4-carboxamide [IUPAC]		Animal: rat (faeces, bile); hen (eggs, muscle, fat, liver, excreta); goat (milk, muscle, fat, liver, kidney, faeces, urine); sunfish (edible parts, viscera) Plant: wheat (straw)
M07 Isoflucypram-desmethyl-1,2-propandiol *N*-[5-chloro-2-(1,2-dihydroxypropan-2-yl) benzyl]-*N*-cyclopropyl-3-(difluoromethyl)-5-fluoro-*1H*-pyrazole-4-carboxamide [IUPAC]		Animal: hen (eggs, muscle, fat, liver, excreta)
M08 Isoflucypram-desmethyl-diOH (isomers)		Animal: rat (faeces)
M09 Isoflucypram-desmethyl-lactic acid 2-{4-chloro-2-[(cyclopropyl{[3-(difluoromethyl)-5-fluoro-*1H*-pyrazol-4-yl] carbonyl}amino)methyl]phenyl}-2-hydroxypropanoic acid [IUPAC]		Animal: rat (faeces, plasma, liver, kidney, bile)
M10 Isoflucypram-lactic acid 2-{4-chloro-2-[(cyclopropyl{[3-(difluoromethyl)-5-fluoro-1-methyl-*1H*-pyrazol-4-yl]carbonyl}amino)methyl] phenyl}-2-hydroxypropanoic acid [IUPAC]		Animal: rat (faeces, plasma, liver, kidney, bile); goat (liver, kidney, faeces, urine) Plant: - Soil: met., aerobic
M11 Isoflucypram-desmethyl-carboxylic acid BCS-CX99799 2-{4-chloro-2-[(cyclopropyl{[3-(difluoromethyl)-5-fluoro-*1H*-pyrazol-4-yl] carbonyl}amino)methyl]phenyl}propanoic acid [IUPAC]		Animal: rat (urine, faeces, plasma, liver, kidney, bile); hen (muscle, fat, liver, excreta); goat (liver, kidney, faeces, urine) Soil: Met., aerobic

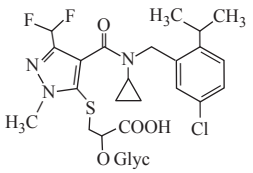
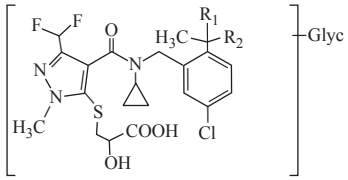
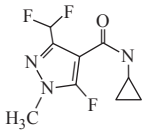
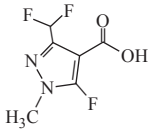
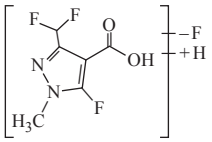
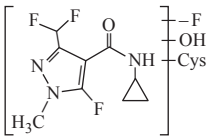
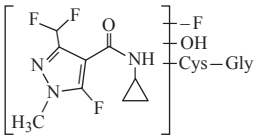
Number Name Identity	Structure	Identified in:
M12 Isoflucypram-carboxylic acid BCS-CY26497 2-{4-chloro-2-[(cyclopropyl{[3-(difluoromethyl)-5-fluoro-1-methyl-*1H*-pyrazol-4-yl]-carbonyl}amino)-methyl]phenyl}-propanoic acid [IUPAC]		Animal: rat (urine, faeces, plasma, liver, kidney, bile); hen (eggs, muscle, fat, liver, excreta); goat (muscle, fat, liver, kidney, faeces, urine) Soil: met., aerobic Water: met., aerobic
M13 Isoflucypram-desmethyl *N*-(5-chloro-2-isopropylbenzyl)-*N*-cyclopropyl-3-(difluoromethyl)-5-fluoro-*1H*-pyrazole-4-carboxamide [IUPAC]		Animal: rat (faeces, plasma, liver, kidney); sunfish (edible parts, viscera)
M14 Isoflucypram-desmethyl-hydroxyphenyl-2-propanol *N*-[5-chloro-4-hydroxy-2-(2-hydroxypropan-2-yl)benzyl]-*N*-cyclopropyl-3-(difluoromethyl)-5-fluoro-*1H*-pyrazole-4-carboxamide [IUPAC]		Animal: rat (urine, faeces, liver, bile)
M15 Isoflucypram-desmethyl-hydroxyphenyl-1,2-propandiol *N*-[5-chloro-2-(1,2-dihydroxypropan-2-yl)-4-hydroxybenzyl]-*N*-cyclopropyl-3-(difluoromethyl)-5-fluoro-*1H*-pyrazole-4-carboxamide [IUPAC]		Animal: rat (faeces)
M16 Isoflucypram-desmethyl-hydroxymethyl-carboxylic acid 2-{4-chloro-2-[(cyclopropyl{[5-fluoro-3-(hydroxymethyl)-*1H*-pyrazol-4-yl]carbonyl}amino)methyl]phenyl}propanoic acid [IUPAC]		Animal: rat (urine, faeces)
M17 Isoflucypram-desmethyl-hydroxymethyl-diOH		Animal: rat (faeces, bile)
M18 Isoflucypram-propanol-Glyc *N*-{5-chloro-2-[1-(hexopyranosyloxy)propan-2-yl]benzyl}-*N*-cyclopropyl-3-(difluoromethyl)-5-fluoro-1-methyl-*1H*-pyrazole-4-carboxamide [IUPAC]		Plant: wheat (hay, straw)

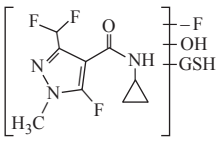
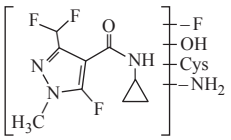
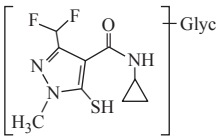
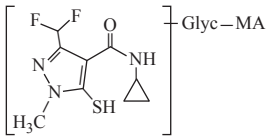
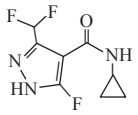
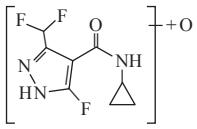
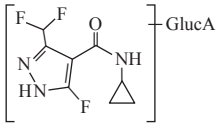
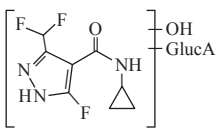
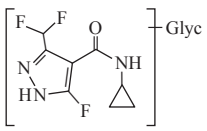
Number Name Identity	Structure	Identified in:
M19 Isoflucypram-propanol-GlucA (isomers 1 and 2) 2-{4-chloro-2-[(cyclopropyl{[3-(difluoromethyl)-5-fluoro-1-methyl-1H-pyrazol-4-yl]carbonyl}amino)methyl]phenyl}propyl glucopyranosiduronic acid [IUPAC]		Animal: rat (faeces, bile); goat (milk, muscle, liver, kidney, faeces, urine); sunfish (edible parts, viscera)
M20 Isoflucypram-2-propanol-GlucA 2-{4-chloro-2-[(cyclopropyl{[3-(difluoromethyl)-5-fluoro-1-methyl-*1H*-pyrazol-4-yl]carbonyl}amino)methyl]phenyl}propan-2-yl beta-D-glucopyranosiduronic acid [IUPAC]		Animal: goat (liver, kidney, faeces, urine)
M21 Isoflucypram-propanol-Glyc-MA 2-{4-chloro-2-[(cyclopropyl{[3-(difluoromethyl)-5-fluoro-1-methyl-*1H*-pyrazol-4-yl]carbonyl}amino)methyl]phenyl}propyl 6-O-(carboxyacetyl) hexopyranoside [IUPAC]		Plant: wheat (hay, straw); oilseed rape (intermediate harvest, mature plants)
M22 Isoflucypram-2-propanol-Glyc-MA 2-{4-chloro-2-[(cyclopropyl{[3-(difluoromethyl)-5-fluoro-1-methyl-1H-pyrazol-4-yl]-carbonyl}amino)-methyl]phenyl}propan-2-yl 6-O-(carboxy-acetyl) hexopyranoside [IUPAC]		Plant: oilseed rape (intermediate harvest, mature plants); potato (leaves)
M23 Isoflucypram-hydroxyphenyl-Gluc-MA 2-chloro-4-[(cyclopropyl{[3-(difluoromethyl)-5-fluoro-1-methyl-*1H*-pyrazol-4-yl]-carbonyl}amino)-methyl]-5-isopropyl-phenyl 6-O-(carboxyacetyl)-beta-D-glucopyranoside [IUPAC]		Plant: oilseed rape (intermediate harvest, mature plants)
M23a Isoflucypram-OH-phenyl-Glyc-MA		Plant: potato (leaves)

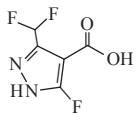
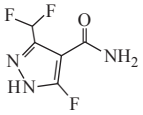
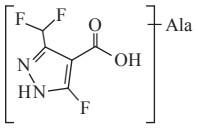
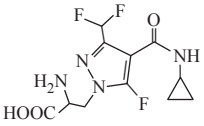
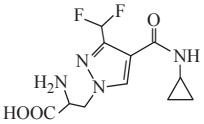
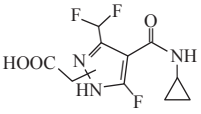
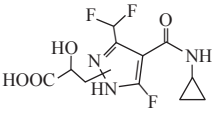
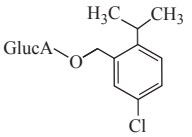
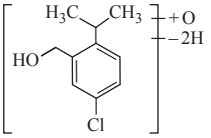
Number Name Identity	Structure	Identified in:
M24 Isoflucypram-hydroxyphenyl-Glyc-MA 2-chloro-4-[(cyclopropyl{[3-(difluoromethyl)-5-fluoro-1-methyl-*1H*-pyrazol-4-yl]-carbonyl}amino)-methyl]-5-isopropyl-phenyl 6-O-(carboxyacetyl)-hexopyranoside [IUPAC]		Plant: oilseed rape (intermediate harvest, mature plants)
M25 Isoflucypram-propenol-GlucA 2-{4-chloro-2-[(cycloprop yl{[3-(difluoromethyl)-5-fluoro-1-methyl-*1H*-pyrazol-4-yl]carbonyl}amino) methyl]phenyl}prop-2-en-1-yl-β*D*-glucopyranosiduronic acid [IUPAC]		Animal: goat (kidney, urine)
M26 Isoflucypram-propanol-SA 2-{4-chloro-2-[(cyclopropyl{[3-(difluoromethyl)-5-fluoro-1-methyl-*1H*-pyrazol-4-yl]carbonyl}amino)methyl] phenyl}propyl hydrogen sulfate [IUPAC]		Animal: hen (liver, excreta)
M27 Isoflucypram-1,2-propandiol-SA		Animal: hen (excreta)
M28 Isoflucypram-carboxylic acid-GlucA		Animal: rat (bile)
M29 Isoflucypram-diOH-GlucA (isomers 1 and 2)		Animal: rat (urine, faeces, bile)
M30 Isoflucypram-oxo-GlucA		Animal: rat (faeces, bile)
M31 Isoflucypram-desmethyl-propanol-GlucA (isomers 1 and 2) 2-{4-chloro-2-[(cyclopropyl{[3-(difluoromethyl)-5-fluoro-*1H*-pyrazol-4-yl] carbonyl}amino)methyl]phenyl}propyl glucopyranosiduronic acid [IUPAC]		Animal: rat (faeces, bile); sunfish (edible parts, viscera)

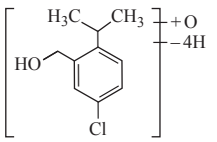
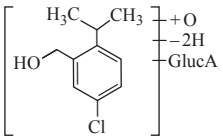
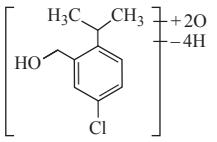
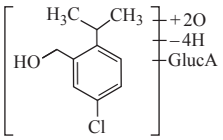
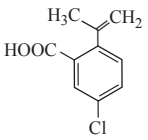
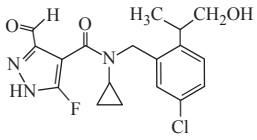
Number Name Identity	Structure	Identified in:
M32 Isoflucypram-desmethyl-OH-GlucA (isomers 1 and 2)		Animal: rat (urine, faeces, bile)
M33 Isoflucypram-desmethyl-diOH-GlucA (isomers 1 to 6)		Animal: rat (urine, faeces, bile)
M34 Isoflucypram-desmethyl-triOH-GlucA		Animal: rat (faeces, bile)
M35 Isoflucypram-desmethyl-GlucA (isomers 1 and 2) *N*-(5-chloro-2-isopropylbenzyl)-*N*-cyclopropyl-3-(difluoromethyl)-5-fluoro-1-(beta-D-glucopyranuronosyl)-*1H*-pyrazole-4-carboxamide [IUPAC]		Animal: rat (bile); sunfish (edible parts, viscera)
M36 Isoflucypram-desmethyl-1,2-propandiol-*N*-GlucA N-[5-chloro-2-(1,2-dihydroxypropan-2-yl) benzyl]-N-cyclopropyl-3-(difluoromethyl)-5-fluoro-1-(glucopyranuronosyl)-1H-pyrazole-4-carboxamide [IUPAC]		Animal: hen (leg muscle, liver, excreta)
M37 Isoflucypram-desmethyl-propanol-N-GlucA *N*-[5-chloro-2-(1-hydroxypropan-2-yl) benzyl]-*N*-cyclopropyl-3-(difluoromethyl)-5-fluoro-1-(glucopyranuronosyl)-*1H*-pyrazole-4-carboxamide [IUPAC]		Animal: hen (eggs, leg muscle, liver, excreta)
M38 Isoflucypram-desmethyl-2-propanol-*N*-GlucA *N*-[5-chloro-2-(2-hydroxypropan-2-yl) benzyl]-*N*-cyclopropyl-3-(difluoromethyl)-5-fluoro-1-(glucopyranuronosyl)-*1H*-pyrazole-4-carboxamide [IUPAC]		Animal: hen (liver, excreta)

Number Name Identity	Structure	Identified in:
M39 Isoflucypram-desmethyl-oxo-GlucA		Animal: rat (bile)
M40 Isoflucypram-desmethyl-carboxylic acid-GlucA (isomers 1 and 2)		Animal: rat (urine, faeces, bile)
M41 Isoflucypram-desmethyl-propanol-Glyc-MA 2-{4-chloro-2-[(cyclopropyl{[3-(difluoromethyl)-5-fluoro-*1H*-pyrazol-4-yl]carbonyl}amino)methyl]phenyl}propyl 6-O-(carboxyacetyl)hexopyranoside [IUPAC]		Plant: wheat (hay, straw)
M42 Isoflucypram-desmethyl-1,2-propandiol-SA		Animal: hen (excreta)
M43 Isoflucypram-desmethyl-SA		Animal: rat (faeces, bile)
M44 Isoflucypram-desfluoro-*homo*GSH gamma-glutamyl-S-{4-[(5-chloro-2-isopropylbenzyl)(cyclopropyl)carbamoyl]-3-(difluoromethyl)-1-methyl-*1H*-pyrazol-5-yl}cysteinyl-beta-alanine [IUPAC]		Plant: soybean (forage, hay, straw)
M45 Isoflucypram-desfluoro-Cys-MA *N*-(carboxyacetyl)-S-{4-[(5-chloro-2-isopropylbenzyl)(cyclopropyl)carbamoyl]-3-(difluoromethyl)-1-methyl-*1H*-pyrazol-5-yl}cysteine [IUPAC]		Plant: soybean (forage, hay, straw)
M46 Isoflucypram-desfluoro-mercapto-lactic acid-OH		Plant: soybean (forage, hay, straw)

Number Name Identity	Structure	Identified in:
M47 Isoflucypram-desfluoro-mercapto-lactic acid-Glyc 3-({4-[(5-chloro-2-isopropylbenzyl)(cyclopropyl)carbamoyl]-3-(difluoromethyl)-1-methyl-*1H*-pyrazol-5-yl}sulfanyl)-2-(hexopyranosyloxy)propanoic acid [IUPAC]		Plant: soybean (forage, hay, straw)
M48 Isoflucypram-desfluoro-mercapto-lactic acid-propyl-OH-Glyc (R_1 and R_2 are an –H and an –OH group)		Plant: soybean (forage, hay, straw)
M49 Isoflucypram-*N*-methyl-cyclopropyl-pyrazole-carboxamide BCS-CR60082 *N*-cyclopropyl-3-(difluoromethyl)-5-fluoro-1-methyl-*1H*-pyrazole-4-carboxamide [IUPAC]		Plant: CRC (wheat forage, wheat hay, wheat straw, Swiss chard immature, Swiss chard at maturity, turnip leaves)
M50 Isoflucypram-*N*-methyl-pyrazole-carboxylic acid BCS-AB72918 BCS-CR73065 CAS: 1255735-09-1 3-(difluoromethyl)-5-fluoro-1-methyl-pyrazole-4-carboxylic acid [IUPAC]		Animal: rat (urine); goat (urine, kidney); sunfish (edible parts, viscera)
M51 Isoflucypram-desfluoro-*N*-methyl-pyrazole-carboxylic acid		Animal: rat (urine)
M52 Isoflucypram-desfluoro-*N*-methyl-cyclopropyl-pyrazole-carboxamide-OH-Cys		Animal: rat (bile) Plant: CRC (wheat forage, wheat hay, wheat straw, Swiss chard immature, Swiss chard at maturity, turnip leaves)
M53 Isoflucypram-desfluoro-*N*-methyl-cyclopropyl-pyrazole-carboxamide-OH-Cys-Gly		Animal: rat (bile)

Number Name Identity	Structure	Identified in:
M54 Isoflucypram-desfluoro-*N*-methyl-cyclopropyl-pyrazole-carboxamide-OH-GSH		Animal: rat (bile); Plant: CRC (wheat forage, wheat hay, wheat straw, Swiss chard immature, Swiss chard at maturity, turnip leaves)
M55 Isoflucypram-desfluoro-*N*-methyl-cyclopropyl-pyrazole-carboxamide-desamino-Cys		Plant: CRC (wheat forage, wheat hay, wheat straw, Swiss chard immature, turnip leaves)
M56 Isoflucypram-desfluoro-*N*-methyl-cyclopropyl-pyrazole-carboxamide-mercapto-Glyc		Plant: CRC (wheat forage, wheat hay, wheat straw, turnip leaves)
M57 Isoflucypram-desfluoro-*N*-methyl-cyclopropyl-pyrazole-carboxamide-mercapto-Glyc-MA		Plant: CRC (wheat forage, wheat hay, wheat straw, turnip leaves)
M58 Isoflucypram-cyclopropyl-pyrazole-carboxamide BCS-CX99798 N-cyclopropyl-3-(difluoromethyl)-5-fluoro-1H-pyrazole-4-carboxamide [IUPAC]		Animal: rat (urine, plasma, liver, kidney); sunfish (edible parts, viscera) Plant: potato (leaves, tubers)
M59 Isoflucypram-cyclopropyl-oxy-pyrazole-carboxamide		Animal: rat (urine)
M60 Isoflucypram-cyclopropyl-pyrazole-carboxamide-GlucA (isomer s1 and 2)		Animal: rat (urine, bile, kidney)
M61 Isoflucypram-cyclopropyl-pyrazole-carboxamide-OH-GlucA		Animal: rat (urine, bile)
M62 Isoflucypram-cyclopropyl-pyrazole-carboxamide-Glyc (isomers 1 and 2)		Plant: CRC (wheat forage, wheat hay, wheat straw, Swiss chard immature, Swiss chard at maturity, turnip leaves)

Number Name Identity	Structure	Identified in:
M63 Isoflucypram-pyrazole-carboxylic acid 3-(difluoromethyl)-5-fluoro-*1H*-pyrazole-4-carboxylic acid [IUPAC]		Animal: rat (urine, faeces, kidney, bile)
M64 Isoflucypram-pyrazole-amide 3-(difluoromethyl)-5-fluoro-*1H*-pyrazole-4-carboxamide [IUPAC]		Animal: rat (urine)
M65 Isoflucypram-pyrazole-carboxylic acid-Ala		Animal: rat (urine, bile)
M66 Isoflucypram-cyclopropyl-pyrazole-carboxamide-Ala 3-[4-(cyclopropylcarbamoyl)-3-(difluoromethyl)-5-fluoro-*1H*-pyrazol-1-yl] alanine [IUPAC]		Plant: CRC (wheat forage, wheat hay, wheat straw, wheat grain, Swiss chard immature, Swiss chard at maturity, turnip leaves)
M67 Isoflucypram-desfluoro-cyclopropyl-pyrazole-carboxamide-Ala 3-[4-(cyclopropylcarbamoyl)-3-(difluoromethyl)-*1H*-pyrazol-1-yl]alanine [IUPAC]		Plant: CRC (wheat forage, wheat hay, wheat straw, wheat grain, Swiss chard immature, Swiss chard at maturity, turnip leaves)
M68 Isoflucypram-cyclopropyl-pyrazole-carboxamide-acetic acid		Plant: CRC (wheat hay, wheat straw)
M69 Isoflucypram-cyclopropyl-pyrazole-carboxamide-OH-lactic acid (isomers 1 and 2)		Plant: CRC (wheat forage, wheat hay, wheat straw, Swiss chard immature, Swiss chard at maturity)
M70 Isoflucypram-benzylalcohol-GlucA 5-chloro-2-*iso*propylbenzyl hexopyranosiduronic acid [IUPAC]		Animal: rat (urine)
M71 Isoflucypram-benzylalcohol-oxo		Animal: rat (urine)

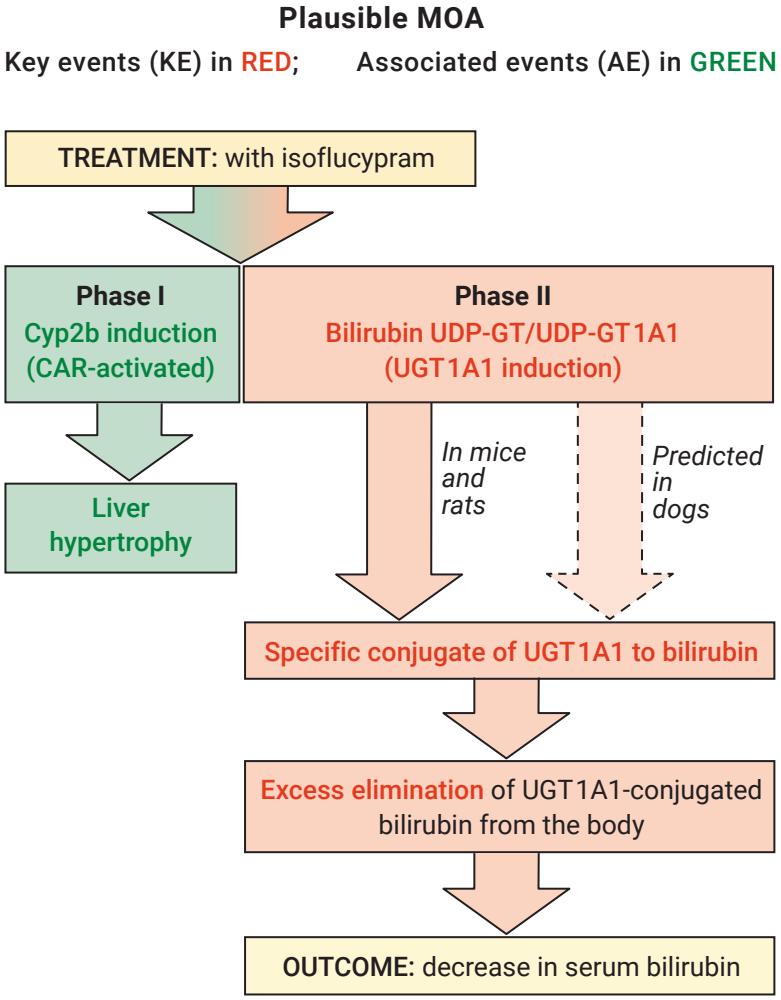
Number Name Identity	Structure	Identified in:
M72 Isoflucypram-benzylalcohol-oxo-desdihydro (isomers 1 and 2)		Animal: rat (urine)
M73 Isoflucypram-benzylalcohol-oxo-GlucA (isomers 1 and 2)		Animal: rat (urine)
M74 Isoflucypram-benzylalcohol-dioxo (isomers 1 and 2)		Animal: rat (urine)
M75 Isoflucypram-benzylalcohol-dioxo-GlucA (isomers 1 and 2)		Animal: rat (urine)
M76 Isoflucypram-phenyl-formyl-olefine CAS: 1006685-15-9 5-chloro-2-(prop-1-en-2-yl)benzaldehyde [IUPAC] benzaldehyde, 5-chloro-2-(1-methylethenyl)- [CA]		Animal: rat (urine)
M77 Isoflucypram-desmethyl-propanol-aldehyde BCS-DH85957 *N*-[5-chloro-2-(1-hydroxypropan-2-yl) benzyl]-*N*-cyclopropyl-5-fluoro-3-formyl- *1H*-pyrazole-4-carboxamide [IUPAC]		Plant: processing (by hydrolysis)

Appendix 2

Postulated MOA for lowered bilirubin in mice, rats and dogs

The decrease in serum bilirubin is a treatment-related change in mice, rats and dogs. Experimental results for these species indicate mice and rats are equally sensitive, and dogs somewhat less so.

Figure 1. Postulated mode of action of lower bilirubin in mice, rats and dogs



Adversity of decrease in serum bilirubin

In animals

An increase in serum bilirubin is an indicator of adverse effects on the hepatobiliary system, or of haemolysis. The decrease itself has been accepted as being of low toxicological significance.

In humans

The decrease is generally of low diagnostic significance, it may have arisen due to serious anaemia, except when haemolytically mediated.

Conclusions on decreased bilirubin in response to isoflucypram

The decrease in this case is treatment-related, but is not adverse unless related toxicity is found in the liver or haematopoietic system.

Methidathion

First draft prepared by
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Explanation

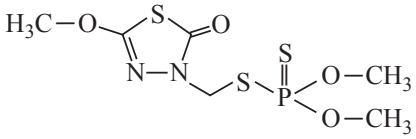
Methidathion is the International Organization for Standardization (ISO)–approved common name for *S*-2,3-dihydro-5-methoxy-2-oxo-1,3,4-thiadiazol-3-ylmethyl-*O,O*-dimethyl phosphorodithioate, with the Chemical Abstracts Service number 950-37-8. Methidathion is a broad-spectrum organophosphate insecticide that acts by inhibiting the activity of acetylcholinesterase (AChE).

The 1992 FAO/WHO Joint Meeting on Pesticide Residues (JMPR, 1993) established an acceptable daily intake (ADI) of 0–0.001 mg/kg body weight (bw) for methidathion on the basis of an overall no-observed-adverse-effect level (NOAEL) of 0.1 mg/kg bw per day in 90-day, one-year and two-year studies in dogs. A 100-fold safety factor was applied. At the 1997 Meeting, an acute reference dose (ARfD) of 0.01 mg/kg bw was established on the basis of a NOAEL of 0.1 mg/kg bw per day, the highest dose tested in a six-week study in humans, and a NOAEL of 1 mg/kg bw in an acute neurotoxicity study in rats. Safety factors were applied: 10 for the volunteer study and 100 for the acute neurotoxicity study in rats.

The evaluation by the present Meeting was conducted for the periodic review programme following a request from the Codex Committee on Pesticide Residues (CCPR). Several toxicity studies were newly submitted to the present Meeting. These had been conducted in compliance with good laboratory practice (GLP) and in accordance with relevant national or international test guidelines, unless otherwise specified.

In a literature search via PubMed Central some new information on acute toxicity and genotoxicity was identified and included in the current toxicological evaluation. There was no new information available on effects in humans, including epidemiology.

Figure 1. Chemical structure of methidathion



Evaluation for acceptable intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

(a) Oral route

Three studies were conducted into absorption, distribution and excretion using rats. In one of these studies a kinetics investigation was performed.

For the current evaluation a study was newly submitted which investigated absorption, distribution and kinetics of methidathion in rats. Carbonyl-^{14}C radiolabelled methidathion (purity 98% or greater; batch C1-XXV-85-1; specific activity 2.07 MBq/mg, equal to 55.95 µCi/mg) was administered to male and female Sprague Dawley rats by gavage. Single oral doses of the radiolabelled methidathion were administered at 0.25 mg/kg bw as a low dose, and 2.5 mg/kg bw as a high dose. Blood was withdrawn from three rats per group of each sex from the low and high dose groups at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 48 and 72 hours after administration. Twelve male rats per group were used to examine selected tissues: heart, lung, liver, kidney, spleen, testes, gastrointestinal (GI) tract with contents, brain, abdominal fat, skeletal muscle, bone, plasma, red blood cells and residual carcass. For the low dose group sampling was carried out at the following times after administration: four hours, the point at which maximum concentration was reached (T_{max}), 12 hours, the point at which half the maximum concentration was reached ($T_{½max}$), 24 hours and 72 hours (termination). For the high dose group sampling was at two hours (T_{max}), 10 hours ($T_{½max}$), 24 hours and 96 hours (termination). A solution of ethanol, polyethylene glycol (PEG) 200 and water in a ratio of 1.5 : 1 : 7.5 by volume was used as a vehicle.

The kinetics properties of methidathion in rats are summarized in Table 1. The time to reach maximum concentration of radioactivity in whole blood was between two and six hours after administration. Clearance of radioactivity from whole blood occurred in two phases, with rapid clearance at 0–24 hours post dose, followed by a slow elimination phase between 48 hours and termination. Assuming first order kinetics, half-lives of depuration of radioactivity from whole blood over the period 0–24 hours were 8.7 and 8.3 hours for males and females respectively following the low dose, and 7.3 and 7.8 hours for males and females respectively following the high dose. Terminal half-lives of elimination following the high dose were 152.6 and 203.7 hours in males and females respectively. The mean values for the area under the concentration–time curve from 0 to 72 hours after dosing ($AUC_{0–72}$) were calculated as 3.937 and 3.976 ppm in males and females respectively at the low dose, and 33.724 and 36.352 ppm in males and females respectively at the high dose.

Table 1. Kinetics of methidathion in rats after a single oral dose

	0.25 mg/kg bw		2.5 mg/kg bw	
	Males	**Females**	**Males**	**Females**
C_{max} (ppm); mean ± SD	0.200 ± 0.017a	0.249 ± 0.019	2.104 ± 0.373	1.540 ± 0.141
T_{max} (hours)	4	2	2	6
$T_{½}$ (hours)	-	-	-	
4–24 hours; mean ± SD	8.7 ± 1.44	8.3 ± 0.4	7.3 ± 0.9	7.8 ± 0.4
AUC (ppm)				
0–72 hours; mean ± SD	3.937 ± 0.577	3.976 ± 0.151	33.724 ± 1,612	36.352 ± 1.755
0–168 hours; mean ± SD	-	-	44.826 ± 1.456	48.857 ± 3.700

AUC: Area under the concentration–time curve; SD: Standard deviation Source: Dunsire, 1994

Distribution in the tissues is shown in Table 2. The highest levels of total radioactivity in tissues following single low and high dose administrations occurred at T_{max} in the liver and kidney. Elimination of radioactivity from whole blood and tissues was biphasic: a rapid elimination phase up to 24 hours was followed by slower elimination from 24 hours and termination at 72 hours (low dose) or 96 hours (high dose). Radioactivity was eliminated much more slowly from red blood cells than from plasma during the second elimination phase. Levels of total radioactivity in testes, brain, fat, skeletal muscle and bone were comparable to those in plasma at termination. Levels in heart, lungs, liver, kidney and spleen were higher than those in plasma. This result may reflect the highly perfused nature of these tissues. Levels of total radioactivity in kidney between 24 hours post dose and termination changed very little or not at all, indicating the possible retention of methidathion and/or its metabolites.

There was evidence of selective association of radioactivity with red blood cells. Depletion was generally rapid, with terminal half-lives of elimination in most tissues in the range 13 to 39 hours. Elimination from blood and also from the highly perfused organs, liver and lung, was slower. There was evidence of retention of methidathion-related residues in the blood cells and kidney, which resulted in a change in the pattern of tissue distribution with time.

Table 2. Tissue residue levels in male rats (% of dose) and residual half-life (hours)

	Single dose level											
	0.25 mg/kg bw						2.5 mg/kg bw					
	Time post dose (hours)				$T_{½}$ of radio-activity over		Time post dose (hours)				$T_{½}$ of radio-activity over	
	4	**12**	**24**	**72**			**2**	**10**	**24**	**96**		
					4–24 h	**24–72 h**					**4–24 h**	**24–96 h**
Heart	0.30	0.14	0.05	0.02	8	29	0.30	0.16	0.05	0.01	9	39
Lungs	0.40	0.17	0.12	0.06	11	39	0.43	0.26	0.10	0.04	11	52
Liver	3.67	2.96	2.05	0.97	15	44	3.94	3.08	2.01	0.74	15	55
Kidney	1.06	0.46	0.20	0.21	8	424	1.07	0.56	0.19	0.19	9	NC

	Single dose level											
	0.25 mg/kg bw						2.5 mg/kg bw					
	Time post dose (hours)				$T_{½}$ of radio-activity over		Time post dose (hours)				$T_{½}$ of radio-activity over	
	4	12	24	72	4–24 h	24–72 h	2	10	24	96	4–24 h	24–96 h
Spleen	0.20	0.10	0.03	0.01	8	25	0.20	0.09	0.03	0.01	9	36
Testes	0.77	0.34	0.10	0.02	6	18	0.67	0.48	0.10	0.01	8	24
GIT	14.20	4.93	1.45	0.26	NA	NA	28.80	5.73	1.42	0.21	NA	NA
Brain	0.55	0.24	0.06	0.01	6	18	0.51	0.32	0.07	0.01	7	25
Fat[a#]	6.11	2.63	1.05	0.33	8	21	6.96	3.65	1.11	0.27	8	32
SM[#]	28.29	12.53	3.52	0.62	7	18	26.44	15.93	3.36	0.52	7	24
Bone[#]	3.30	1.67	0.60	0.20	8	28	2.61	2.17	0.50	0.14	9	35
Plasma[#]	2.88	1.36	0.45	0.04	7	12	3.01	1.76	0.45	0.06	8	22
BC[#]	1.75	1.01	0.51	0.29	10	44	1.71	1.25	0.46	0.26	11	68
Carcass	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Total	49.28	23.61	8.73	2.78	NA	NA	47.93	29.72	8.43	2.22	NA	NA

[a] abdominal fat; NA: Not applicable; GIT: Gastrointestinal tract; Source: Dunsire, 1994

SM: Skeletal muscle; BC: Blood cells; NC: Kidney value was not suitable for calculating $T_{½}$

#: Percentage dose values were estimates based on total tissue weights of muscle, fat, bone and blood cells representing 43%, 11%, 11%, 3.6% and 2.4% of body weight respectively

In conclusion, the kinetics results indicated that methidathion was rapidly absorbed, and reached maximum concentration (C_{max}) at two to four hours after a low dose, and two to six hours after a high dose. No clear differences related to sex or dose level were observed. After its absorption, methidathion was rapidly circulated systemically, and was distributed widely throughout the body, and particularly high levels were seen in the liver and kidney at T_{max}. The pattern of distribution changed with time due to the retention of methidathion-related residues in the kidney and in blood cells. Elimination from the blood and tissues was biphasic, with a rapid initial rate followed by a slower terminal phase (Dunsire, 1994).

Another study (Simoneaux, 1987a) was conducted in rats to compare the elimination and tissue distribution of radiolabel with respect to sex, dose rate and pretreatment regimen. [*Carbonyl-^{14}C*]methidathion (purity 99%; specific activity 47.6 µCi/mg; lot No. not reported) was administered to three groups of five male and five female Charles River CD rats by gavage as a 3% corn starch suspension, using 0.5% polysorbate-80 as a vehicle. The first and second groups were given a single dose at a rate of either 0.314 mg/kg bw or 2.985 mg/kg bw respectively. The third group was preconditioned for 14 days with unlabelled methidathion (purity and lot No. not reported) at a rate of 0.25 mg/kg bw per day. At the end of this pretreatment period, 0.309 mg/kg bw ^{14}C-labelled methidathion was administered orally. Individual samples of urine and faeces were collected for all animals at 4, 8, 12, 24, 36, 48, 72, 96, 120, 144 and 168 hours after dosing. All rats were euthanized at the end of seven days and the distribution of ^{14}C radiolabel was determined in selected tissues including heart, lung, spleen, kidney, liver, fat, gonads, uterus, muscle, brain, bone and carcass. Samples of cage wash were collected on the day of termination. This study followed the USEPA guideline (U.S. EPA, 1982). No information concerning GLP was obtained. Data for elimination via carbon-dioxide were obtained from a related study (Simoneaux, 1987b). Data from both studies on rats, for radioactive material from methidathion in tissues, urine, faeces, cage wash and expired carbon-dioxide are summarized in Table 3.

Regarding tissue distribution at necropsy, the residue levels in the tissues were dose-related. The total mean radioactivity in the tissues ranged from 0.53 ± 0.09% of administered dose (AD) for high-dose females to 1.14 ± 0.12% AD for low-dose males. The tissues receiving the highest proportion of distribution were liver and bone. Values in the liver, ranged from 0.05 ± 0.03 % for the low-dose females to 0.08 ± 0.02% for low-dose males. There were no apparent differences in the distribution pattern or tissue concentrations between males and females, or between the single- and repeat-dose animals.

Table 3. Radioactive material from methidathion in tissue, urine, faeces, cage wash and expired carbon-dioxide in rats (mean ± standard deviation as percentage of administered dose)

	Dose (mg/kg bw)					
	0.25; single dose		0.25; for 14 days		2.5; single dose	
	Male	Female	Male	Female	Male	Female
Tissues	1.14 ± 0.12	0.72 ± 0.08	0.84± 0.23	0.66 ± 0.18	0.78 ± 0.07	0.53 ± 0.09
Urine	30.28 ±2.10a	37.09 ± 6.74	35.26± 3.46	36.91± 4.81	41.84 ± 3.50	57.00 ± 7.46
Faeces	2.32 ± 0.49	2.24 ± 0.74	2.47 ± 0.47	2.28± 0.73	2.60± 0.52	2.33 ± 0.43
Cage wash	0.21 ±0.14	0.32 ± 0.17	0.23 ± 0.17	0.42 ± 0.39	0.32 ± 0.17	0.83± 0.66
CO_2 [a]	41.18	43.50	41.18 [b]	43.50 [b]	34.39	32.22
Total	75.13	83.87	79.98	83.77	79.93	92.91

[a] data from carbon-dioxide exhalation assay; Source: Simoneaux, 1987a, b

[b] data from the same data as single dose

The results of this study indicated that the urine was the preferred route of elimination for methidathion, followed by respiratory carbon-dioxide, regardless of sex or dose rate. The half-life of elimination by both renal and pulmonary routes was approximately eight hours. Renal and pulmonary (that is, as carbon-dioxide) excretions were consistent with one-compartment open linear model and exhibit first-order kinetics at both dose levels (Simoneaux, 1987a).

A related study (Simoneaux, 1987b) was conducted to determine to the extent of carbon-dioxide evolution and thus support the tissue distribution and elimination study described above (Simoneaux, 1987a), also to determine the route of elimination in rats based on sex and dose rate. Male and female Sprague Dawley rats (two rats per group for each sex) were administered by gavage [*carbonyl-^{14}C*]methidathion (purity 99% or greater; specific activity 10.0 µCi/mg; batch No. not reported) as a single dose of 0.295 or 2.949 mg/kg bw. Urine, faeces and volatiles were collected daily; expired carbon-dioxide at 4,8,12, 24, 36, 48, 72 and 96 hours post dose. At 168 hours all surviving rats were terminated. No blood or tissues were taken. Both blood and tissues were included in the carcass. Faeces and carcass were frozen in liquid nitrogen and then homogenized. Eight aliquots from the carcass homogenate and two aliquots from the faecal homogenate were analysed to determine the representative radioactivity. No information on GLP was provided.

The accidental death of one high-dose male was found on the third day.

The distribution of radioactivity is summarized in Table 4. The level of radioactivity in the expired carbon-dioxide at the low dose was 41.2–43.5% compared with 32.2–34.4% at the high dose. No sex-related difference was found. This study indicated that the elimination of the radioactivity began via urine and was followed by expiration in carbon-dioxide. The data confirmed the fragmentation of the thiadiazole ring, which started within four hours of dosing (Simoneaux, 1987b).

Table 4. The radioactivity of ^{14}C-methidathion exhaled by rats in carbon-dioxide (% AD)

	0.295 mg/kg bw (single dose)		2.949 mg/kg bw (single dose)	
	Female average	Male average	Female average	Male average [a]
Time post dose				
4 hours	6.77[b]	7.45	3.27	6.38
8 hours	7.97	8.44	3.34	6.05
12 hours	7.08	7.34	4.10	6.02
24 hours	14.95	11.11	11.69	10.38
36 hours	4.61	3.94	6.21	3.75
48 hours	1.64	1.21	2.50	1.27
72 hours	0.48	0.50	1.11	0.54

	0.295 mg/kg bw (single dose)		2.949 mg/kg bw (single dose)	
	Female average	Male average	Female average	Male average[a]
Time post dose				
96 hours[a]	-[c]	1.19	-[c]	-[c]
120 hours	-[c]	-[c]	-[c]	-[c]
144 hours	-[c]	-[c]	-[c]	-[c]
168 hours	-[c]	-[c]	-[c]	-[c]
Total[b]	43.50	41.18	32.22	34.39

AD: Administered dose Source: Simoneaux, 1987b

[a] One rat died between 72 and 96 hours; [b] Total values are the same as those for carbon-dioxide in Table 3;
[c] Limits of detection and quantitation were obtained as described in analytical method AG-276

1.2 Biotransformation

Information on biotransformation was available from two studies. One of the studies is a published article, written by researchers of the developer of methidathion, in which a metabolic pathway in rats was proposed (Dupuis, Muecke & Esser, 1971). The second study was performed to characterize metabolites in rat urine (Simoneaux, 1987c), and used the same samples as the related study described above (Simoneaux, 1987b). As the information from both studies was limited, available data including information on urinary and pulmonary elimination was integrated when considering the metabolism pathways in rats.

In the published article (Dupuis, Muecke & Esser, 1971), rats were administered 0.17 mg of ^{14}C-carbonyl-labelled methidathion in 0.5 mL of water:ethanol mix (4 : 1). Neither purity nor batch No. were reported, and dosing, by gavage and intravenously, was continued for 10 days. Urine and faeces of male rats were sequentially collected up to 96 hours post dose to analyze for metabolites. To identify metabolites, the following compounds were synthesized:

- methidathion (code GS-13005),
- the oxygen analogue of methidathion (code GS-13007),
- RH (2,3-dihydro-5-methoxy-1,3,4-thiadiazol-2-one (code GS-12956),
- RH-sulfide (code GS-28368),
- RH-sulfoxide (code GS-28370),
- RH-sulfone (code GS-28369).

In vitro experiments were also conducted to investigate the molecular basis of the degradation reaction, especially the origin of the methyl group.

Results indicated that methidathion was rapidly metabolized, and its metabolites were eliminated with the expired air and in the urine as the main routes of excretion. Carbon-dioxide was considered the final product of oxidation for all fragments of the molecule which may originate during metabolic cleavage. Carbon-dioxide was the main metabolite (up to 36% of AD), followed by RH-sulfoxide (up to 25% of AD) and RH-sulfone (up to 7% of AD). The intermediate methyl-thiomethyl derivative (RH-sulfide) did not appear in the urine in significant amounts.

In the in vitro study incubation was with [^{14}CO]methidathion (^{14}CO, 1.995 µCi/µmol), in a 10 000 g supernatant of male Wistar WU rat liver homogenate at 37°C under a nitrogen or air atmosphere for one hour. Liberated ^{14}C-carbon-dioxide was in the range 0.005 µmol/g liver (wet weight) per hour: this corresponded to up to 3% of the total dose from incubation. Higher recoveries of ^{14}C-carbon-dioxide were obtained when nitrogen was substituted for air. In identical experiments under air using [$^{14}CH_3$]*L*-methionine (56.8 µCi/µmol) and nonlabelled methidathion, small amounts [^{14}C] RH-sulfone and in some cases traces of [^{14}C] RH-sulfoxide were isolated. These results suggested that during metabolism the hydrolysis of the thiol bond of the phosphorus ester is followed by a methylation of the thiol group and a subsequent oxidation of the sulfur atom. (Dupuis, Muecke & Esser, 1971).

Another study investigated the metabolites of methidathion as a function of the position of ^{14}C labelling of the carbonyl group. Since for this study the materials (and methods, in part) were the same as the study described above, for details of dose preparation, animals, dosing and sample collection refer to the study of Simoneaux, (1987b). Urine was obtained from Sprague Dawley rats (one male and one female) administered by gavage [*carbonyl-^{14}C*]methidathion (specific activity 10.0 µCi/mg) as a single dose of 2.949 mg/kg bw. This was analyzed using two-dimensional thin layer chromatography (TLC) and anion exchange column chromatography: when analysing aqueous soluble metabolites urine from the male only was used.

The results are summarized in Tables 5a and 5b. In analysis by two-dimensional TLC and anion exchange column chromatography, the urine the urine was shown to consist of mainly organic soluble metabolites which accounted for 79.0% and 66.0% of the administered radioactivity in the male and female, respectively. The remaining radioactivity (21% and 34%) was considered aqueous soluble metabolites. The organic phase was separated into five zones: RH-sulfide, RH-sulfoxide, RH-sulfone and RH derivatives of methidathion, along with unaltered methidathion. The oxygen analogue was not detected. The most abundant metabolite was the sulfide derivative, which contributed 44.0–45.0% of radioactivity in urine regardless of sex. Second most abundant were the sulfone and sulfoxide derivatives. More of these two metabolites was found in males than in females. Radioactivity from the RH compound and unchanged methidathion was very low. The oxygen analogue of methidathion was not detected. On the basis of the elution pattern of the cysteine conjugate and the desmonomethyl derivative of methidathion from the same anion exchange column, the aqueous portion of peak 2 (2%) should be the cysteine conjugate and the aqueous portion of peak 7 (14.0%) should be the desmonomethyl derivative of methidathion. Peaks 3, 4, 5, representing 5.0% in combination of the radioactivity in urine, were not further characterized. (Simoneaux, 1987c)

Table 5a. Organic soluble metabolites in urine of rat (% of radioactivity in urine)

		Male[a]	Female[a]	Average
Sulfoxide,	(GS-28370)	3.3	11.1	7.2
Oxygen analogue,	(GS-13007)	ND	ND	ND
Sulfone,	(GS-28369)	8.0	14.2	11.1
RH compound,	(GS-12956)	1.8	2.9	2.4
Methidathion,	(GS-13005)	ND	0.7	0.7
Sulfide,	(GS-28368)	44.6	45.2	44.9
Characterized		57.7	74.1	65.9
Uncharacterized		8.3	4.9	6.6
Total		66.0	79.0	72.5

ND: Not detected; Source: Simoneaux, 1987c

[a] data from two males and two females at single high dose

Table 5b. Aqueous soluble metabolites in urine of rat (% of radioactivity in urine)

	Whole urine[a]	Aqueous soluble[a]	Organic soluble[a]
Peak 1	ND	ND	ND
Peak 2 (Methidathion cysteine conjugate)	68.0	2.0	66.0
Peak 3	1.5	1.0	0.5
Peak 4	4.7	2.9	1.8
Peak 5	1.6	1.1	0.5
Peak 6	ND	ND	ND
Peak 7 (desmonomethyl-methidathion, desmethyl-methidathion)	24.2	14.0	10.2
Total	**100**	**21.0**	**79.0**

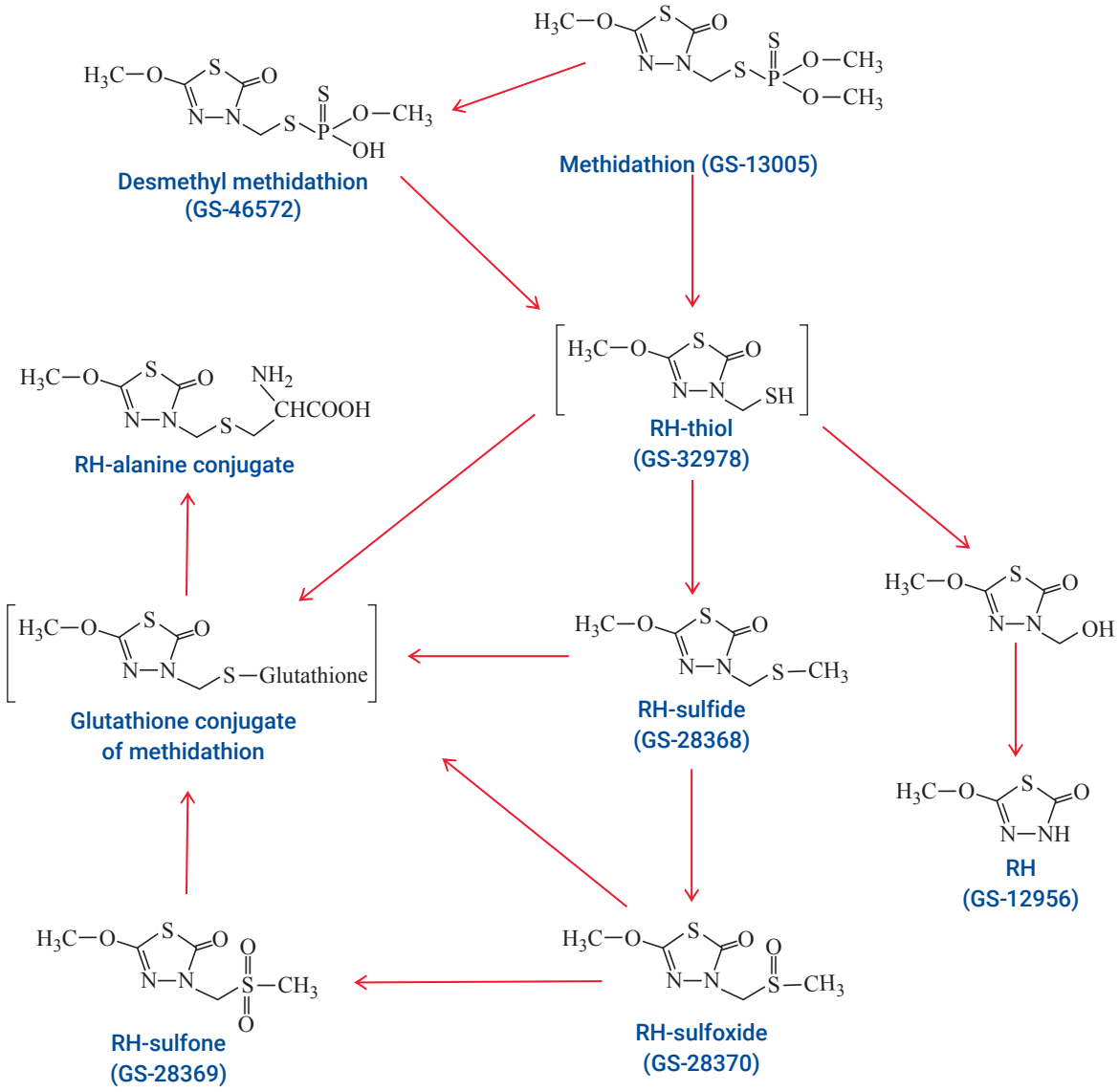
ND: Not detected Source: Simoneaux, 1987c

[a] Data from one male and one female at single high dose;

A more toxic metabolite than methidathion, the oxygen analogue of methidathion, was not identified in rats from the data submitted to the present Meeting by the sponsor. The Meeting, however, noted that very low levels (0.2% and 0.6%) of the oxygen analogue were identified in rat urine in two metabolism studies in JMPR's previous 1992 evaluation of methidathion (JMPR, 1993). There is no reason to believe that the oxygen analogue does not follow the same metabolic pathway as the parent, via dealkylation of the $P-O-CH_3$ bonds, which is possibly desmethylation with a subsequent rapid cleavage of the P−S (not P=S) bond like the parent. Hence the oxon metabolites are not considered to be different from those of methidathion. The hydrolysis of the P−S (as opposed to a P=S) bond will give rise to (di)methyl(thio)phosphates from methidathion, and (di)methylphosphates from the oxygen analogue, which are all devoid of significant toxicity.

In conclusion, the main metabolic pathway of methidathion in rats was considered to be the hydrolysis of the thiol bond of the phosphorus ester, followed by methylation of the thiol group and subsequent oxidation of the sulfur atom, resulting in the liberation of carbon-dioxide. Also proposed was a metabolic route common to other species, where the heterocyclic moiety of methidathion is cleaved after hydrolysis of the ether bond, and further oxidized to carbon-dioxide. Proposed metabolic pathways for methidathion in rats is shown in Fig. 2.

Figure 2. Proposed metabolism pathway in rats



[ ] indicates tentative identification only

(Modified and redrawn from a figure provided by sponsor, ZEN-NOH)

1.3 Effects on enzymes and other biochemical parameters

There was no information available on other biochemical parameters.

2. Toxicological studies

2.1 Acute toxicity

Acute toxicity, dermal and ocular irritation and dermal sensitization studies are summarized in Table 6.

Table 6. Summary of acute toxicity, irritation and sensitization studies with methidathion

Species	Strain	Sex	Route	Vehicle	purity (Batch)	LD_{50}/LC_{50}	Reference
Rat	Sprague Dawley (Charles River CD)	F	Oral	Dimethyl sulfoxide (DMSO)	99% (SMO9C597)	50–300 mg/kg bw	Buda, 2011
Rat	Sprague Dawley (Charles River CD)	M/F	Dermal	-	99% (SMO9C597)	> 2000 mg/kg bw	Zelenák, 2011a
Rat	Tif:RAI (SPF) hybrids	M/F	Inhalation	Ethanol	96.3% (OP.909749)	140 mg/m^3 air (range 105–195) (combined sexes)	Hartmann, 1993
Rabbit	New Zealand White	M	Skin irritation	Moistened with distilled water	99% (SMO9C597)	Not irritant	Zelenák, 2011b
Rabbit	New Zealand White	M	Eye irritation	-	99% (SMO9C597)	Not irritant	Tavaszi, 2011
Guinea pig	Ibm:GOHI	M/F	Skin sensitization (MK)	-	96.3% (802658)	Sensitizing	Arcelin, 2000

M: Male; F: Female; LD_{50}: Median lethal dose; LC_{50}: Median lethal concentration; MK: Magnusson & Kligman

(a) Lethal doses

Oral

The acute oral toxicity of methidathion was investigated in female CRL:CD (Sprague Dawley) rats. The animals were dosed by gavage with 50 mg/kg bw (six rats) or 300 mg/kg bw (three rats) and observed for 14 days for clinical signs and mortality. Dimethyl sulfoxide (DMSO) was used as a vehicle. Body weights were recorded and necropsy was performed on all animals.

At a dose of 300 mg/kg bw all animals were found dead at 30 minutes post dose. All animals survived a dose of 50 mg/kg bw, but showed clinical signs such as decreased activity, hunched back, prone position, tremors (intermittent or continuous), piloerection, reddish material around the eyes or noisy breathing within 30 minutes post dose. The rats recovered from these signs within four days post dose. There was no treatment-related effect on body weight gain or macroscopic findings. Under the conditions of this study, the acute oral median lethal dose (LD_{50}) of methidathion was found to be between 50 and 300 mg/kg bw in female CRL: CD (SD) rats (Buda, 2011).

In the previous JMPR evaluation three acute oral studies on methidathion were reported in male and female Tif, RAIf rats. These were not provided by the sponsor to the present Meeting. The LD_{50} values in these studies were 43.8 mg/kg bw (sample purity not stated), 26 mg/kg bw (purity 96.9%) and 26 mg/kg bw (purity 92.7%) (JMPR, 1993).

In the biotransformation study discussed above (Dupuis, Muecke & Esser, 1971; see metabolite toxicity), oral LD_{50} in rats (sex or strain, not reported) was reported as 35 mg/kg bw.

In an open literature search, information on the LD_{50} of methidathion in weaning rats was obtained. Male and female adults (90 days old) and weaning (4–6 weeks old, sex not mentioned) Sherman strain rats (a specific pathogen-free strain) were given methidathion, technical grade, purity and batch No. not given. The test article was suspended in peanut oil and administered by gavage. The LD_{50}s were 31, 32 and 21 mg/kg bw for adult males, adult females and weanlings, respectively. Confidence limits (95%) for the LD_{50} values were 26–35, 30–34, and 19 –25 mg/kg bw for adult males, adult females and

weanlings, respectively. The ratio for adults to weanlings was 1 : 1.48, with a statistically significance of $p < 0.05$ (Gaines & Linder, 1986).

Dermal

An acute dermal toxicity study was performed with methidathion using CRL:CD(Sprague Dawley) rats. A limit test was carried out in both sexes (five rats per sex) at 2000 mg/kg bw. The test substance was applied (as provided) in a single dermal 24-hour exposure, followed by a 14-day observation period. Methidathion was placed onto a gauze pad which was fixed with a hypoallergenic plaster to the shaved skin of the rats. The entire trunk of the animal was then wrapped with semi-occlusive plastic wrap for 24 hours.

All animals survived. There was no indication of any treatment-related effect on body weight gain nor any macroscopic findings. No local dermal signs were observed up to 14 days after treatment. The acute dermal LD_{50} of the test article methidathion was found to be higher than 2000 mg/kg bw in male and female CRL:CD(SD) rats (Zelenák, 2011a).

Inhalation

An acute inhalation toxicity study (four-hour exposure, nose-only) was performed with methidathion in Tif:RAI (SPF) hybrids of RII/I and RII/s) rats, applying aerosol concentrations of 0, 54, 134 and 297 mg/m^3. An aerosol of the test substance GS-13005 (tech.) in ethanol was generated in a pneumatic nebulizer with a small aspirating reservoir (1–2 mL) and an attached bulk fluid container (to minimize solvent evaporation). The nebulizer was operated at 2, 3, and 3 L/minute (input pressure 4, 8, and 8 kPa) at 54, 134, and 297 mg/m^3, respectively: the aerosol was diluted with filtered, humidified air to yield a total flow of 64, 64, and 32 L/minute, respectively. The animals were examined for clinical symptoms and mortalities for up to 14 days, body weights were recorded and necropsy performed on all animals.

At a concentration of 134 mg/m^3 four females died. At 297 mg/m^3 all five males and three females died between four hours and three days of exposure. The two remaining females of the 297 mg/m^3 exposure group had to be euthanized for humane reasons. A concentration-dependent body weight loss was recorded for the groups exposed to 134 and 297 mg/m^3 at least on day 1 after exposure. At necropsy four females of the 134 mg/m^3 group displayed a spotted thymus and one male of the 297 mg/m^3 group exhibited haemorrhage in the lungs.

The mass median aerodynamic diameter (MMAD) of the particles was between 2.5 and 4.6 µm, with a geometric standard deviation (GSD) of 1.7 to 2.7 µm.

Values of LC_{50} (with 95% confidence limits) for methidathion were:

173 mg/m^3 of air (104–503 mg/m^3) for males,

109 mg/m^3 of air (51–174 mg/m^3) for females, and

140 mg/m^3 of air (105–195 mg/m^3) for combined sexes

(Hartmann, 1993).

(b) Dermal irritation

To assess the skin corrosion/irritation potential, 0.5 g of methidathion was applied to intact, clipped skin of New Zealand White rabbits (three males) for four hours. Skin reactions were examined according to the Draize scheme at 1, 24, 48 and 72 hours after patch removal. Animals were observed for mortality and clinical signs and body weights were recorded.

No mortality or clinical signs were observed. There were no treatment-related body weight changes. Examination of skin reactions revealed very slight erythema (score 1) in one animal from one hour until up to 48 hours after patch removal. The individual mean Draize score for this animal was 0.67. All other animals were free of skin reactions.

Under the conditions of this study, methidathion was not considered irritating to skin (Zelenák, 2011b).

(c) Ocular irritation

To assess the eye corrosion/irritation potential, 0.1 g of methidathion was administered to New Zealand White rabbits (3 males) to the conjunctival sac of the left eye for 1 hour. Eye reactions were examined

according to the Draize scheme at 1, 24, 48 and 72 hours after removal of the test substance. Animals were observed for mortality and clinical signs and body weights were recorded.

No mortality and clinical signs were observed. There were no treatment related body weight changes. Examination of eye reactions revealed conjunctival redness (score 1 or 2) in all animals from 1 up to 24 hours after patch removal. The individual mean scores for these animals were 0.33, 0.67 and 0.67.

Under the conditions of this study, methidathion was not considered irritating to the eyes of rabbits (Tavaszi, 2011).

(d) Dermal sensitization

In order to assess the cutaneous allergenic potential of GS-13005 (tech.), the Magnusson & Kligman maximization test was performed using lbm:GOHI Guinea pigs (control group, five animals per sex; treated group 10 animals per sex). Intradermal induction was performed at a test substance concentration of 5% in a vehicle consisting of polyethylene glycol (PEG 300) and Freund′s Complete Adjuvant. The skin was pretreated with sodium lauryl sulfate (10%) on day 7 after induction. Epidermal induction started on day 8 with the application of a test substance concentration of 50% (same vehicle) for 48 hours. Two weeks after induction the animals were challenged by epidermal application of the substance at 15% (same vehicle) and skin reactions were evaluated 24 and 48 hours after removal of the dressing.

Neither mortality nor any toxic signs were observed. All animals of the dose group showed discrete/patchy to moderate/confluent erythema at the 24- and 48-hour examinations after the treatment with methidathion at 15%. No skin effect was observed in the control group.

On the basis of results above, methidathion was considered a skin sensitizer (Arcelin, 2000).

2.2 Short-term studies of toxicity

(a) Oral administration

Rat

A ninety-day oral toxicity study was conducted in rats. Methidathion (purity 96.3%; batch No. 802658) was administered to 10 male and 10 female bm:WI(Han) rats per dose level for 90 days at dietary concentrations of 0, 2, 6, 30 or 100 ppm (equal to 0, 0.13, 0.40, 2.05 and 7.04 mg/kg bw per day for males, 0, 0.16, 0.48, 2.50 and 9.02 mg/kg bw per day for females). Clinical signs, body weight, feed consumption, water consumption and mortality were monitored throughout the study for all animals. Ophthalmological examinations were performed on all animals during pretest and in animals at 0 and 100 ppm toward the end of treatment. Haematological, blood chemistry (including plasma), erythrocyte and brain AChE, and urine analyses were performed at the end of the treatment period on all animals. Blood was collected from the orbital plexus under light isoflurane anaesthesia early in the morning after overnight fasting. At termination, animals were examined macroscopically and organ weights were recorded. Organs and tissues were collected and prepared for histopathological examination.

None of the animals was found dead nor were any euthanized when moribund. At 100 ppm, tremor, abnormal gait, hunched posture and/or piloerection were observed in all females. These clinical sings appeared from weeks 2 and 3 to the end of treatment. In males, tremor was observed in four rats during weeks 3 to 5 and disappeared at weeks 4 to 8. Piloerection was observed in two male rats from week 10 to treatment end. Similar clinical signs were not observed in other treated groups of either sex. Body weight gains in both sexes at 100 ppm were markedly reduced during the first several weeks. Afterwards the gains recovered to a level comparable with controls. However, lower body weights continued throughout the treatment period. Overall body weight gain (days 1–91) was significantly reduced (19%) in males and females (24%). No consistent change in body weight gain was observed in other treatment groups. Feed consumption was lower (statistically significant) in females at 100 ppm during the first several weeks. Water consumption was not affected. No treatment-related changes were observed in haematology, urinalysis and ophthalmology.

Dose-related findings that were observed are summarized in Table 7. At 100 ppm blood biochemistry results indicated increased urea and aspartate transaminase (AST) in males, and decreased glucose in females. The decreased urea was not accompanied by any histopathological finding or change in urinalysis. No effect on the kidney was observed at 100 ppm in two-year study of rats (see 2.3 Long-

term studies..., *Rat*). Therefore, this change was not considered treatment-related. The increased AST was approximately 20% higher than the control value. However, alanine aminotransferase (ALT), a more sensitive enzyme than AST for detecting hepatocyte damage, was not increased with statistical significance. No morphological changes indicating hepatobiliary damage were found in the liver. Therefore, the increase in AST was not considered toxicologically significance. The decrease in glucose might be a secondary change consistent with body weight changes in this group.

Erythrocyte and brain AChE activities showed treatment-related decreases of 20% or more compared to controls at doses of 30 ppm and above in both sexes, with statistically significance. Erythrocyte AChE was decreased just 20% in females at 6 ppm without statistical significance. In males the decreased level was 13%. The Meeting noted that it was possible that the sampling time, (early morning after overnight fasting) might be past the time of peak effect for methidathion on AChE activity. The Meeting also noted that in the 90-day neurotoxicity study, 10 ppm (equal to 0.608 mg/kg bw per day in males, 0.659 mg/kg bw per day in females) was a clear cause of a decrease of 20% or more in erythrocyte and/or brain AChE activity at most of time points in rats, but no information on fasting before blood or tissue sampling was reported in that 90-day neurotoxicity study (Chow, 1995). The Meeting considered the decrease in erythrocyte AChE activity in females at 6 ppm to be adverse because the possibility could not be excluded that AChE activity at peak inhibition was in fact depressed more than was apparent after the overnight delay, and because it was part of a consistent dose–response relationship.

No treatment-related differences in organ weights including liver weight were found. No dose-related changes were observed during macroscopic examination. Incidences of hepatocyte hypertrophy were increased at 100 ppm in both sexes. The hypertrophy was considered an adaptive change but not evidence of toxicity, due to no corresponding finding that indicated damage to the liver.

Table 7. Summary of main findings in 90-day toxicity study of methidathion in rats

		Males (dose in ppm)					Females (dose in ppm)				
		0	**2**	**6**	**30**	**100**	**0**	**2**	**6**	**30**	**100**
Number of rats		10	10	10	10	10	10	10	10	10	10
Blood biochemistry											
Glucose (mol/L)	Mean	7.250	8.291	8.406*	8.764**	7.862	7.180	7.071	6.080*	6.778	5.861**
	SD	0.836	0.517	0.482	1.373	1.091	1.136	0.761	0.862	0.908	0.574
Urea (mol/L)	Mean	5.492	5.736	6.265	5.945	6.578**	7.106	7.735	8.266**	7.070	7.886
	SD	0.699	1.053	0.364	0.698	0.542	0.836	1.142	0.831	0.964	0.708
AST (U/L)	Mean	68.07	74.43	73.49	76.04	88.50**	87.30	87.19	73.54	77.65	76.28
	SD	8.08	8.62	7.26	20.08	15.25	34.15	30.76	13.10	17.71	17.32
ALT (U/L)	Mean	32.89	32.81	33.45	34.71	41.29	38.66	38.75	23.85	28.11	28.28
	SD	6.67	6.61	4.54	11.10	17.89	22.67	22.34	5.44	6.82	10.46
Histopathology											
Hepatocyte hypertrophy	0	0	0	2	9	NA	0	0	0	0	4
Acetylcholinesterase measurement											
Erythrocyte (U/L)	Mean	951.2	831.4	825.0	398.5**	159.4**	759.4	696.5	605.9	164.8**	95.8**
	SD	122.9	82.5	78.7	98.4	29.4	56.5	69.0	46.2	31.8	22.7
	%[a]	100	88	87	42	17	100	92	80	22	13
Brain (U/g)	Mean	2.30	2.56	2.41	1.72**	0.73**	2.26	2.60	2.32	1.03*	0.37**
	SD	0.25	0.31	0.23	0.21	0.11	0.21	0.35	0.23	0.13	0.08
	%[a]	100	111	104	75	32	100	115	103	46	16

AST: Aspartate transaminase; ALT: Alanine transaminase; SD: Standard deviation; Source: Altmann, 2001

[a] Percentage of control value; Statistically significant from control value at: * $p < 0.05$ ** $p < 0.01$

The NOAEL for 90-day toxicity in rats was 2 ppm (equal to 0.16 mg/kg bw per day) based on a decrease in erythrocyte AChE in females at 6 ppm (equal to 0.48 mg/kg bw per day) (Altmann, 2001).

Dog

A ninety-day toxicity study was conducted using Beagle dogs. Groups of four dogs per sex were fed methidathion (purity up to 97%; batch No. FL-862491) for 90 days at dietary concentrations of 0, 0.5, 4, 45 or 140 ppm (equal to 0, 0.021, 0.160, 1.944 and 5.689 mg/kg bw per day for males, 0, 0.021, 0.191, 2.111 and 7.016 mg/kg bw per day for females). In addition, methidathion was administered to a group of four dogs per sex in gelatin capsules (vehicle corn oil) at 0.14 mg/kg bw per day. This dose was equivalent to a dietary concentration of 4 ppm. The dietary concentration levels were chosen based on results from a previous two-year chronic toxicity dog study of inadequate design, (see Johnson's 1976 study in JMPR, 1993; not submitted to the current Meeting), in which the dietary concentrations were 0, 4, 16 and 64 ppm. Mortality, clinical signs, body weight, feed consumption, water consumption and mortality were monitored throughout the study for all animals. Ophthalmology was performed pretest and prior to termination. For all animals haematological analysis, blood chemistry (including plasma), erythrocyte and brain (cerebellum at terminal examination only) AChE, and urine analyses were performed pretest, at eight weeks and at the end of the treatment period. Blood was collected from the jugular vein after at least 16 hours fasting. At termination animals were examined macroscopically and organ weights recorded. Organs and tissues were collected and prepared for histopathological examination.

No deaths occurred during this study. Two males, (one each in the 45 and 140 ppm groups) showed decreased activity after feeding from day 50. The male at 140 ppm showed tremor from day 78. No other effects regarded as cholinergic were observed. There were no significant differences in body weight in either sex in all the treated groups. Males in the capsule-treated group at 0.14 mg/kg bw per day, showed a significant decrease in body weight at weeks 1 and 4. No dose-related changes were revealed by ophthalmoscopy, haematology and urinalysis. No treatment-related change was found in organ weights.

With respect to blood biochemistry, parameters indicating hepatotoxicity, such as increases in alkaline phosphatase (ALP), AST, ALT, γ-glutamyl transpeptidase (GGTP) and sorbitol dehydrogenase (SDH), were observed in males and females at 45 ppm and above at eight weeks and/or termination. Macroscopically the colour of the liver changed to dark red in males and females at 45 ppm and above. At these doses histopathological examination also revealed cholestasis, characterized by bile plugs in distended bile canaliculi and bile retention distributed diffusely throughout the liver, prominently in the centrilobular to midzonal area. Total bilirubin was not affected by the treatments.

Erythrocyte AChE measurements showed dose-related decreases at eight weeks and 14 weeks of 20% or more compared to controls, seen at 45 ppm and above in males, and in all treated groups in females. Statistically significant decreases in both sexes at 140 ppm, and decreases in brain AChE of 20% or more at 140 ppm in females were both considered to be adverse. Approximately 40% decreases in erythrocyte AChE at 45 ppm at both time points were also considered to be adverse, because the decreases were also observed at 40 ppm in one-year study of male dogs (Chang, 1991; see Table 8). The decreases at 0.5 ppm and 4 ppm found at both time points were not considered adverse, because the changes seen in the 90-day study were inconsistent with the changes at the same dose administered by capsule in the same study, and the same concentration level in the one-year study by Chang & Wyand (1991). No decrease was observed in dogs at 0.5 ppm or 2 ppm in the one-year study (Chang, 1991). The Meeting noted that values were 50% higher in the controls at 8 and 14 weeks compared to their pretest values (Mean ± SD, 1630 ± 525 U/L; 24 dogs examined) and that this might influence the decreases observed at 4 ppm and lower. The one-year dog study (Chang & Wyand, 1991) was conducted one year later in the same laboratory as the 90-day study (Chang, 1990).

No significant changes in either sex were observed after administration of 0.14 mg/kg bw per day via capsule. Major changes observed during the 90-day dog study (Chang, 1990) are summarized in Table 8.

Table 8. Summary of 90-day toxicity study of methidathion in dogs

		Males (dose in ppm)						Females (dose in ppm)					
		0	**0.5**	**4**	**45**	**140**	**0.140[a]**	**0**	**0.5**	**4**	**45**	**140**	**0.140[a]**
Number of dogs		4	4	4	4	4	4	4	4	4	4	4	4
ALP (U/L)													
8 weeks	Mean	206	186	211	606**	515**	188	177	175	187	567**	670**	175
	SD	31	29	34	139	98	37	37	40	39	184	344	34
14 weeks	Mean	177	167	198	625**	609**	172	153	161	170	691**	731**	178
	SD	29	29	38	151	114	32	58	32	32	203	463	174
AST (U/L)													
8 weeks	Mean	23	22	26	34*	35*	26	27	21	27	34	36	26
	SD	5	1	2	4	10	7	5	1	5	5	5	5
14 weeks	Mean	20	22	28	34*	35**	25	20	19	25	32**	31*	28
	SD	3	1	2	4	12	3	2	2	5	8	5	2
ALT (U/L)													
8 weeks	Mean	16	14	37	173**	201**	39	19	15	23	163**	191**	34
	SD	4	4	18	29	63	24	4	2	7	61	24	13
14 weeks	Mean	15	13	39	148**	162**	54	15	13	23	164**	155**	39
	SD	4	3	14	20	48	28	3	2	6	65	27	12
GGTP (U/L)													
8 weeks	Mean	7	7	7	10**	9**	7	7	8	8	10**	10**	7
	SD	1	1	0	1	1	1	1	1	1	1	1	0
14 weeks	Mean	6	7	7	10**	9**	7	6	6	7	11**	11**	7
	SD	1	1	1	1	1	0	1	1	1	1	2	0
SDH (U/L)													
8 weeks	Mean	3	4	6	10*	11*	5	4	3	5	12*	8	4
	SD	2	1	2	3	7	4	1	1	1	6	4	1
14 weeks	Mean	4	5	7	17*	16**	7	6	6	7	20**	14	8
	SD	1	2	2	4	9	2	1	1	2	11	7	1
Erythrocyte acetylcholinesterase (U/L[d])													
8 weeks	Mean	1220	1245	1320	1205	300*	1335	1860	1365	1510	1065	320**	1625
	SD	439	337	538	464	156	368	399	428	624	184	91	671
	%	-	102	108	99	25	109	-	73	81	57	17	87
14 weeks	Mean	1485	1265	1440	1210	175**	1450	2105	1435	1650	1175	290**	1795
	SD	402	504	368	357	350	4304	321	455	646	237	335	817
	%	-	85	97	81	18	98	-	68	78	56	14	85
Brain acetylcholinesterase (U/g tissue)													
14 weeks	Mean	1.45	1.83*	1.57	1.60	1.36	1.70	1.83[b]	2.13	1.73	1.65	1.34	2.06
	SD	0.08	0.31	0.13	0.15	0.23	0.08	0.45	0.66	0.23	0.11	0.13	0.65
	%	-	126	108	110	94	117	-	116	94	90	73	113
Histopathology													
Cholestasis		0	0	0	4	4	1	0	0	0	4	4	0

ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; SD: Standard deviation Source, Chang 1990
GGTP: γ-glutamyl transferase; SDH: Sorbitol dehydrogenase; ALT: Alanine aminotransferase;
[a] Capsule treatment (as mg/kg bw per day); [b] Three dogs were examined; [c] Percentage of control value;
[d] RBC AChE activity measured as U/L of packed RBCs; Statistically significant from control value at: * $p < 0.05$ ** $p < 0.01$

The NOAEL for 90-day toxicity in dogs was 4 ppm (equal to 0.160 mg/kg bw per day) based on decreased erythrocyte AChE, hepatotoxicity-related changes in blood biochemistry and pathological finding in females at 45 ppm (equal to 1.944 mg/kg bw per day) (Chang, 1990).

A one-year toxicity study was conducted in Beagle dogs. Groups of four dogs per sex were fed daily for 12 months with dietary concentrations of methidathion (purity 96%; batch No. FL-890331) at 0, 0.5, 2, 4, 40 or 140 ppm (equal to 0, 0.02, 0.07, 0.15, 1.33 and 4.51 mg/kg bw per day for males, 0, 0.02, 0.07, 0.15, 1.39 and 4.90 mg/kg bw per day for females). Mortality, clinical signs, body weight, feed and water consumption were monitored throughout the study for all animals. Ophthalmology was performed at pretest and prior to termination. Haematological analysis, blood chemistry (including plasma), erythrocyte and brain AChE (cerebellum at terminal examination only), and urine analyses were performed for all animals pretest and at three, six and 12 months. Blood was collected from the jugular vein after at least 16 hours fasting. At termination animals were examined macroscopically and organ weights recorded. Organs and tissues were collected and prepared for histopathological examination.

None of the animals was found dead nor were any euthanized when moribund during the study. No dose-related changes were observed in body weight or feed consumption. Increased salivation was seen at all doses including the controls, except for the 0.5 ppm groups. In females, all dogs at 140 ppm showed salivation. The salivation observed in both sexes was considered to be treatment-related. No significant effects due to treatment at any time point were observed in the results of ophthalmology, haematology or urinalysis

Treatment-related changes for the one-year study in dogs (Chang & Wyand, 1991) are summarized in Table 9.

Considering blood biochemistry, indicators of hepatotoxicity, such as increased ALP, AST, ALT and SDH were observed in males and females at 40 ppm and above at most of time points. Very slight increases in GGTP in females and bilirubin in males were also observed at 140 ppm.

At necropsy, discolouration of the liver was found at 40 ppm and above, although no treatment-related change was seen in organ weights, including liver weight. Microscopic examination revealed cholestasis in males and females at 40 ppm and above. Grades of lesion severity increased with increasing dose.

Decreases in AChE activity that appeared treatment-related were observed at 40 ppm and above in both sexes. Erythrocyte AChE activity was decreased at 140 ppm by 50% or more compared to control values in males and females all time points. At 40 ppm however, dose-related decreases were observed at all time-points in males only. In females, the decreases were smaller, not exceeding a 20% reduction compared to controls. Brain AChE activities were decreased at 140 ppm in both sexes. The decrease was by 20% or more compared with control values in the vermis of the brain of both sexes, and approximately 15% lower than the controls in hemisphere minus vermis. These effects on AChE did not increase in severity with treatment duration.

Table 9. Summary of one-year toxicity study of methidathion in dogs

		Males (dose in ppm)						Females (dose in ppm)					
		0	**0.5**	**2**	**4**	**40**	**140**	**0**	**0.5**	**2**	**4**	**40**	**140**
Number of dogs		4	4	4	4	4	4	4	4	4	4	4	4
ALP (U/L)													
3 months	Mean	175	148	199	158	465**	500**	179	149	167	184	503**	615**
	SD	46	39	68	32	74	175	38	20	51	64	76	290
6 months	Mean	130	112	141	106	383**	469**	184	111	134	172	415	755**
	SD	31	44	44	29	56	161	77	16	70	62	95	483
12 months	Mean	126	122	103	99	297**	371**	152	133	114	190	353	623*
	SD	21	101	41	34	38	152	81	66	60	40	120	467

		Males (dose in ppm)						Females (dose in ppm)					
		0	**0.5**	**2**	**4**	**40**	**140**	**0**	**0.5**	**2**	**4**	**40**	**140**
AST (U/L)													
3 months	Mean	26	25	22	26	33	35*	25	22	25	25	29	41**
	SD	4	4	2	6	5	4	2	1	5	3	5	8
6 months	Mean	24	25	25	22	31*	34**	22	18	22	19	25	30*
	SD	4	3	2	2	5	3	5	1	4	2	5	4
12 months	Mean	23	23	21	23	29	32*	24	25	21	22	28	35*
	SD	3	3	2	3	7	4	2	6	3	3	6	7
ALT (U/L)													
3 months	Mean	19	20	18	41	158**	164**	17	14	16	41	130**	157**
	SD	3	3	4	17	42	67	5	2	4	23	26	65
6 months	Mean	18	22	22	40	159**	154**	21	15	15	27	121**	146**
	SD	3	2	3	15	43	74	7	1	4	8	22	45
12 months	Mean	15	21	21	40	134**	140**	17	15	15	26	126**	134**
	SD	4	3	4	16	37	70	5	1	4	7	42	58
GGTP (U/L)													
3 months	Mean	5	5	6	5	6	6	5	5	5	5	7*	7*
	SD	2	1	1	1	1	1	1	1	1	1	1	1
6 months	Mean	7	7	7	7	7	8	5	6	6	6	7	9*
	SD	1	1	1	1	1	1	1	1	1	1	2	3
12 months	Mean	5	5	6	5	6	6	5	6	6	6	7	8
	SD	1	1	1	0	1	1	1	0	1	1	2	5
SDH (U/L)													
3 months	Mean	5	7	5	9	14*	20**	6	6	6	9	15**	14*
	SD	1	2	1	2	4	9	1	1	1	2	6	5
6 months	Mean	2	5	3	5	7**	10**	5	5	4	4	7	6
	SD	1	2	1	1	3	2	1	1	1	1	5	2
12 months	Mean	4	6	3	6	11**	13**	5	6	6	6	13**	10*
	SD	1	1	1	3	3	5	1	2	1	1	4	3
Organ weights, macroscopic and microscopic change													
Liver weight (g)	Mean	291	275	237	277	297	266	259	259	246	278	251	265
	SD	24.4	21.7	38.8	35.4	60.2	26.4	31.1	48.6	37.0	37.2	35.1	57.0
Liver discolouration		0	0	0	0	1	2	0	0	0	0	2	3
Cholestasis		0	0	0	0	4	4	0	0	0	0	4	4

		Males (dose in ppm)						Females (dose in ppm)					
		0	0.5	2	4	40	140	0	0.5	2	4	40	140
Erythrocyte acetylcholinesterase (U/L [b])													
3 months	Mean	1405	1565	1190	1535	980	185**	185**	1515	1655	1895	1245	245**
	SD	203	205	352	392	298	130	130	746	264	428	232	177
	% [a]	-	111	85	109	70	13	13	105	115	131	86	17
6 months	Mean	1410	1510	1290	1495	1035	260**	260**	1465	1565	1695	1190	300*
	SD	185	165	373	356	216	191	191	664	179	386	208	82
	% [a]		107	91	106	73	18	18	118	126	137	96	24
12 months	Mean	1385	1605	1315	1565	1105	315**	315**	1405	1610	1715	1220	310**
	SD	262	204	242	475	302	123	123	490	385	462	198	101
	% [a]	-	116	95	113	80	23	23	109	125	133	95	24
Brain acetylcholinesterase (U/g tissue)													
Brain A	Mean	2.14	1.90	2.34	2.07	2.04	1.57**	2.15	2.18	2.16	2.29	2.06	1.68*
	SD	0.20	0.14	0.34	0.17	0.07	0.16	0.19	0.09	0.19	0.20	0.17	0.30
	% [a]		89	109	97	95	73	-	101	100	106	96	78
Brain B	Mean	1.53	1.50	1.57	1.60	1.52	1.29	1.57	1.56	1.59	1.62	1.58	1.31*
	SD	0.06	0.18	0.12	0.19	0.16	0.08	0.12	0.15	0.12	0.17	0.08	0.09
	% [a]		98	103	105	99	84	-	99	101	103	100	83

ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; Source: Chang & Wyand, 1991

GGTP: γ-glutamyl transferase; SDH: Sorbitol dehydrogenase; ALT: Alanine aminotransferase;

Brain A: Measured in homogenized fraction from the vermis of the cerebellum;

Brain B, Measured in homogenized fractions from the right hemisphere minus vermis ;

[a] Percentage of control value; [b] Erythrocyte AChE activity measured as units per litre of packed red blood cells (RBC)

Statistically significant from control value at: * $p < 0.05$ ** $p < 0.01$

The NOAEL for one-year toxicity in dogs was 4 ppm (equal to 0.15 mg/kg bw per day) based on decrease in erythrocyte AChE activity, hepatotoxicity related changes in blood biochemistry and pathological finding at 40 ppm equal to 1.33 mg/kg bw per day (Chang & Wyand, 1991).

The last periodic evaluation of methidathion was conducted in JMPR 1992. The ADI was established 0.001 mg/kg bw based on the (overall) NOAEL of 3 dog studies, applying 100 as a safety factor. The NOAEL in 2-year study was suggested 0.14 mg/kg bw per day. Whereas the levels of dietary concentration of 3 studies were the same level, 4 ppm. the NOAELs for 90-day and 1-year study were 0.16 or 0.15 mg/kg bw per day, respectively. The Meeting concluded the 2-year study was inappropriate to use an endpoint for ADI establishment because the treatment methods was inadequate (starved of diet one day each week and received a double ration on the next day) (JMPR 1993).

(b) Dermal application

The sponsor submitted no information on toxicity via the dermal route for the current Meeting. The previous 1992 JMPR evaluation (JMPR, 1993) considered two toxicity studies on dermal application in male and female New Zeeland White rabbits. In the first study, conducted using occlusive application, the rabbits were treated with methidathion (purity unknown) at doses of 0, 1, 5, or 20 mg/kg bw for 22 consecutive days. No significant adverse effects, including any on AChE activity, were observed in either sex up to 20 mg/kg bw per day of methidathion, except a minimal increase in hypoactivity in a rabbit at 20 mg/kg bw per day. The second study was conducted using occlusive application of methidathion (purity 95%) at doses of 0, 1,10, 40 or 80 mg/kg bw for 21 days. the death of approximately half of the of animals with various clinical signs was observed spanning all treatment doses in males and 10 mg/kg bw per day and above in females. The AChE activity in erythrocytes and the brain

was decreased by 40–80% and 37–88% in males and females respectively, at 10 mg/kg bw per day and above. In the previous Meeting it was reported that factors such as the occlusive rubber or neck collar might have resulted in the differences in toxic effect seen between the first and second study. (JMPR, 1993).

The United States Environmental Protection Agency (U.S. EPA) has performed a risk assessment of methidathion. In this is mentioned a species-specific metabolic enzyme in rabbits and its effect on estimation of toxicity. In the report, the short-term dermal end-point is based on a NOAEL of 20 mg/kg bw per day, established from a 21-day dermal toxicity study in rabbits. It is stated that generally, dermal toxicity studies with thio-organophosphates (such as methidathion) tend to underestimate the toxicity of these chemicals because rabbits possess high concentrations of plasma carboxylesterase, which deactivates the chemical before it is converted into the active oxon. They also mention that in the case of methidathion, the weight of evidence from the oral and dermal toxicity data in rats and rabbits indicates that the dose used in risk assessment would not underestimate any potential dermal risk from methidathion exposure (U.S. EPA, 2006).

(c) Exposure by inhalation

There was no available information on toxicity by inhalation

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

A two-year combined chronic toxicity and carcinogenicity study was conducted in male and female CD-1 mice. purity and batch were not reported for the test article used, however the same batch number as that for the two-year rat study (533131 M; 202444) described below was indicated on the label provided together with the test material. This was administered for two years to 170 mice per sex in each dose group at dietary concentrations of 0, 3, 10, 50 or 100 ppm (equal to 0, 0.4, 1.4, 6.7 and 13.1 mg/kg bw per day in males, 0, 0.5, 1.6, 8.1 and 15.9 mg/kg bw per day for females). Mice in each group were allocated to either chronic toxicity phase or carcinogenicity phase groups. For the toxicity phase group, 20 mice per sex (3, 6, 12 or 12+1 month groups) or 40 mice per sex (18 month group) were further allocated to the time point subgroups for 3, 6, 12 and 18 months treatment and the group that underwent a one-month recovery period after12 months of treatment. For the carcinogenicity phase group, 50 mice per sex were assigned to each group and treated for 23 months. All mice were checked for clinical signs, mortality, body weight, food and water consumption and ophthalmoscopy. Ten randomly selected mice per sex were examined for haematology, clinical biochemistry, and changes in erythrocyte and brain AChE activity. Blood samples were collected from the orbital sinus plexus of mice fasted prior to blood collection. Blood from non-fasting mice was collected for haematological analysis and AChE measurement. At each necropsy, various organs were weighed after macroscopic examination. Microscopic examination of selected tissues was performed. This study was conducted in compliance with GLP. The guideline followed for the study was not specified. However, the study design was comparable to the Organisation for Economic Co-operation and Development (OECD) Guideline 453 for combined chronic toxicity and carcinogenicity testing.

An increase in mortality was observed in male mice only at 100 ppm. The body weights in the treated groups means were comparable to those in the control groups in both sexes during the study.

Hepatotoxicity was induced by the treatment with methidathion in males at 50 ppm and above and in females at 100 ppm. In blood biochemistry, increases in ALP, AST and ALT were observed at the most intervals in analysis in males at 100 ppm. The AST and ALT values were essentially recovered after 1-month. In females ALP and ALT were increased in only one or two intervals of analysis. At 100 ppm, ALP and ALT values were increased at a few intervals of analysis, mostly in males. Treatment related macroscopic changes were detected in the liver at 50 and 100 ppm, being most prominent in male mice. They consisted of enlarged liver, firm mass, foci, nodules, discolouration and mottling. In males at the 50 and 100 ppm, absolute and relative liver weights were increased with treatment related histopathological findings of bile ducts in gall bladder and liver and hepatitis at these doses. It indicated that main targets of toxicity in the hepatobiliary system were bile ducts as well as hepatocytes in mice.

Histopathological changes in the gall bladder and liver, including cholestasis and hyperplasia of the gall bladder and bile duct hyperplasia, bile stasis and cholangiofibrosis in the liver, were found in both sexes after three months treatment at 100 ppm. Lesions were more severe in males than in females. Hepatobiliary damage developed in severity at 100 ppm, and was also found at 50 ppm and above in both sexes at 12-months (termination). The results of toxicity were reversed to some extent after the one month's recovery period that followed, although the results of toxicity were still detectable at 100 ppm in both sexes.

In males the incidence of hepatocellular carcinoma and combined incidence of hepatocellular adenoma and carcinoma were increased at 50 and 100 ppm, displaying a dose–effect relationship. Increases at 50 and 100 ppm were considered treatment-related. Incidence of the adenomas was increased at 3 ppm and above (statistically significant) in males, although the incidence at 3 ppm and 50 ppm was very similar (9/47 and 7/47 respectively) and the incidence of adenoma at 0 ppm was low (1/50). Hepatocellular adenomas and carcinomas were apparent among the 12-month interim terminations, and incidences of both were comparable among all groups, including males in the control group. The incidence of hepatocellular adenomas at termination in control males was 2/20 and 2/19 at 12 months and at 12 + 1 months, respectively. Historical control data were not provided. However, incidence of hepatocellular adenoma in CD-1 mice was elsewhere reported to be approximately 10% in males (Charles River, 2005; Chandra & Frith, 1992) or higher (Maita et al., 1988). In addition, one postulated mode of action (MOA) for liver tumour initiation was considered to be the continuous stimulation of hepatobiliary damage observed at 50 ppm and above in males as described below. Taken together, the increased incidence of adenomas at 3 and10 ppm were not considered treatment-related but probably due to an unusually low incidence in the control group. Similar treatment-related liver tumours were not observed in females. No other tumours were treatment-related.

An absolute and relative increase in weight of spleen and related extramedullary haematopoiesis in the spleen were observed in males at 100 ppm. These findings were considered a reactive change.

With regard to AChE measurement, a statistically significant decrease in erythrocyte and brain AChE activity of 20% or more than that of controls was observed at most sampling points at 100 ppm in males and females. In females, a decrease in erythrocyte AChE activity was noted at 50 ppm at most sampling points. Decreases in AChE activity essentially recovered to normality after one month's withdrawal treatment. These effects on AChE did not increase in severity with treatment duration.

The major findings in mice are summarized in Tables 10 and 11.

Table 10. Hepatobiliary lesions observed in mice treated with methidathion after three and 12 months of treatment, and a further one month recovery period

	Males (dose in ppm)					**Females (dose in ppm)**				
	0	**3**	**10**	**50**	**100**	**0**	**3**	**10**	**50**	**100**
					At 3 months					
Liver [No. of mice]	[20]	NE	NE	NE	[20]	[20]	NE	NE	NE	[20]
Bile stasis	**0**	-	-	-	17	0	-	-	-	1
Cholangiofibrosis	0	-	-	-	17	0	-	-	-	1
Hypertrophy	0	-	-	-	18	0	-	-	-	1
Necrosis	0	-	-	-	15	3	-	-	-	3
Bile duct hyperplasia	0	-	-	-	17	0	-	-	-	0
					At 12 months					
Gall bladder [No. of mice]	[19]	NE	NE	NE	[20]	[20]	NE	NE	NE	[20]
Cholecystitis	0	-	-	-	11	1	-	-	-	1
Hyperplasia	0	-	-	-	5	0	-	-	-	1

	Males (dose in ppm)					Females (dose in ppm)				
	0	**3**	**10**	**50**	**100**	**0**	**3**	**10**	**50**	**100**
Liver [No. of mice]	[20]	[20]	[20]	[20]	[20]	[20]	[20]	[20]	[20]	[20]
Bile stasis	0	0	0	8	20	0	0	0	5	10
Cholangiofibrosis	0	1	0	3	19	0	0	0	0	8
Bile duct hyperplasia	0	2	1	4	19	0	0	0	0	7
Hepatitis, chronic	13	10	13	14	20	14	4	15	18	14
Hepatocellular adenoma	2	1	3	0	2	1	0	0	0	2
Hepatocellular carcinoma	0	0	0	0	3	0	0	0	0	0
	At 13 months [a]									
Gall bladder [No. of mice]	[16]	NE	NE	NE	[15]	[19]	NE	NE	NE	[20]
Cholecystitis	0	-	-	-	6	1	-	-	-	3
Hyperplasia	0	-	-	-	5	0	-	-	-	2
Liver [No. of mice]	[19]	[20]	[19]	[20]	[19]	[19]	[20]	[20]	[20]	[20]
Bile stasis	0	0	0	6	19	0	2	0	2	9
Cholangiofibrosis	0	0	0	3	19	0	0	0	1	5
Bile duct hyperplasia	1	0	0	4	19	1	1	0	2	4
Hepatitis chronic	13	11	13	13	19	15	10	15	17	11
Hepatocellular adenoma	2	2	3	2	3	0	0	0	0	0
Hepatocellular carcinoma	3	0	1	1	1	0	0	0	0	0

NE: Not examined. Source: Goldenthal, 1986

[a] 13 months includes one month recovery period after 12 months of treatment.

Table 11. Major findings observed in two-year study of methidathion in mice

	Dose (ppm)									
	0		**3**		**10**		**50**		**100**	
	MALES									
Males used for carcinogenicity	50		50		50		50		50	
Number of survivors at week 99	29		30		29		30		16	
Blood biochemistry										
ALP (U/L), [No. of mice]	[10]		[10]		[10]		[10]		[10]	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
3 months	37	12.0	52	31.3	51	34.6	35	5.6	496	676.1
6 months	47	39.3	30	8.9	31	6.9	71	126.2	256	248.4
12 months	42	15.6	41	28.6	30	10.8	29	10.7	446	319.1
13[b] months	45[a]	28.8	33	5.7	40	22.2	51	28.3	448	697
18 months	49	30.5	36	12.2	34	8.8	64	51.1	981[b]**	1254
24 months	32	8.0	44	19.5	26	5.3	86	109	265**	119.1
ALT (U/L), [No. of mice]	[10]		[10]		[10]		[10]		[10]	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
3 months	57	25.3	55	21.8	51	11.1	47	8.3	140**	55.8
6 months	55	34.1	52	18.4	43	22.5	77	40.5	137*	92.8
12 months	45	23.8	48	15.1	50	21.5	129*	93.2	161	51.9

	Dose (ppm)									
	0		**3**		**10**		**50**		**100**	
13[b] months	55	20.1	50	25.2	56	34.0	45	26.0	100	86.1
18 months	58	39.5	51	28.9	57	39.1	104	69.7	130**	47.6
24 months	44	36.8	35	19.7	21	5.0	98*	70.3	136**	62.6
Histopathology[d]	**Carcinogenicity phase**									
Gall bladder [No. of mice]	[49]		[46]		[47]		[48]		[48]	
Cholecystitis	4 (0/4/0/0)		0		1 (0/1/0/0)		21 (1/17/2/1)		38 (0/14/13/11)	
Hyperplasia	0		0		0		15 (13/1/1)		33 (11/18/4)	
Liver [No. of mice]	[50]		[47]		[47]		[49]		[48]	
Bile duct hyperplasia	0		1 (0/1/0/0		0		21 (3/16/1/1)		42 (0/22/16/4)	
Cholangiofibrosis	1 (0/1/0/0)		0		0		18 (1/6/7/4)		47 (0/7/18/22)	
Hepatitis, chronic	3		1		2		24		47	
Bile stasis	0		0		0		25 (1/11/10/3)		49 (0/5/12/32)	
Hepatocellular:										
adenoma	1		9		7		8		**24**	
carcinoma	8		6		4		**13**		**17**	
as above, combined	9		15		11		21		**41**	
	FEMALES									
Females used for carcinogenicity	50		50		50		50		50	
Number of survivors at week 99	22		28		31		25		25	
Blood biochemistry										
ALP (U/L), [No. of mice]	[10]		[10]		[10]		[10]		[10]	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
3 months	46	5.9	51	16.5	54	21.7	54	23.4	78	100.2
6 months	47	16.3	58	29.5	49	18.7	44	7.8	63	50.1
12 months	41	17.8	43	13.6	37	12.2	39	21.8	120	107.4
13[b] months	74	40.5	53	21.0	44[a]	15.5	49	17.2	83	78.9
18 months	46	27.3	54	32.5	45	21.9	35	9.4	71	58.4
24 months	34	13.3	41	24.0	52	32.8	34	17.3	47	32.9
ALT (U/L), [No. of mice]	[10]		[10]		[10]		[10]		[10]	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
3 months	50[a]	13.6	44	12.6	40	12.0	45	16.3	54	16.6
6 months	34	8.4	41	22.9	58	81.8	60*	30.4	67**	29.2
12 months	41	17.4	43	18.6	39	14.7	60	48.7	116*	82.5
13[b] months	77	75.6	70	74.9	49	33.3	54	39.4	48	34.5
18 months	51	47.4	92	110.5	32	14.1	48	30.0	100	109.2
24 months	35	18.6	81	111.1	29	15.6	33	16.7	81	75.9

	Dose (ppm)				
	0	**3**	**10**	**50**	**100**
Histopathology [d]	**Carcinogenicity phase**				
Gall bladder [No. of mice]	[45]	[46]	[46]	[44]	[46]
Cholecystitis	1 (0/1)	1 (1/0)	0	2 (2/0)	11 (10/1)
Hyperplasia	0	0	0	0	6 (5/1)
Liver [No. of mice]	[46]	[48]	[47]	[44]	[46]
Bile duct hyperplasia	0	1 (0/1/0)	2 (1/1/0)	0 (0/0/0)	4 (0/3/1)
Cholangiofibrosis	1 (1/0/0)	0	2 (2/0/0)	3 (3/0/0)	8 (2/5/1)
Hepatitis, chronic	3	2	2	4	8
Bile stasis	1 (1/0/0/0)	1 (0/1/0/0)	1 (0/1/0/0)	2 (0/2/0/0)	12 (2/5/4/1)
Hepatocellular:					
adenoma	3	7	3	1	3
carcinoma	2	3	2	2	5
as above, combined	5	10	5	3	8

Acetylcholinesterase activity in males (% of control shown in brackets)

Number of mice	[10]		[10]		[10]		[10]		[10]	
Erythrocyte AChE activity (μM/mL per minute)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
3 months	2.2	0.45	2.3	0.93	2.1	0.47	1.7 (77)	0.85	1.2** (55)	0.61
6 months	3.3	1.33	3.2	0.87	2.9	1.19	2.8 (85)	0.55	2.2 (67)	0.84
12 months	4.5	1.21	4.3	1.33	4.1	1.51	3.3 (73)	1.27	2.8* (62)	1.50
13[c] months	[4.2]	2.28	3.4	0.83	3.1[a]	0.59	3.5 (83)	1.26	5.3[a] (126)	4.00
18 months	3.3[a]	2.15	2.8	0.66	3.0[a]	0.55	2.4 (72)	0.70	3.0 (91)	2.21
24 months	3.7	0.85	4.4	0.97	3.5	1.47	2.8b (76)	0.72	3.0 (81)	0.57
Brain AChE activity (μM/mL per minute)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
3 months	25.5	2.15	27.8	4.26	27.3	1.92	21.8 (85)	3.75	15.5** (61)	4.22
6 months	23.5	2.74	25.2	3.34	23.1	4.87	17.9* (76)	3.92	14.7** (63)	5.30
12 months	22.4	3.18	23.2	4.55	22.1	2.58	19.5 (87)	3.52	11.5** (51)	2.96
13[c] months	26.6[a]	2.36	25.9	2.33	26.9[a]	2.36	24.7 (93)	2.87	22.5[a] **(85)	3.43
18 months	23.8	4.11	23.7	4.77	25.4	4.51	21.4 (90)	3.05	15.8** (66)	3.39
24 months	24.6	2.35	26.7	2.16	27.3*	2.28	21.0 (85)	5.69	15.7** (64)	3.92

	Dose (ppm)									
	0		3		10		50		100	
Acetylcholinesterase activity in females (% of control shown in brackets)										
Number of mice	[10]		[10]		[10]		[10]		[10]	
Erythrocyte AChE activity (μM/mL per minute)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
3 months	2.0	0.63	2.1	1.05	2.1	0.85	1.4* (70)	0.62	1.1** (55)	0.30
6 months	3.3	0.64	3.1	0.85	3.2	0.62	2.1** (64)	0.68	2.2** (67)	0.58
12 months	4.2	1.32	4.5	1.38	4.4	1.51	3.4 (81)	1.16	2.4** (55)	0.71
13[c] months	2.7	0.73	2.8	0.67	2.8	0.68	2.7 (100)	0.57	3.1 (115)	1.03
18 months	2.6	0.64	2.6	0.84	2.6	0.56	1.9* (73)	0.32	1.7** (66)	0.47
24 months	3.1	0.51	3.2	0.73	3.1	0.70	2.3* (74)	0.60	2.2** (71)	0.43
Brain AChE activity (μM/mL per minute)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
3 months	25.4	3.87	25.8	3.32	25.0	3.03	24.3 (96)	4.04	19.5** (77)	2.05
6 months	21.7	3.41	23.6	5.24	22.2	3.44	22.5 (104)	2.92	16.5* (76)	2.82
12 months	22.2	3.76	23.6	2.79	21.8	3.21	20.3 (91)	2.18	16.5** (74)	2.38
13[c] months	25.4	2.90	27.7	3.26	25.7	1.84	25.3 (100)	4.38	24.2 (95)	3.57
18 months	24.5	2.95	26.8	3.80	25.3	2.53	22.0 (90)	3.32	19.1** (78)	3.67
24 months	25.3	2.90	23.8	2.88	23.4	2.14	21.2* (84)	4.65	16.7** (66)	4.05

[a] Nine mice examined; [b] Eight mice examined; SD: Standard deviation; Source: Goldenthal, 1986

[c] 13-month interval was one month recovery after 12 months treatment;

[d] Histopathology severity scores; each number in parenthesis subclassifies the incidence of the lesions as to severity as follows: (trace/mild/moderate/severe) when 4 subclassified; (mild/moderate/severe) when 3 subclassified; (mild/moderate) when 2 subclassified;

Statistically significant from control value at: * $p < 0.05$ ** $p < 0.01$

The NOAEL for two-year chronic toxicity in mice was 10 ppm (equal to 1.4 mg/kg bw per day for males, 1.6 mg/kg bw per day for females) based on evidence of hepatotoxicity from blood biochemistry and histopathological findings in males, and decreases in erythrocyte AChE activity in both sexes at 50 ppm (equal to 6.7 mg/kg bw per day).

The NOAEL for carcinogenicity in mice was 10 ppm (equal to 1.4 mg/kg bw per day) based on increased incidence of hepatocellular adenoma and carcinoma, and a combined incidence of these tumours in males at 50 ppm (equal to 6.7 mg/kg bw per day) . No carcinogenicity was observed in female mice (Goldenthal, 1986).

A study on the mechanism of liver tumour induction in male mice was conducted. Methidathion (purity 99.5%; batch No. ZE1607-4C4M) was administered for 4, 7 and 14 days to male CD-1 mice (eight mice per group) at dietary concentrations of 0, 100 and 300 ppm (equal to 0, 13.7 and

37.5 mg/kg bw per day). The CD-1 mice were of the same strain as in the two-year study in mice, and the dose concentration of 100 ppm was the same as the dose that where liver tumours were seen in the two-year mouse study (Goldenthal, 1986). Phenobarbital sodium (PB) was administered at dietary dose level of 300 ppm (equal to 42.0 mg/kg bw per day) as a positive control. At each time point liver tissues (left lobe) were collected from all necropsied animals to measure mRNA of hepatic enzymes involved in phase I drug metabolism, including Cyp1a2, Cyp2b10, Cyp3 and Cyp4a14, using reverse transcription in a quantitative polymerase chain reaction (qPCR). Cell proliferation activity in the liver was measured on day 4. The number of cells that were positive for proliferating cell nuclear antigen (PCNA) was counted immunohistochemically in approximately 1000 hepatocytes from each of the mice in the control, methidathion-treated and PB groups. No measurements were conducted at other time points. No routine histopathological examination was conducted. This study was conducted in compliance with GLP. There was no applicable guideline available for this study.

No treatment-related changes were apparent in mortality, general clinical observations or body weight. Feed consumption was decreased (statistically significant) at 300 ppm after one and two weeks. At necropsy the livers were seen to have darkened in all mice at 300 ppm on day 4. Liver weight showed a significant increase, or at least a tendency to increase, at all time points in both treatment groups. In the analysis of hepatic metabolism enzyme mRNAs, there were significant increases after 14 days of treatment in the mRNA of Cyp2bl0 by five-fold, 10-fold and 50-fold compared to controls in the100 ppm, 300 ppm methidathion, and the 300 ppm PB groups, respectively. Enzymes Cyp1a2, Cyp3a11 and Cyp4a14 showed no significant increase at any time point in any of the methidathion-treated groups, although Cyp1a2 increased two-fold in the PB group. On day 4 PCNA-positive hepatocytes were increased 14-fold and 20-fold in the 100 and 300 ppm methidathion groups, although the increase was only five-fold for the 300 ppm PB group. This study suggested very short-term dietary treatment with methidathion induced Cyp2b10 and cell proliferation (Kitazawa, 2017)

The study author proposed that patterns of metabolic enzyme induction and cell proliferation activity in the methidathion-treated groups were similar to those in resulting from PB. However, wide discrepancies were apparent between the toxic profiles observed in the two-year mouse study (Goldenthal, 1986) and what was proposed. Major toxicological changes in the two-year mouse study mainly indicated damage to the hepatobiliary system, such as the gall bladder and liver, and related inflammatory changes. By contrast, the proposal was for a similar pattern to the effects of PB, the typical morphological feature of which is liver hypertrophy. Both treatments increased cell proliferation activity as seen on day 4, but there were no data provided on the effect of more prolonged treatment. Taken together, the MOA of liver tumour induction by methidathion was considered to be different to that of PB. Although the precise MOA remains undetermined, continuous stimulation by damage to the hepatobiliary system might be postulated as one possible MOA.

Rat

A study was conducted to investigate chronic effects and carcinogenicity due to methidathion (purity 97.3%; batch No. 533131/M202444) in Sprague Dawley rats. A group of 80 male and 80 female rats were administered methidathion in the diet for two years at concentrations of 0, 4, 40 or 100 ppm (equal to 0, 0.16, 1.72 and 4.91 mg/kg bw per day for males, 0, 0.22, 2.20 and 6.93 mg/kg bw per day for females) . Interim termination was conducted at 52 weeks (10 rats per sex) and 93 weeks (five rats per sex). All rats were monitored for survival and clinical signs. Body weight, food and water consumption were recorded. Ophthalmology, haematology, blood biochemistry, urinalysis, organ weights, and incidence of palpable tissue masses at necropsy were recorded as well as a histopathological examination of neoplastic and non-neoplastic lesions. Blood was collected from the orbital sinus plexus after overnight fasting under light ether anaesthesia. Haematological and/or blood biochemical examinations were conducted at weeks 26, 52, 78, 93 and 104. Erythrocyte AChE activity was measured at weeks 26, 52, 78, 93 and 104, and brain AChE activity at weeks 52, 92 and 104.

Survival rates were comparable for all treated groups and controls. An increased incidence of alopecia, hypersensitivity to touch, lesion/sores and tremors was observed and regarded as treatment-related clinical signs in males and/or females at 40 ppm and above. An increased incidence of chromorhinorrhea, fasciculation and hyperactivity were observed in males and/or females at 100 ppm. At 100 ppm body weight gains were suppressed from weeks 72 to 92 in males, and throughout the study in females.

Haematological examination revealed slight anaemia in females at 100 ppm. Blood biochemistry results indicated that at 100 ppm cholesterol was slightly decreased in males and total protein decreased in both sexes. Increased neutrophils and decreased lymphocytes were found in females. However, leukocyte counts were not increased. Therefore the changes in neutrophils and lymphocytes were not considered to be treatment-related.

Erythrocyte and brain AChE activities were consistently decreased in both sexes at 40 ppm and/or 100 ppm.

At 12 months relative liver weights were increased in males at 40 ppm and above. This increase was not considered to be toxicologically significant due to the absence of any other changes indicating hepatotoxicity, and no similar increase being observed at the termination. In the carcinogenicity group, incidences of the accumulation of foamy macrophages in the lung, and skin lesions including chronic purulent inflammation, acute purulent inflammation and ulcer were increased with statistical significance in both sexes at 100 ppm. The incidence of tumours was not increased in either sex by treatment with methidathion.

The results of measurements of AChE in erythrocytes and the brain, and treatment-related histopathological changes are summarized in Tables 12a and b.

Table 12a. Summary of erythrocyte and brain ChE findings in a two-year feeding study in rats treated with methidathion (Mean ± standard deviation)

		Dose (ppm)			
		0	**4**	**40**	**100**
				Males	
Erythrocyte AChE (mU/mL)					
26 weeks	[*n* = 10]	2490 ± 82.26	2670 ± 47.26	2280 ± 62.89	2130 ± 115.52** (86)
52 weeks	[*n* = 10] [a]	2442 ± 56.36	2435 ± 52.45	2090 ± 22.83	**1860 ± 54.48** (76)**
78 weeks	[*n* = 10]	2060 ± 63.6	2170 ± 55.88	1933,3 ± 55.28	1670 ± 73.11** (81)
93 weeks	[*n* = 5]	1296 ± 24.82	1300 ± 52.82	1374 ± 102	1116 ± 72.5 (86)
104 weeks	[*n* = 10]	1985 ± 72.28	1900 ± 107.5	**1550 ± 115.47** (78)**	**1525 ± 62.47**(77)**
Brain AChE (mU/mg)					
52 weeks	[*n* = 10] [a]	3017.8 ± 116.37	3414 ± 111.19	**1759 ± 125.21**(58)**	**1347 ± 41.07**(45)**
93 weeks	[*n* = 5]	2712 ± 78.51	2634 ± 143.97	**1513 ± 79.99**(56)**	**1472 ± 105.26**(54)**
104 weeks	[*n* = 10] [b]	2597 ± 169.97	2389 ± 158.58 (92)	**1283.8 ± 95.65**(49)**	**885.6 ± 61.96**(34)**
				Females	
Erythrocyte AChE (mU/mL)					
26 weeks	[*n* = 10] [c]	1720 ± 126.32	1840 ± 123.11	**1090 ± 127.33** (63)**	**1070 ± 81.72** (62)**
52 weeks	[*n* = 10]	2425 ± 49.14	2379 ± 43.65	2365 ± 208.7	2050 ± 38.04** (85)
78 weeks	[*n* = 10]	2280 ± 59.25	2470 ± 85.70	1940 ± 77.75** (85)	**1760 ± 92.14** (77)**
93 weeks	[*n* = 5]	1348 ± 220	1434 ± 78.84	1204 ± 84.12 (89)	1322 ± 84.52
104 weeks	[*n* = 10] [d]	1875 ± 114.56	1905 ± 159.61	1545 ± 86.98* (82)	1513 ± 56.45** (81)
Brain AChE (mU/mg)					
52 weeks	[*n* = 10]	3423 ± 91.14	2935 ± 190.97** (86)	**1148 ± 82.58** (34)**	**1064 ± 27.13** (31)**
93 weeks	[*n* = 5]	2992 ± 162.63	2825 ± 109.62	**1618 ± 66.44** (54)**	**1180 ± 105.27** (39)**
104 weeks	[*n* = 10]	2718 ± 146.81	2595 ± 179.29 (93)	**1294 ± 109.36** (48)**	**716.8 ± 26.93** (26)**

n: The number of rats examined; AChE: Acetylcholinesterase; Source: Yau, 1986

[a] The number of rats at 0 ppm was nine; [b] The numbers of rats examined at 40 and 100 ppm was eight;

[c] The number of rats at 4 ppm was nine; [d] The number of rats examined at 100 ppm was 11;

( ): Bracketed values are % compared to relevant control value;

Statistically significant from control value at: * $p < 0.05$ ** $p < 0.01$

Table 12b. Summary of histopathological findings in the two-year feeding study in rats treated with methidathion

	Dose (ppm)							
	0		4		40		100	
	M	F	M	F	M	F	M	F
Lung [Number of rats]	[65]	[65]	[65]	[65]	[65]	[65]	**[65]**	**[65]**
Accumulation of foamy cell	9	12	6	8	**12**	**7**	**20***	**25****
Skin [Number of rats]	[65]	[65]	[65]	[65]	**[65]**	**[65]**	**[65]**	**[65]**
Chronic purulent inflammation	2	1	0	1	**3**	**1**	**8***	**2**
Acute purulent inflammation	2	1	4	1	**7**	**5**	**3**	**7***
Ulceration	3	1	1	1	**6**	**2**	**11***	**3**

Statistically significant from control value at: * $p < 0.05$ ** $p < 0.01$ Source: Yau, 1986

The NOAEL for chronic toxicity in rats was 4 ppm (equal to 0.16 mg/kg bw per day) based on the decreases in erythrocyte and brain AChE activity, histopathological changes in the skin and the lung at 40 ppm (equal to 1.72 mg/kg bw per day). The NOAEL for carcinogenicity in rats was 100 ppm (equal to 4.91 mg/kg bw per day), the highest dose tested (Yau, 1986).

2.4 Genotoxicity

An adequate number of studies were performed to assess the genotoxicity of methidathion. There were six in vitro studies and two in vivo studies. Results of the battery of genotoxicity tests are summarized in Table 13.

The genotoxic potential of methidathion was evaluated in two bacterial reverse mutation assays (Newell, 1977; Sudo, 1979), in an in vitro chromosomal aberration test in Chinese hamster ovary (CHO) cells (Strasser, 1990) and in an in vivo bone marrow micronucleus test in mice (Honarvar, 2001). These studies were considered acceptable despite some limitation as listed in the footnote of Table 13; they produced negative results. This foregoing combination of tests fulfils the basic requirements to cover the three genetic end-points (that is, gene mutations, structural and numerical chromosome aberrations). The following additional studies were available and are included in Table 13: rec-assay (Sudo, 1979); in vitro unscheduled DNA synthesis (UDS) assay (Hertner, 1990); in vitro sister chromatid exchange (SCE) assay (Kevekordes et al., 1996); gene mutation in yeasts (Arni 1981); host-mediated assay (Newell, 1977); in vivo nuclear anomaly test (Kevekordes et al., 1996): These studies, generally negative, were not included in the evaluation since they were considered no longer relevant or obsolete as listed in the footnote below. The bone marrow micronucleus test (Kevekordes et al., 1996) was also not included in the evaluation due to its significant limitations.

Overall, the results obtained indicate that there is no concern with respect to the genotoxicity of methidathion.

Table 13. Summary of in vitro and in vivo genotoxicity tests with methidathion

Test system	Test object	Concentration range	purity	Result	Reference
In vitro					
Bacterial mutation assay	*S. typhimurium* strains TA1535, TA1537, TA1538, TA98 and TA100	Experiment 1: 0 10, 50, 100, 500, 1000 and 5000 µg/plate (± S9)	NA	Negative[a]	Newell, 1977
Bacterial mutation assay	*S. typhimurium* strains TA 1535, TA100, TA1537, TA1538, TA98 *E. coli* strain Wp2 Hcr-Try-	0, 10, 50, 100, 500, 1000 and 5000 µg/plate (± S9)	99.95%	Negative[b]	Sudo, 1979

Test system	Test object	Concentration range	purity	Result	Reference
Rec-assay	*Bacillus subtilis* strains H17 and M45	0, 5, 25, 100 mg/mL	99.95%	Negative[c]	Sudo, 1979
Chromosomal aberration test	Chinese hamster ovary cells, strain CCL61	Short-term treatment (–S9): 87.5, 175, and 350 µg/mL Short-term treatment (+S9): 43.8, 87.5 and 175 µg/mL Long-term treatment (–S9): 43.8, 87.5 and 175 µg/mL	96.0%	Negative[d]	Strasser, 1990
Unscheduled DNA synthesis (UDS) assay	Primary rat hepatocytes	Experiments 1 and 2: 1.85, 5.56, 16.67, 50, 100 and 200 µg/mL	Not stated	Negative[e]	Hertner, 1990
Sister chromosomal exchange test	Human lymphocytes	Experiments 1 and 2, (–S9): 0, 1, 10 and 100 µmol/L Experiments 3, (+S9) 0, 1, 10 and 100 µmol/L of rat liver	99.9%	Positive[f] (–S9) Negative (+ S9)	Kevekordes et al., 1996
Gene mutation assay in yeasts	*Saccharomyces cerevisiae*	0, 675, 1250, 2500, 5000 and 10 000 µg/mL (± S9)	93.4%	Inconclusive[g]	Arni, 1981
In vivo					
Host-mediated assay	Male Swiss Weber mice and *S. typhimurium* strains TA1535 and TA1538.	Single oral administration: 0, 10, 20 and 40 mg/kg bw Administration on five consecutive days: 0, 5, 10 and 20 mg/kg bw	Not stated	Negative[h]	Newell, 1997
Nucleus anomaly test	Chinese hamster	0, 17, 34 and 68 mg/kg bw	96.9%	Negative[i]	Hool, 1980
Micronucleus assay in bone marrow cells	Mice	0 plus 27.5 and 25 mg/kg bw; the highest dose for males and females respectively	99.9%	Negative	Kevekordes et al., 1996
Micronucleus assay in bone marrow cells	NMRI mice	0, 7.5, 15 and 30 mg/kg bw	96.3%	Negative[j]	Honarvar, 2001

bw: body weight; S9: The 9000 × *g* supernatant fraction from rat liver homogenate;

[a] Study with limitations due to incomplete set of bacterial strains (*S. typhimurium* TA102 or *E. coli* WP2 uvrA strains missing). Only one experiment was performed (plate incorporation assay).

[b] Only one experiment performed (plate incorporation assay) and two plates per experimental point employed. The Meeting noted that the methods implemented were sufficiently robust to support the results reported.

[c] The test system employed has not received further validation and is presently considered obsolete. The results of this study were not included in the assessment.

[d] Short-term treatment included a three-hour exposure, following by 21 hours in fresh medium with and without S9. Long-term treatment included exposure for 24 hours without S9. Major deviations from current OECD Guideline 473 include scoring 200, instead of 300 metaphases and the use of mitotic index as a cytotoxicity parameter. However, the Meeting noted that the methods implemented were sufficiently robust to support the results reported.

[e] The OECD Guideline 472 for this test was deleted (2 April 2014) following an OECD Council decision, since it was no longer considered relevant. The results of this study were not included in the current evaluation.

[f] The result was considered positive by the authors based on significant increases over concurrent controls at the high-dose level (100 µmol/L). The Meeting considered this conclusion questionable since the increase observed did not reach twice the concurrent negative control value. The Meeting further noted that the OECD Guideline 479 for this test was deleted (2 April 2014) following an OECD Council decision, since it was longer relevant. The results of this study were not included in the assessment.

[g] The test system employed is presently considered obsolete. Results of this study were not included in the current evaluation

[h] The test system employed has not received further validation and is presently considered obsolete. The results of this study were not included in the current evaluation.

[i] The study did not follow internationally accepted scientific protocol or relevant test guidelines and the Meeting concluded that the methods implemented were not sufficiently robust to support the results reported.

[j] The study meets the requirements of the OECD Guidelines for Testing of Chemicals No. 474 Mammalian erythrocyte micronucleus test, adopted 21 July 1997. Major shortcomings includes the scoring of 2000 immature erythrocytes per animal instead of 4000, but five male and five female animals were used. However, the Meeting considered that the methods implemented were sufficiently robust to support the results reported. The observed slight reduction in immature to mature erythrocytes ratio (PCE : NCE ratio) and the death of one female animal at the high dose (30 mg/kg bw, at 48 h) indicate that the bone marrow was systemically exposed.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

A two-generation study was conducted in rats. A group of 15 male and 30 female Sprague Dawley rats were administered methidathion (purity 95%; batch not reported) in the diet at concentrations of 0, 5, 25 or 50 ppm (equal to 0, 0.43, 2.21 and 4.33 mg/kg bw per day in males, 0, 0.47, 2.38 and 4.96 mg/kg bw per day in females) during premating (12 weeks) and mating (three weeks) in both sexes, and gestation (three weeks) and weaning (three weeks) in females, all throughout two generations. Mortality, clinical signs, body weight, feed consumption and reproductive indices were recorded for both of the parental generations. Parental animals and a group of 10 male and 10 female progeny were subjected to complete necropsy. Selected organs/tissues were weighed and examined microscopically. Growth of offspring including in life observations, developmental abnormalities and gross lesion were checked. Neither erythrocyte nor brain AChE measurements were made during this study.

In the parental generations, the treatment did not affect mortality. Significant treatment-related general observations were made in females at 25 ppm and above. The incidence of alopecia was increased in the F0 females at 25 and 50 ppm. During the F1a lactation period, slight and/or intermittent muscle tremors were observed in 2 of 19 dams at 25 ppm and 17 of 22 dams at 50 ppm. During the F2a lactation period, slight and/or intermittent muscle tremors were observed in 15 of 20 dams at 50 ppm. No treatment-related clinical sign were seen in males.

In the F0 generation, no treatment-related change was observed in body weights at premating or postmating. In the F1 generation, body weight was lower (approximately 15%; statistically significant) in males at 50 ppm through week 8 of the premating period. In females of the F1 generation the initial body weights at weaning, in 25 ppm and 50 ppm groups, were lower (statistically significant) than the controls. With the F1a and F2a litters, statistically significant body weight decreases were seen in the dams at 50 ppm on lactation days (LDs) 14 and 21. Body weights of dams fed at 50 ppm during the gestation/lactation periods and LD 0, 4 and 7 were not affected.

There was a statistically significant reduction in absolute and relative ovary weights in F0 and F1 parental females in the 50 ppm group. At 25 ppm, relative ovary weight was reduced to a statistically significant extent. However, on histopathological examination no treatment-related findings were observed in any organs/tissues including the ovaries. No effect related to the decreased ovary weight was observed in the fertility index. Therefore the ovary weight change was not considered to be toxicologically significant.

With regard to reproductive performance analysis, no statistically significant differences were seen in either the F0 or F1 generations, except for the mating index for the F1 generation at 25 ppm and above. The mating index at these doses was lower, and showed a dose–effect relationship, although the mating indexes for all dose groups, including the control group, were under 90% in both generations. The study author reported no effects on reproductive performance. However, the statistically significant depression in mating index at 25 ppm and above in F1 generation was considered treatment-related. The Meeting noted that the low mating index might be a secondary effect due to AChE inhibition of the nervous system, since a decrease in AChE activity in the brain or erythrocytes was observed in both sexes at 30 ppm and above in the short-term rat study (see Table 6).

There were no significant differences in delivery and population data between the treated groups and control group in the F1A generation. Slight depressions in offspring survival were observed at 25 ppm and 50 ppm in the F1a offspring. Statistically significant body weight decreases were observed at 25 and 50 ppm in F1A and F2A offspring. Changes in several organ weights at 50 ppm were considered an effect secondary to reduced body weight in these generations. No treatment-related structural anomalies were observed in F1 and F2 offspring.

General observations related to poor maternal care were observed in F1 and F2 offspring at 25 and 50 ppm. Among the F1 progeny, 22 pups from 2/19 litters at 25 ppm, and 35 pups from 7/23 litters at 50 ppm felt cool to the touch. In addition, 33 pups from three litters at 25 ppm among the F1A offspring, and 11 pups from two litters at 50 ppm among the F1A offspring appeared to be starving. In addition, eight pups from two litters at 50 ppm among the F1A offspring were weak and/or lethargic. Pups from the F2A generation were also affected. Two pups from two litters at 25 ppm, and seven pups from five litters at 50 ppm were weak and/or cool to the touch. Also in the F2A offspring, 29 pups from five litters at 50 ppm appeared to be starving. No macroscopic changes were observed at necropsy in selected F1A and F2A offspring.

Results of the rat multigenerational study are summarized in Table 14.

Table 14. Summary of reproductive toxicity in rats treated with methidathion

	Dose (ppm)			
	0	**5**	**25**	**50**
	PARENTAL ANIMALS			
Clinical signs/antemortem				
Females [Number examined]	[30]	[30]	[30]	[30]
F0, antemortem: alopecia	3	4	7	12
F0 lactation: intermittent muscle tremors	0	0	2	16
F1 lactation: intermittent muscle tremors	0	0	0	15
Body weight (mean ± standard deviation)				
F1 males [Number examined]	[15]	[15]	[15]	[15]
Initial (at weaning)	49.0±8.5	48.3±7.7	40.6±9.0	34.9±9.9**
Week 1	165.3±26.2	143.1±43.3	136.3±26.9	130.1±42.7*
Week 2	224.4±28.4	199.0±46.3	192.7±31.6	184.1±48.3*
Week 4	324.0±28.7	298.2±40.3	288.7±35.8	283.5±51.0*
Week 8	436.1±31.2	416.5±33.6	403.7±45.9	395.9±45.5*
Week 9	455.2±32.5	439.1±34.3	423.5±48.8	417.5±48.1
Premating weight gain	463.7±34.4	448.7±35.5	439.3±52.5	436.2±44.0
Total weight change	568.3±46.8	569.4±51.1	549.8±68.0	539.5±65.6
F1 females [Number examined]	[30]	[30]	[30]	[30]
Initial (at weaning)	45.6±8.6	43.1±8.3	39.4±9.4*	32.3±7.7**
Week 1	121.8±23.5	117.1±29.7	112.5±24.4	111.6±25.4
Premating weight gain	233.1±25.3	238.9±32.3	244.8±44.0	251.5±40.9
Total weight change	200.4±33.8	286.9±38.6	291.8±62.4	299.7±53.3
Organ weight (mean ± standard deviation)				
F0 parent (females) [Number]	[30]	[30]	[30]	[30]
Ovary weight (mg)	99.1±28.7	102.3±28.0	82.7±30.9	77.6±21.5**
F1 parent (females) [No.]	29	29	29	30
Ovary weight (mg)	113.4±25.1	113.8±17.6	103.6±20.3	91.0±17.0**

	Dose (ppm)			
	0	**5**	**25**	**50**
Reproductive performance				
F0 generation				
Mating index (%)	73.2	60.0	65.2	60.9
Fertility index (%)	93.3	83.3	63.3	82.1
Gestation index (%)	100.0	100.0	100.0	100.0
F1 generation				
Mating index (%)	87.9	81,1	**69.0***	**65.9***
Fertility index (%)	86.2	96.7	93.1	79.3
Gestation index (%)	100.0	100.0	88.9	95.7
PROGENY				
Delivery and population				
F0 progeny: [number of dams]	[28]	[25]	**[19]**	**[23]**
Number of progeny, PND 0	13.1±3.41	13.3±3.63	13.7±3.07	13.2±3.29
Viable progeny, PND 7[a]	7.8±0.97	7.7±1.60	7.8±0.69	**7.3±1.85**
Viable progeny, PND 21	7.8±0.97	7.5±1.64	7.0±2.16	6.6±2.39
F1 progeny: [number of dams]	[25]	[29]	**[24]**	**]22]**
Number of progeny, PND 0	12.6±3.40	12.9±3.64	13.0±3.11	12.1±3.24
Viable progeny, PND 4	12.1±3.21	12.2±3.54	12.3±2.95	**8.5±4.71****
Viable progeny, PND 7[a]	7.7±0.89	7.5±1.66	7.7±0.86	5.3±3.44**
Viable progeny, PND 14	7.7±0.89	7.4±1.70	7.7±0.86	4.3±3.48**
Viable progeny, PND 21	7.7±0.90	7.4±1.70	7.6±0.93	4.1±3.50**
F1 progeny: body weights				
PND 0	6.2±0.9	6.2±0.7	6.3±0.8	6.0±0.9
PND 4	9.4±2.1	9.8±1.7	9.0±2.2	8.6±2.1**
PND 7	15.6±3.3	15.9±2.5	14.3±3.6**	13.0±3.4**
PND 14	28.5±5.5	29.2±4.4	25.3±5.6**	21.4±4.7**
PND 21: for males	47.3±9.2	47.5±7.1	40.0±9.1**	33.4±8.7**
PND 21: for females	44.4±8.1	44.8±7.1	40.6±9.0**	31.2±8.1**
F2 progeny: body weights				
PND 0	6.1±0.7	6.1±0.7	5.9±0.8*	5.9±0.8*
PND 4	9.5±1.5	9.2±1.5	8.6±1.7**	7.4±1.6**
PND 7	15.4±2.3	14.9±2.6	138.6±2.8**	10.6±2.9**
PND 14	27.9±3.7	28.2±3.8	23.9±4.9**	19.1±4.4**
PND 21: for males	46.0±6.6	47.5±6.2	39.2±9.5**	28.4±8.4**
PND 21: for females	43.9±6.0	44.7±6.4	35.1±7.3**	26.2±5.5**
General observations				
F1 progeny: [No. litters in F1]	[28]	[25]	[19]	[23]
Cool to the touch	0	0	**2**	**7**
Appeared to be starving	0	0	**3**	**2**
F1 progeny: [No. litters in F1]	[25]	[29]	**[24]**	**[21]**
Weak/cool to the touch	0	0	**2**	**5**
Appeared to be starving	0	0	**0**	**5**

[a] Adjusted on postnatal day 4; Source: Salamon, 1987

Statistically significant from control value at: * $p < 0.05$ ** $p < 0.01$

The NOAEL for parental and offspring toxicity in rats was 5 ppm (equal to 0.47 mg/kg bw per day) based on the clinical signs in lactating mothers during F1 and F2 generations, lowered body weight of females at 25 ppm (equal to 2.38 mg/kg bw per day), and poor maternal care at 25 ppm equal to 2.38 mg/kg bw per day). The NOAEL for reproductive toxicity in rats was 5 ppm (equal to 0.43 mg/kg bw per day) based on lower mating index in the F1 generation at 25 ppm (equal to 2.21 mg/kg bw per day) (Salamon, 1987).

(b) Developmental toxicity

Rat

Two studies on developmental toxicity were conducted in rats.

Methidathion (purity 96.6%; batch No. 533131 M202444) was administered to three groups of pregnant Sprague Dawley rats (25 rats per group) at doses of 0, 0.25, 1.0 or 2.5 mg/kg bw once daily by gavage during gestation days (GDs) 6 to 15. The vehicle was a mixture of 3% aqueous cornstarch with 0.5% Tween 80. The dams were observed for changes in appearance or behaviour. Body weights were recorded on GDs 0, 6, 8, 12, 16, and 20. Feed consumption was recorded for the one week period from day 0 to day 6, and daily thereafter throughout gestation. All surviving dams were euthanized by carbon-dioxide asphyxiation on GD 20 and examined for gross pathology. The fetuses were weighed and examined for gross abnormalities. Approximately half of the fetuses from each litter were placed into Bouin's fixative for subsequent visceral examination. The remaining half of the litter was placed in 95% ethanol for skeletal examination. Fetal dissection and visceral examination were performed. Neither erythrocyte nor brain AChE measurements were conducted in this study.

Treatment-related mortality was limited to the death of one dam on GD 12 at 2.5 mg/kg bw per day. Clinical signs attributable to treatment were: lethargy (24/25); tremors (25/25); salivation (12/25); lacrimation (4/25); exophthalmia (10/25); raspy respiration (3/25); vaginal bleeding (4/25), and chromodacryorrhea (2/25), all at 2.5 mg/kg bw per day. Treatment-related reductions in food consumption were observed at 2.5 mg/kg bw per day from GD 8 to GD 17. Treatment-related decreases in body weight and body weight gain compared to controls were observed during gestational days in the group receiving 2.5 mg/kg bw per day.

Treatment-related effects were not observed in reproduction or embryo/fetus parameters, including post-implantation loss, pregnancy rate, number of corpora lutea, number of implantation sites or number of dead fetuses. Fetal weight and fetal sex ratio were comparable among control and treatment groups. No treatment-related gross, visceral or skeletal malformations were observed in the fetuses.

The NOAEL for maternal toxicity in rats was 1.0 mg/kg bw per day based on cholinergic clinical signs, decreased body weight gain and reduced food consumption at 2.5 mg/kg bw per day. The NOAEL for embryo/fetal effects was 2.5 mg/kg bw per day, the highest dose tested. No teratogenic effect was observed (Mainiero, 1987).

Another developmental toxicity study in rats (Fritz, 1976) was reported in a very brief form. The Meeting judged that the information obtained from this study report was inadequate for the evaluation of developmental toxicity.

Rabbit

Two studies of developmental toxicity in rabbits were reported.

In the first study, methidathion (purity 92.6%; batch No. OP. 26-768) was administered by gavage to pregnant Chinchilla rabbits (20 does/group) from GD 6 until GD 18 inclusive. The daily doses were 0, 0.5, 1.5 or 3.0 mg/kg bw. During the period of treatment, clinical signs, body weight and body weight gains were checked daily. Feed consumption was measured on GDs 6, 11, 15, 19, 24 and 28. Does were euthanized by cervical dislocation and fetuses removed by caesarean section on GD 28. At necropsy, the numbers of corpora lutea, mucosa and contents, including amniotic fluid and placentae as well as abortions and resorption sites in the uterus were countered. The removed fetuses were weighed, checked for external changes and subjected to visceral and skeletal examination. Neither erythrocyte nor brain AChE measurements were conducted in this study.

No treatment-related changes in mortality, clinical signs, body weight, body weight gain, food consumption or gross pathology were observed in does. The two does, one each at 0 and 1.5 mg/kg bw per day were found dead on days 7 and 25, respectively. The cause of the control death was an intubation error. The cause of death for the doe at 1.5 mg/kg bw per day was reported as spontaneous; no further explanation was given. Values for lethality of embryo/fetuses (resorptions) were comparable between all groups. The mean numbers of corpora lutea and of implantation sites, as well as the ratio of corpora lutea to implantation sites were also comparable between all groups, as were fetal body weights and sex ratios. Fertility was low in all groups, including controls: 78.9%, 50%, 73.7% and 50% at 0, 0.5, 1.5 and 3.0 mg/kg bw, respectively.

Gross and/or visceral examination of live fetuses revealed one multiple malformation (aneucephaly and craniofacial dysplasia, such as absence of a jaw) at 3.0 mg/kg bw per day. A single occurrence of the multiple malformation observed in one fetus from one litter was considered to be incidental and not treatment-related. Similar malformations were not observed in another study (Hummel et al., 1987) conducted at higher doses (6 and 12 mg/kg bw per day), although the strain used was different from this study. In skeletal examinations, irregular ossification of sternebrae was observed in one fetus from each group including the controls. Instances of sternebral irregular ossification found in all experimental groups were not considered treatment-related. Abnormalities and malformations are shown in Table 15.

Table 15. The list of abnormalities and malformations observed in developmental toxicity study of methidathion in Chinchila rabbits

		Dose (mg/kg bw per day)			
		0	**0.5**	**1.5**	**3.0**
Abnormality[a]					
Irregular-shaped 5th sternebra	Affected litters/total litters	ND	1/10[#]	1/14	1/9
	Affected fetuses/all live fetuses (%)		1/84[#] (1.2)	1/114 (0.9)	1/77 (1.3)
Irregular-shaped 1st sternebra	Affected litters/total litters	1/15	ND	ND	ND
	Affected fetuses/all live fetuses (%)	1/127 (0.8)	-	-	-
Irregular-shaped 6th sternebra	Affected litters/total litters	1/15	ND	ND	ND
	Affected fetuses/all live fetuses (%)	1/127 (0.8)	-	-	-
Asymmetrically ossified sternum	Affected litters/total litters	1/15	-	-	-
	Affected fetuses/all live fetuses (%)	1/127 (0.8)	-	-	-
Irregular ossified sternum	Affected litters/total litters	ND	1/10	ND	ND
	Affected fetuses/all live fetuses (%)	-	1/84 (1.2)	-	-
Partial synostosis of sternum	Affected litters/total litters	ND	ND	1/14	ND
	Affected fetuses/all live fetuses (%)	-	-	1/114 (0.9)	-
Malformation[b]					
Multiple malformations	Affected litters/total litters	ND	ND	ND	1/9
	Affected fetuses/all live fetuses (%)	-	-	-	1/77 (1.3)

ND: Not detected; Source: Giese, 1981

[a] In cumulative control data (51 months Nov 1976–Feb 1981), hypoplasia of the heart was 1 in 1504 live fetuses; total incidence of sternebral irregular ossifications (supernumerary sternebra between sternebra 5 and 6, synostosis of sternebrae 4 and 5, partial synostosis of rib 4 and 5 and irregular ossification of 6th sternebra) was 5/1504 (0.33 %).

[b] Multiple malformation characterized by aneucephaly, craniofacial dysplasia (absence of upper jaw), hypoplasia of heart, skeleton generally poorly ossified

The NOAEL for maternal toxicity and embryo/fetal toxicity in rabbits was 3.0 mg/kg bw per day, the highest dose tested. Methidathion showed no teratogenicity in rabbits (Giese, 1981)

In another developmental toxicity study in rabbits, a group of pregnant New Zealand White rabbits (19 does/group) were administered methidathion (purity 96.6%; batch No. 53313 M202444) by gavage at dose levels of 0, 2, 6 or 12 mg/kg bw per day from GD7 to GD 19, inclusive. Methidathion was suspended in 3% cornstarch containing 0.5 % Tween 80 as a vehicle; the volume administered

was 5.0 mL/kg bw per day. The does were checked for changes in general appearance, behaviour and mortality. Body weights were measured on GDs 0, 7, 10, 14, 20, 24, and 29, and food consumption daily from GD 5 to GD 29. All surviving does were necropsied on day 29 of presumed gestation following an injection of T-6l euthanasia solution. The ovaries were examined and the corpora lutea were counted. The uteri, including their contents, were weighed. Live fetuses, dead fetuses and intrauterine resorption sites were counted. Implantations were numbered in order of their position in the uterus. Viable fetuses were weighed, examined for gross abnormalities and placed in 95 % ethanol. Visceral examination for organs of various system and skeletal examination were performed. Neither erythrocyte nor brain AChE activities were measured in this study.

There were no treatment-related deaths in this study. One doe in each of the 2, 6 and 12 mg/kg bw per day groups was euthanized prior to scheduled necropsy due to abortion on GDs 20, 23 and 26, respectively. Two does were terminated due to a dosing incident and occurrence of a back injury. Treatment-related clinical signs were observed at 12 mg/kg bw per day. The signs (statistically significant) were increased incidences of ataxia, salivation and tremors. These clinical signs were observed shortly after dosing. In addition, isolated incidences of miosis and blood in the cage pan were observed at the same dose; these were considered to be treatment-related. No compound-related effect on food consumption was observed in this study. No statistically significant differences in maternal body weight, or maternal body weight gain were observed among controls and treated groups. No treatment-related effects on reproductive parameters, including post-implantation loss, pregnancy rate, number of corpora lutea, number of implantation sites or number of dead fetuses were observed.

No treatment-related changes were observed in either fetal body weights or fetal sex ratio. A total of 476 fetuses from 60 litters were examined externally for the presence of external malformations and/or variations. One fetus at 2 mg/kg bw per day showed hyperflexure of the forepaws. This malformation was the only external malformation seen; due to lack of dose-dependency it was not considered to be treatment-related. A total of four fetuses from four litters were found to have visceral malformations and one additional fetus was found to have a visceral variation. Two fetuses in the control group showed renal malformations: bilateral renal agenesis in one fetus and a hollow and misshapen kidney in the other. Two other fetuses showed malformations of the head region: hydrocephalus in one at 2 mg/kg bw per day, and microencephalia in one at 12 mg/kg bw per day. A rudimentary omphalomesenteric duct observed in one control group fetus was the only visceral variation seen in this study. All of these malformations and the one variation were considered to be spontaneous in nature.

The NOAEL for maternal toxicity in rabbits was 6 mg/kg bw per day based on cholinergic clinical signs observed immediately after dosing at 12 mg/kg bw per day. The NOAEL for embryo/fetal effects in rabbits was 12 mg/kg bw per day, the highest dose tested. Methidathion showed no teratogenicity in rabbits. (Hummel et al., 1987).

2.6. Special studies

(a) Neurotoxicity

Acute neurotoxicity

An acute neurotoxicity study was conducted in Sprague Dawley rats, ca eight-weeks old at start of study. A single dose of methidathion (purity 93.2%; lot No. FL-921219) was administered by gavage to a group of 20 or 25 rats per sex at doses of 0, 1, 4, 8 or 16 mg/kg bw after overnight fasting. Corn oil was used as vehicle. A group of 10 rats per sex was given a single injection intraperitoneally at 30 mg/kg bw of carbaryl (SEVIN®, batch No. 29-144A) as positive control AChE inhibitor. The methidathion-treated and vehicle control rats were fasted for approximately 18 hours prior to dosing. The rats injected with carbaryl were not fasted. Body weight, food consumption, and clinical observations were recorded. Complete functional observation battery (FOB) and figure-B maze activity counts were performed at pretest, 1.5 hours after dosing for methidathion and 0.5 hour after dosing for carbaryl, as well as seven and 14 days after dosing. The peak time, 1.5 hours, was selected based on the peak effect having occurred around two hours after dosing when cholinergic clinical signs were found in two previous acute studies. The peak time post dosing. of 1.5 hours was selected for erythrocyte AChE activity, which was measured at pretest, time of peak effect, and 14 days post dosing. Blood was collected from the orbital plexus of unfasted animals at pretest and 14 days post dosing (week 2). The blood at peak-time

was withdrawn from the rats that did not receive access to food between the time of dosing and the sampling at time of peak effect (around two hours). AChE activity in regional brain samples including the cerebellum, striatum, and cerebral cortex was measured at the time of peak effect, then one and two weeks post dosing. Necropsy and histopathological examination were performed on selected tissues from the nervous system.

All animals survived to termination at14 days. Cumulative body weight gain was significantly lower in males at 16 mg/kg bw than for controls. Food consumption significantly decreased in both sexes at 16 mg/kg bw during the first week post dosing. Treatment-related effects were seen to show a dose–effect relationship only at the time of peak effect were: clinical observations, FOB parameters, and figure-B maze activity counts. These effects were observed in males at 8 mg/kg bw and above and in females at 4 mg/kg and above, and were attributed to AChE inhibition. They consisted of autonomic signs (impaired respiration, lacrimation and salivation), central nervous system signs (tremors, reduced arousal, and decreased activity, convulsions, muscle fasciculation and repeated opening/closing of mouth), effects on the sensorimotor system (decreased touch and tail-pinch response), impaired neuromuscular functions (ataxic and/or abnormal gait, impaired righting reflex, reduced hindlimb extensor strength, and reduced forelimb and hindlimb grip strength) and depressed body temperature. Similar effects were observed in the rats treated with carbaryl.

Measured AChE activities were markedly inhibited at the time of peak effect, with recovery apparent at two weeks after dosing. At the time of peak effect, the activity of erythrocyte and brain AChE was inhibited in both sexes at 4 mg/kg bw and above. The decreases observed in both sexes at 4 mg/kg bw were considered to be treatment-related. At 1 mg/kg bw the cerebral cortex AChE in males was decreased by 20% or more compared to negative control values, with dose-dependency at the time of peak effect. Changes in erythrocyte and brain AChE are summarized in Table 16.

Table 16. Erythrocyte and brain* acetylcholinesterase (*AChE) activity in an acute neurotoxicity study of methidathion in rats.

		Males (dose in mg/kg bw)					Females (dose in mg/kg bw)				
		0	**1**	**4**	**8**	**16**	**0**	**1**	**4**	**8**	**16**
Erythrocyte AChE (U/L)											
Pretest	Mean	1608	1528	1572	1578	1539	1684	1676	1680	1718	1696
	SD	228	280	195	209	197	241	195	276	155	235
1.5 hours[a]	Mean	1530	1482	646**	394**	248**	1852	1690	610**	336**	260**
	SD	242	295	273	148	74	384	411	307	103	100
	%	100	97	**42**	**26**	**16**	100	91	**33**	**18**	**14**
Week 2	Mean	1488	1516	968*	1224	1084	1548	1456	1440	1208	1252
	SD	381	366	107	249	212	322	196	203	174	342
Cerebellum AChE (U/g)											
1.5 hours[a]	Mean	3.13	2.74	1.48**	0.99**	0.69**	3.10	2.69	1.22**	0.77**	0.53**
	SD	0.39	0.21	0.41	0.16	0.12	0.14	0.66	0.18	0.07	0.19
	%	100	88	**47**	**32**	**22**	100	87	**39**	**25**	**17**
Week 2	Mean	3.31	3.20	3.26	3.09	2.79	3.10	2.95	2.95	2.84	2.81
	SD	0.55	0.27	0.99	0.31	0.36	0.40	0.36	0.34	0.52	0.10
Cortex AChE (U/g)											
1.5 hours[a]	Mean	8.22	4.81**	2.60**	1.01**	0.51**	7.78	6.74	2.26**	0.82**	0.50**
	SD	1.91	1.18	0.64	0.71	0.17	3.28	1.07	0.63	0.35	0.16
	%	100	**59**	**32**	**12**	**6**	100	87	**29**	**11**	**6**
Week 2	Mean	6.24	8.07	8.09	6.50	5.94	7.26	7.11	7.41	6.63	6.16
	SD	1.13	0.88	1.63	0.94	0.81	1.35	1.85	0.95	1.63	0.90

		Males (dose in mg/kg bw)					Females (dose in mg/kg bw)				
		0	**1**	**4**	**8**	**16**	**0**	**1**	**4**	**8**	**16**
Striatum AChE (U/g)											
1.5 hours[a]	Mean	25.32	27.00	7.08**	4.12**	2.32**	28.96	26.52	7.76**	2.32**	1.52**
	SD	7.55	5.65	5.95	2.56	0.47	4.15	5.82	3.11	1.37	0.77
	%[b]	100	107	**28**	**16**	**9**	100	92	**27**	**8**	**5**
Week 2	Mean	27.95	30.80	37.60	19.12	17.88	15.36	35.84	17.92	28.68	21.52
	SD	19.61	22.04	31.77	12.16	10.99	9.55	24.05	6.97	19.71	5.72

SD: Standard deviation; Source, Chang, 1994

[a] 1.5 hours was the time of peak effect for AChE; [b] % represents percentage of relevant control value

Statistically significant from control value at: * $p < 0.05$ ** $p < 0.01$

The Meeting considered that the decrease in cerebral cortex was marginal. The Meeting noted that AChE activity in other regions of the brain and in erythrocytes did not decrease by 20% or more compared to corresponding control values. It was also noted that in the single dose study of Glaza (1994) the AChE in erythrocytes and brain was not decreased 20% or more compared with corresponding control values at the same dose (1 mg/kg bw). Therefore the Meeting considered the decrease in AChE in the cerebral cortex as inconsistent. The NOAEL for neurotoxicity was 1 mg/kg bw based on AChE activity decreases in erythrocytes and the brain at 4 mg/kg bw (Chang, 1994).

A single dose study was conducted to investigate clinical, behavioural and body weight effects (Phase I), and erythrocyte and brain AChE inhibition (Phase II), of methidathion in young adult Sprague Dawley rats.

In Phase I methidathion (purity 93.2%; batch No. 909749) was administered to a group of five males per group at dose levels of 0, 10 or 35 mg/kg bw after an overnight fasting period, and to five females per group at dose levels of 0, 3, 5, 10 or 20 mg/kg bw. The animals were treated at day 0 and necropsied at the end of the observation period (day 14). As a result of observations in Phase 2, Phase 1 was repeated with five males at10 mg/kg bw, and an additional dose level of 5 mg/kg bw was given to five males. Based on the results, five males were treated with 3 mg/kg bw of methidathion. Rats in the control group were not assigned. Corn oil was used as the vehicle.

All males died by day 3 at 35 mg/kg bw, and four females were dead on day 1. Treatment-related clinical signs were miosis at 3 mg/kg bw and above, hypoactivity, tremors and fasciculations at 10 mg/kg bw and above in males. In females, the signs were tremors, excessive salivation, absence of pain reflex and hypoactivity at 10 mg/kg bw. These signs appeared within four hours of dosing. Soft stool was observed within eight hours at 3 and 5 mg/kg bw in females; this was considered due to gavage treatment with corn oil, rather than any toxicity due to methidathion treatment. Recovery from all clinical signs was observed on day 2, except for the rats at 20 and 35 mg/kg bw. On the basis of the treatment-related clinical signs observed in males at 5 mg/kg bw, the NOAEL for clinical signs by single oral administration was 3 mg/kg bw.

In Phase II, methidathion was given to rats (five per sex in each group) by gavage at single dose levels of 0, 0.5, 1.0, 2.5, 5.0 or 10 mg/kg bw after overnight fasting. Corn oil was used as the vehicle. After approximately four hours post dosing the animals were anaesthetized and necropsied. Erythrocyte and brain ChE activity was measured. In the 10 mg/kg bw group, tremors in both sexes, excessive salivation in males and absence of pain reflex in females were observed as treatment-related clinical signs within four hours of dosing. Soft stool was observed within four hours post dosing in one rat from each of the 0, 0.5 and 1.0 mg/kg bw dose groups. The changes were considered to be due to gavage treatment with corn oil, rather than toxicity due to methidathion itself.

Changes in erythrocyte and brain AChE activity are summarized in Table 17. In males, decreases of 20% or more in erythrocyte and brain AChE activity were observed at 5 mg/kg bw and above. In females, erythrocyte AChE activity decreased by 20% or more compared with control values at 5 mg/kg bw and above, and brain AChE activity decreased by 20% or more at 2.5 mg/kg bw and above. All decreases in AChE activity showed dose-dependency.

Table 17. Erythrocyte and brain acetylcholinesterase (AChE) activity in a single oral dose study in rats treated with methidathion.

		Males (dose in mg/kg bw)						Females (dose in mg/kg bw)					
		0	**0.5**	**1**	**2.5**	**5**	**10**	**0**	**0.5**	**1**	**2.5**	**5**	**10**
Erythrocyte AChE (mU/mL)													
4 hours	Mean	1411	1329	1282	1177	1119	1037	944	1107	979	769	559*	618*
	SD	145.1	268.2	386.6	328.0	238.0	190.5	126.6	170.0	126.3	48.9	97.5	134.4
	%[a]	100	94	90	83	**79**	**73**	100	117	104	81	**59**	**65**
Brain AChE (mU/mg)													
4 hours	Mean	1593	1715	1687	1676	1138*	744*	1659	1549	1626	1016*	605*	477*
	SD	260.9	152.6	178.1	423.1	249.3	91.1	63.2	56.6	57.6	102.8	98.9	135.2
	%[a]	100	108	106	105	**71**	**47**	100	93	98	**61**	**36**	**29**

SD: Standard deviation; [a] % represents percentage of relevant control value; Source: Glaza, 1994
* Statistically significant from control value at $p < 0.05$

Subchronic neurotoxicity

This study was newly submitted to the current Meeting.

A subchronic neurotoxic toxicity study was conducted. Methidathion (purity 94.3%; lot No. FL-931472) was administered to a group of 30 male and 30 female Sprague Dawley rats for 90 days at dietary concentrations of 0, 3, 10, 30 or 100 ppm (equal to 0, 0.182, 0.608, 1.86 and 6.36 mg/kg bw per day for males, 0, 0.198, 0.659, 2.01 and 7.19 mg/kg bw per day for females). One group of 10 males and 10 females was administered by gavage five daily doses of acrylamide per week at 16 mg/kg bw as a positive control. The first 10 animals from each group were subjected to a battery of tests to assess neurological function approximately one week prior to test substance exposure, and during weeks 4, 8 and 13 of treatment. Tests included an FOB and motor activity assessment in a figure-8 maze. The remaining 20 animals from each of these groups (satellite animals) were sampled for determination of erythrocyte and serum AChE activity. Subsequently, five of these 20 rats from each group were sampled following seven days of exposure, and during weeks 4, 8 and 13 of exposure, for erythrocyte and serum AChE activity as well as regional brain and spinal cord AChE activity. In addition, the same procedure was performed on rats from the positive control group. Blood was collected from the orbital plexus under isoflurane anaesthesia in unfasted animals.

No animal died during the study. Treatment-related clinical signs were observed only in females at 100 ppm. These females showed infrequent stool (seen in five animals during the second half of the study), transient tremors (mostly in weeks 2 and 3), and chromorhinorrhea. Body weights in males were not affected by the treatment. Females at 100 ppm showed dramatic decreases in body weight change during the first two weeks of treatment. This effect receded and the body weights in this group were comparable to those of the controls from the fourth week onwards. No treatment-related effects in either sex were observed in food consumption or by ophthalmoscopy. With regard to neurological examinations, in males findings from the FOB examinations were not attributable to treatment. In females, effects on neuromuscular, central nervous system (CNS) excitability and sensorimotor parameters were found at 100 ppm. There appeared an abnormal gait at week 4, and a persistent (all test intervals) and statistically significant reduction in forelimb and hindlimb grip strength. Tremors, stereotypy (compulsive sniffing), and bizarre behaviour (hyper-responsive) were observed in a considerable number of females in week 8 at 100 ppm. At week 13 three females also became slightly more difficult to handle than the controls. In addition, increases in the response to touch at weeks 4 and 8, click at week 4, and tail pinch at weeks 4 and 8 were also observed in females at 100 ppm. The treatment did not affect figure-8 maze activity.

The change in erythrocyte and brain AChE activity is summarized in Table 18. Inhibition of AChE in erythrocytes and various parts of the brain were observed in both sexes to a similar extent. For both sexes, duration enhancement of the decreased AChE levels was not clear in either erythrocytes or the brain. Statistically significant inhibition of erythrocyte AChE was detected at as low as 10 ppm in both sexes. Inhibition of AChE activity in various CNS regions was seen in both sexes, generally at 30 ppm and above with a few exceptions. At 10 ppm, statistically significant inhibition was also noted in

the male cerebral cortex, female striatum, and female hippocampus. In males at 100 ppm and in females at 30 ppm and above, statistically significant inhibition was noted as early as in week 2. At the lower dose levels in both sexes, statistically significant inhibition was generally seen after a more prolonged period of exposure. The extent of CNS AChE inhibition varied (statistically significant) among regions even within the same sex and the same dose group. In general, the cerebral cortex, striatum and hippocampus were inhibited to a greater extent than the cerebellum and spinal cord in both sexes.

Table 18. The change in erythrocyte (RBC)and brain acetylcholinesterase (AChE) activity in a 90-day neurotoxicity study of methidathion in rats

	Males (ppm)					Females (ppm)				
	0	**3**	**10**	**30**	**100**	**0**	**3**	**10**	**30**	**100**
					Pretest					
No. of rats	20	20	20	20	20	20	20	20	20	20
Mean AChE activity										
RBC, (U/L[a])	1502	1474	1481	1610	1536	1664	1755	1622	1702	1674
					Week 2					
No. of rats	5	5	5	5	5	5	5	5	5	5
Mean AChE activity										
RBC, (U/L[a])	2052	2032	1756	1356**	400**	1860	1772	1708	996**	284**
%[b]			85	**66**	**19**			92	**54**	**15**
Cerebellum (U/g)	3.95	3.66	3.67	3.57	2.29**	3.79	3.71	3.57	2.98**	1.54**
Cortex, (U/g)	8.58	7.67	6.38*	6.65	2.77**	6.34	6.16	6.25	3.82**	1.16**
Ratio (%)			**74**	**77**	**32**			99	**60**	**18**
Striatum (U/g tissue)	29.20	30.24	33.08	26.60	10.12**	35.56	32.72	32.20	19.76**	3.32**
Hippocampus (U/g)	5.76	5.60	5.81	6.24	2.34**	5.07	4.93	4.50	3.05**	0.85**
Spinal cord (U/g tissue)	5.74	5.94	5.18	5.04	2.78**	5.22	5.34	5.40	3.74**	1.56**
					Week 4					
No. of rats	5	5	5	5	5	5	5	5	5	5
Mean AChE activity										
RBC, (U/L[a])	1176	1092	948*	468**	120**	1260	1096	940**	308**	112**
%[b]		93	81	**40**	**10**		87	**75**	**24**	**9**
Cerebellum (U/g)	3.63	3.49	3.48	3.16**	1.69**	3.49	3.61	3.48	2.39**	1.17**
Cortex, (U/g)	8.29	7.14	6.10**	4.62**	1.26**	7.88	6.96	6.77	3.24**	0.84**
%[b]		86	**74**	**56**	**15**		88	86	**41**	**11**
Striatum (U/g tissue)	38.00	33.80	37.40	31.80	6.04**	34.80	36.68	33.44	12.00**	2.20**
Hippocampus (U/g)	6.17	5.91	5.99	5.01**	1.44**	5.76	6.01	5.55	4.08**	0.72**
Spinal cord (U/g tissue)	4.86	5.98	5.64	4.74	1.74**	5.66	6.54	5.28	2.92**	1.10**

	Males (ppm)					Females (ppm)				
	0	**3**	**10**	**30**	**100**	**0**	**3**	**10**	**30**	**100**
					Week 8					
No. of rats	5	5	4	4	5	5	5	5	5	5
Mean AChE activity										
RBC, (U/L[a])	1852	1580	1365**	685**	312**	1612	1712	904**	324**	232**
%[b]		85	**74**	**37**	**17**			**56**	**20**	**14**
Cerebellum (U/g)	3.05	3.11	3.06	2.69	1.48**	3.14	3.19	2.57	2.51	0.63**
Cortex, (U/g)	5.18	5.82	3.75	3.37	1.18**	5.93	6.15	6.04	2.18**	0.65**
Striatum (U/g tissue)	37.80	43.4	33.85	23.05**	6.44**	33.40	33.24	29.32	10.40**	1.00**
Hippocampus (U/g)	6.23	5.57	6.05	4.23**	1.33**	6.21	6.36	5.16	2.01	0.43**
Spinal cord (U/g tissue)	5.28	5.48	4.95	4.23	1.86**	4.76	4.84	4.10	2.26**	0.84**
					Week 13					
No. of rats	5	5	5	5	5	5	5	5	5	5
Mean AChE activity										
RBC, (U/L[a])	1656	1480	1328*	680**	196**	1476	1452	1004**	416**	136**
Ratio (%)		89	**80**	**41**	**12**		98	**68**	**28**	**9**
Cerebellum (U/g)	3.59	3.55	3.07	3.09	1.73**	3.65	3.35	2.96	2.33**	1.16**
Cortex, (U/g)	6.34	5.16	4.78	3.72*	1.23**	7.93	7.37	6.76	2.69**	0.66**
%[b]		81	**75**	**59**	**19**		93	85	**34**	**8**
Striatum (U/g tissue)	37.04	34.40	33.36	21.84**	4.96**	32.56	31.04	20.36**	11.08**	1.20**
Hippocampus (U/g)	5.49	5.79	5.28	4.17**	1.40**	5.96	6.15	4.55**	2.63**	0.56**
%[b]			96	**76**	**26**			**76**	**44**	**9**
Spinal cord (U/g tissue)	4.36	3.74	3.78	3.34*	1.02**	3.82	4.14	3.78	2.46**	0.66**

[a] Erythrocyte AChE activity measured as units per litre of packed red blood cells (RBC) (Source, Chow,1995)

[b] % represents percentage of relevant control value

Statistically significant from control value at: * $p < 0.05$ ** $p < 0.01$

The NOAEL for the 90-day neurotoxicity study in rats was 3 ppm (equal to 0.182 mg/kg bw per day) based on the decrease in AChE activity in the brain cortex at two weeks, cortex and erythrocytes at four and 13 weeks, and erythrocytes at eight weeks in males, and in females erythrocytes at four and eight weeks, and erythrocytes and hippocampus at 13 weeks, all of these effects at 10 ppm (equal to 0.608 mg/kg bw per day) (Chow, 1995).

Delayed neurotoxicity

A delayed neurotoxicity study was conducted using domestic chicken hens. The hens were treated with methidathion (purity 96.5%; batch No. OP709514 FL890331) to calculate the LD_{50} and to investigate any potential delayed neurotoxicity.

In advance of examining the LD_{50} for methidathion in the main study on potential acute delayed neurotoxicity, various pretests were conducted, including a positive control confirmation test, a dose range-finding study to help select doses for the main LD_{50} determination, development of protection

schedules and an initial study administering 165 mg/kg bw of methidathion to 20 hens on day 0 and treating the surviving six hens with the test substance again on day 21. The initial study was terminated prior to completion on day 26 due to insufficient number of surviving hens.

In the main study, the test group (60 hens) received 145 mg/kg bw of methidathion in corn oil on days 0 and 21 by gastric intubation. The vehicle control (10 hens) received corn oil on days 0 and 21. The positive control group (eight hens) received an intramuscular injection of 500 mg/kg bw of tri-*O*-cresyl phosphate (TOCP; a known delayed neurotoxicant), on day 0. Atropine (100 mg/kg bw) administered by intramuscular injection was used to provide protection from acute cholinergic effects by treating all animals at 5, 20.5, 25.5, and 29 hours after dosing.

All hens were observed at least once daily for signs of toxicity. In addition, each hen was observed and scored for signs of delayed neurotoxicity beginning five days after dosing. Positive control hens were necropsied on day 22, and all surviving treated and vehicle control hens were necropsied on day 42.

Twenty-two treated hens were found dead by day 4 of the study. One positive control hen showed slight unsteadiness in walking on day 7, and by day 14 all positive control hens exhibited some signs of delayed neurotoxicity. There were eight treated hens with sporadic and varying degrees of unsteadiness, but only one hen showed any persistent symptoms.

Since no definitive neurotoxic response was noted in the test group a second dose was administered on day 21. Six treated hens were found dead within two days of dosing, but no definitive signs of delayed neurotoxicity were noted during the three weeks following the second dose.

At necropsy no remarkable findings were recorded for any of the hens terminated on day 22 or day 42. With regard to the histopathological examination, no lesions were found in the treated hens terminated on day 42.

The LD_{50} for methidathion in unprotected hens was 132.5 mg/kg bw. There was no evidence of delayed neurotoxicity from methidathion after administration of two doses, 145 mg/kg bw each, which exceeded the LD_{50} (Kuhn, 1989).

(b) Immunotoxicity

There was no available information on the immunotoxicity of methidathion.

(c) Studies on metabolites

Studies submitted addressed acute oral toxicity in rats and inhibition effects on cholinesterase.

Acute toxicity of metabolites

In the open literature on metabolism of methidathion, information about acute toxicity and in vitro AChE inhibitory effects of its metabolites was obtained, but such information was limited. The information described here comes from same study as the metabolism covered in section 1.2 (Dupuis, Muecke & Esser, 1971). In addition to the parent and four metabolites found in rats, RH (GS-12956), RH-sulfoxide (GS-28368), RH-sulfide (GS-28370), RH-sulfone (GS-28369), one plant metabolite, the oxygen analogue of methidathion (GS-13007) was investigated. For oral acute toxicity in rats, no further information to that shown in Table 19 was obtained.

The LD_{50} of the parent compound was similar to that reported in other studies (see acute toxicity). The LD_{50} of the oxygen analogue of methidathion was 10 mg/kg bw, indicating that its acute toxicity is approximately four-folds greater than that of the parent. No detailed information on the study design of this test was obtained from the literature. The other metabolites (RH, RH-sulfide, RH-sulfoxide, RH-sulfone and RH) showed weak acute toxicity, with approximately 2.5% to 10% of the parent compound's toxicity (Dupuis, Muecke & Esser, 1971).

The oral LD_{50}s of methidathion and its metabolites are summarized in Table 19.

Effect of metabolites on acetylcholinesterase inhibition in vitro

In the same report on the acute oral toxicity study of metabolites described above, inhibition of AChE by methidathion and its metabolites was also examined. Measurement of inhibition, expressed as an IC_{50}, represented the molecular concentration of inhibitor causing 50% inhibition of an enzyme derived from bovine erythrocytes (obtained from Sera, Heidelberg, Germany). No further information was obtained from the literature.

The inhibitory activity in vitro of methidathion and its metabolites is summarized in Table 19. The in vitro inhibitory action of the oxygen analogue (GS-130007) according to its IC_{50} displayed an effect around three orders of magnitude greater (5.4×10^{-7}) in bovine erythrocytes than the parent, for which the IC_{50} was greater than 10^{-4}. The other four metabolites demonstrated weaker inhibition than the oxygen analogue or parent methidathion. The RH compound (GS-12956) displayed approximately 1000-fold lower inhibition than the oxygen analogue, while RH-sulfide, RH-sulfoxide and RH-sulfone had an IC_{50} for AChE around 1/100 of that for the parent (Dupris, Muecke & Esser, 1971).

Table 19. Oral LD_{50} in rats and in vitro effects on inhibition of acetylcholinesterase (AChE) by methidathion and its metabolites

Compound	Where found (data from FAO)	Code name	IC_{50} for inhibition of AChE (M)	Acute oral toxicity in rats: LD_{50} (mg/kg bw)
Methidathion	Parent compound	GS-13005	$>1 \times 10^{-4}$	35
Oxygen analogue	Plants	GS-13007	$>5.4 \times 10^{-7}$	10
RH compound	Plants/animals	GS-12956	$>6.0 \times 10^{-3}$	750
Sulfoxide	Plants/animals	GS-28368	$>1 \times 10^{-2}$	1110
Sulfide	Plants/animals	GS-28370	$>1 \times 10^{-2}$	1750
Sulfone	Plants/animals	GS-28369	$>1 \times 10^{-2}$	535

Source: Dupuis, Muecke & Esser, 1970

The study provided no information about the relationship between the AChE inhibitory effects seen from in vitro tests and data obtained from in vivo studies. However, the AChE inhibitory effects of the oxygen analogues of other organophosphorus compounds are generally stronger than that of their parents. If the greater acute toxicity seen here for the oxygen analogue of methidathion is in general related to AChE inhibitory effects, the oxygen analogue of methidathion might be expected to have around four times as strong an inhibitory effect on AChE activity as its parent in vivo. However with oral exposure the parallel between the two values will also depend on the respective toxicokinetics of the two compounds.

Genotoxicity of metabolites

In silico predictions of the genotoxicity (bacterial gene mutation) of methidathion and its metabolites were evaluated in two studies.

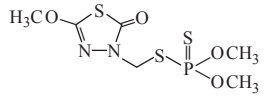
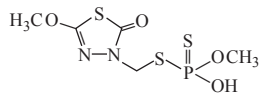
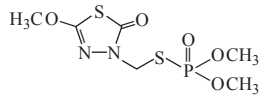
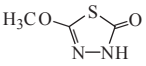
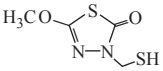
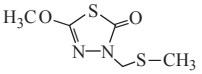
In the first study, using quantitative structure activity relationship, (QSAR), the genotoxic potential of methidathion, desmethyl methidathion and the oxygen analogue of methidathion were predicted using three software tools:

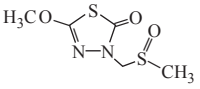
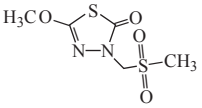
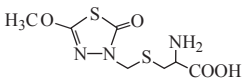
- Toxtree v. 3.1.0, a system for knowledge-based prediction of toxic effects,
- U.S. EPA T.E.S.T v. 4.1, a system for the predictions within the respective applicability domains,
- Derek Nexus version 6.0.1 (Derek KB 2018 1.1)/v. 6.1.0 (Derek KB 2020.1.0), a system of either hypothesis relating to mechanism of action of a chemical class or on observed empirical relationships.

The results are summarized in Table 20. For the oxygen analogue of methidathion, a read-across for in vitro mutagenicity (Ames test) with or without metabolic activation was predicted using the OECD QSAR Toolbox, Version 4.4.1, due to an equivocal alert for mutagenicity using Derek Nexus and other alerts. The result was summarized in Table 20 (de Amanqui, Harder & Hofer, 2020)

In the second study using QSAR, predictions were made for the three compounds in the first study, and additionally RH, RH-thiol, RH-sulfide, RH-sulfoxide, RH-sulfone and the cysteine conjugate of methidathion using Derek Nexus (program version: Derek Nexus, 6.1.0, Nexus, 2,3.0), and CASE ULTRA (Model: GT 1 BMUT [bacterial mutagenicity by OECD 471 Test]), Model, Version 1.7.0.3.13514.500; Tested by CASE Ultra, Version 1.8.0.1). These results are also summarized in Table 20.

Table 20. Summary of in silico prediction of genotoxicity for methidathion and its metabolites

Name, structure and code	Toxtree, v. 3.1.0 1 [a]	U.S. EPA T.E.S.T. v. 4.1 [a]	Derek Nexus [b]	Case Ultra [b]	OECD QSAR Toolbox v. 4.4.1 [a]
Methidathion (GS-13005) 	Negative No structural alert	Positive Predictive value, 0.67	Equivocal Alert group: 305 alkyl ester of phosphoric or phosphonic acid	Inconclusive Predicted potency is 0.52 Binary Score QSAR calculated probability 52.3%, inside the grey zone (40–60%) around threshold (50.0%)	No alert
Desmethyl methidathion (GS-46572) 	Negative No structural alert	Negative Predictive value, 0.49	Inactive No alerts fired	Negative QSAR calculated probability 26.4%	No alert
Oxygen analogue of methidathion (GS-13007) 	Negative No structural alert	Negative Predictive value, 0.44	Equivocal Alert group: 305 alkyl ester of phosphoric or phosphonic acid	Inconclusive QSAR calculated probability 50.4%, inside the grey zone (40–60%) around threshold (50.0%)	Profiling alert Alkyl (C<5) or benzyl ester of sulphonic or phosphonic acid; three closest analogues [c] showed negative predictions for Ames test ± S9
RH (GS-12956) 	NE	NE	Inactive No alerts fired	Negative QSAR calculated probability 22.9%	NE
RH-thiol (GS-32978) 	NE	NE	Inactive	Negative QSAR calculated probability 14.5%	NE
RH-sulfide (GS-28368) 	NE	NE	Inactive	Negative	NE

Name, structure and code	Toxtree, v. 3.1.0 1 [a]	U.S. EPA T.E.S.T. v. 4.1 [a]	Derek Nexus [b]	Case Ultra [b]	OECD QSAR Toolbox v. 4.4.1 [a]
RH-sulfoxide (GS-28370) 	NE	NE	Inactive	Negative QSAR calculated probability 14.5%	NE
RH-sulfone (GS-28369) 	NE	NE	Inactive	Negative QSAR calculated probability 14.5%	NE
Cysteine conjugate of methidathion 	NE	NE	Inactive	Negative QSAR calculated probability 11.4%	NE

[a] From first study); [b] From combined first and second studies due to the same results obtained from different versions. Derek Nexus version 6.0.1 (Derek KB 2018 1.1)/v.6.1.0 (Derek KB 2020.1.0 for de Amanqui, Harder & Hofer (2020), and Program version: Derek Nexus, 6.1.0, Nexus, 2,3.0

[c] Closest analogues: ethion; *O,O,S*-trimethylphosphorothiate; *O,O,O*-trimethylphosphorothiate; NE: Not examined

The parent methidathion and its oxygen analogue produced the same alerts in Derek Nexus and other systems. In Derek Nexus the alert group for methidathion was the same as its oxygen analogue. Using the read-across of the OECD QSAR Toolbox, the three closest analogues of the oxygen analogue of methidathion produced negative predictions for in vitro mutagenicity (Ames test) with and without metabolic activation. Since methidathion itself yielded negative results in the available genotoxicity studies (Table 13) and the structural alerts triggered were identical for both parent its oxygen analogue, it can be assumed that the oxygen analogue is not mutagenic, like the remaining metabolites, (desmethyl-methidathion, RH, RH-thiol, RH-sulfide, RH-sulfoxide, RH-sulfone, and the cysteine conjugate of methidathion) for which no structural alerts were triggered by either the Derek Nexus or CASE Ultra in silico systems.

On this basis it was assumed that the above metabolites are unlikely to be mutagenic (de Amanqui, Harder & Hofer, 2020).

Toxicity-related information on other metabolites

Desmonomethyl methidathion (GS-46572) found in rat urine at greater than 10% of the absorbed dose of the parent methidathion has a chemical structure in common with other organophosphorus pesticides, indicating that this metabolite possesses AChE inhibitory activity (see Fig. 2). Its toxicity is considered to be covered by the toxicity of the parent compound.

No other information on toxicity of methidathion metabolites was obtained.

(d) Published data

A literature research was conducted in PubMed on 15th April 2020 using a keyword of 'methidathion′: 207 items were returned by this search. Several articles on acute toxicity in rats and genotoxicity in vitro and in vivo were available for the current evaluation. They have been described in relevant sections of this monograph. The remaining reports on toxicity were studies on mixture effects of various organophosphorus compounds, or studies using commercial formulations. No further information which might influence health-based guidance values was found in them.

3. Microbiological data

There was no information available in the public domain and no experimental data were submitted which addressed the possible impact of methidathion residues on the human intestinal microbiome.

4. Observations in humans

(a) Volunteer study

A human study had been evaluated by an earlier JMPR meeting. The current Meeting was made aware of new information relating to the performance of human studies by the author of that report. No evidence of informed consent was found and the full protocol was not available for the study.

The present Meeting was unable to confirm that the study complied with the declaration of Helsinki and was ethically valid, and concluded that it would be inappropriate to use this study for the assessment of methidathion.

(b) Published data

In a literature research at PubMed using the keyword of "methidathion" conducted on 15th April 2020; 207 items were returned by this search.. One report was found on observations in humans was a report of poisoning.

There was no information on epidemiological or other any adverse effect on human health due to methidathion where data of sufficient quality was available for its use in toxicological evaluation for dietary risk assessment.

Comments

Biochemical aspects

When radiolabelled methidathion was administered orally to rats, the radioactivity was rapidly absorbed to the extent of approximately 80%. No sex- or dose level-related differences were observed. The absorbed radioactivity was rapidly circulated systemically. The time to maximum concentration (T_{max}) in rats was 2–4 hours at a dose level of 0.25 mg/kg bw, and 2–6 hours at 2.5 mg/kg bw. Absorbed radioactivity was distributed widely but especially to the liver and kidneys. Elimination from the blood and tissues was biphasic, with a rapid initial phase (within 24 hours) followed by a slower terminal phase (72 hours at 0.25 mg/kg bw; 96 hours at 2.5 mg/kg bw). Main elimination routes were urine and as carbon-dioxide via the lungs. There were no significance differences in elimination patterns with regards to dose levels administered, pretreatment or sex.

The major metabolic pathway of methidathion was cleavage of the heterocyclic moiety followed by further reactions. In rats, major metabolites were: RH (2,3-dihydro-5-methoxy-1,3,4-thiadiazol-2- one)-sulfide (GS-28368), RH-sulfoxide (GS-28370), RH-sulfone (GS-28369), and desmethyl-methidathion. (Dunsire, 1994; Dupuis, Muecke & Esse, 1971; Simoneaux, 1987a, b, c)

Toxicological data

The acute oral median lethal dose (LD_{50}) for methidathion in rats was 50–300 mg/kg bw. The acute dermal LD_{50} was greater than 2000 mg/kg bw. The acute inhalation median lethal concentration (LC_{50}) was 0.105–0.195 mg/L. Methidathion was not irritating to the skin or eyes of rabbits. Methidathion was sensitizing in a maximization test in Guinea pigs (Arcelin, 2000; Hartmann, 1993; Tavaszi, 2011; Zelenak, 2011a, b)

In single and repeat-dose oral toxicity studies with methidathion in mice, rats, rabbits and dogs, AChE inhibition in erythrocyte and brain and its related clinical signs were the major effects. Hepatotoxicity was observed at similar dose levels to those of AChE inhibition in mice and dogs.

In a 90-day oral toxicity study, rats were given methidathion at dietary concentrations of 0, 2, 6, 30 or 100 ppm (equal to 0, 0.13, 0.40, 2.05 and 7.04 mg/kg bw per day for males, 0, 0.16, 0.48, 2.50 and 9.02 mg/kg bw per day for females) The NOAEL was 2 ppm (equal to 0.16 mg/kg bw per day) based on a decrease in erythrocyte AChE at 6 ppm (equal to 0.48 mg/kg bw per day). (Altmann, 2001).

In a 90-day oral toxicity study in dogs given methidathion at dietary concentrations of 0, 0.5, 4, 45 or 140 ppm (equal to 0, 0.02, 0.16, 1.94 and 5.69 mg/kg bw per day for males, 0, 0.02, 0.19, 2.11 and 7.02 mg/kg bw per day for females) the NOAEL was 4 ppm (equal to 0.16 mg/kg bw per day) based on decreased erythrocyte AChE, hepatotoxicity-related changes in blood biochemistry and pathological finding at 45 ppm (equal to 1.94 mg/kg bw per day).

In a one-year oral toxicity study, dogs were given methidathion at dietary concentrations of 0, 0.5, 2, 4, 40 or 140 ppm (equal to 0, 0.02, 0.07, 0.15, 1.33 and 4.51 mg/kg bw per day for males, 0, 0.02, 0.07, 0.15, 1.39 and 4.90 mg/kg bw per day for females). The NOAEL was 4 ppm (equal to 0.15 mg/kg bw per day) based on a decrease in erythrocyte AChE activity, hepatotoxicity-related changes in blood biochemistry and pathological findings at 40 ppm (equal to 1.33 mg/kg bw per day). (Chang & Wyand, 1991).

The overall NOAEL for the 90-day and one-year dietary toxicity studies in dogs was 0.16 mg/kg bw per day, and the overall LOAEL 1.33 mg/kg bw per day.

In a two-year feeding toxicity study, mice were administered methidathion at dietary concentrations of 0, 3, 10, 50 or 100 ppm (equal to 0, 0.4, 1.4, 6.7 and 13.1 mg/kg bw per day for males, 0, 0.5, 1.6, 8.1 and 15.9 mg/kg bw per day for females). An interim kill in the 100 ppm groups at three months showed extensive liver toxicity. The NOAEL for chronic toxicity was 10 ppm (equal to 1.4 mg/kg bw per day) based on the hepatotoxic parameters of blood biochemistry and histopathological findings in males, and decreases in brain and erythrocyte AChE activity in both sexes at 50 ppm (equal to 6.7 mg/kg bw per day). The NOAEL for carcinogenicity in mice was 10 ppm (equal to 1.4 mg/kg bw

per day) based on increased incidences of hepatocellular adenomas and carcinomas, and a combined incidence of these tumours in males at 50 ppm (equal to 6.7 mg/kg bw per day). No carcinogenicity was observed in female mice (Goldenthal, 1986).

The Meeting considered that as preneoplastic lesions were seen in male mice during shorter duration studies, continuous stimulation by damage to the hepatobiliary system might be one factor leading to methidathion-induced hepatocarcinogenesis in male mice.

In a two-year combined chronic toxicity and carcinogenicity study, rats were administered methidathion at dietary concentrations of 0, 4, 40 or 100 ppm (equal to 0, 0.16, 1.72 and 4.91 mg/kg bw per day for males, 0, 0.22, 2.20 and 6.93 mg/kg bw per day for females). The NOAEL for chronic toxicity in rats was 4 ppm (equal to 0.16 mg/kg bw per day) based on decreases in erythrocyte and brain AChE activity, histopathological changes in the skin and lung at 40 ppm (equal to 1.72 mg/kg bw per day). The NOAEL for carcinogenicity in rats was 100 ppm (equal to 4.91 mg/kg bw per day) the highest dose tested (Yau, 1986).

The Meeting concluded that methidathion is carcinogenic in male mice but not in rats or female mice.

Methidathion was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found (Hertner, 1990; Honarvar, 2001; Hool, 1980; Newell, 1977; Strasser, 1990; Sudo, 1979).

The Meeting concluded that methidathion is unlikely to be genotoxic.

In view of the lack of genotoxicity, and the absence of carcinogenicity in rats and female mice, and the fact that the carcinogenicity in male mice exhibits a threshold, the Meeting concluded that methidathion is unlikely to pose a carcinogenic risk to humans via exposure from the diet.

In a two-generation study in rats methidathion was administered in the diet at concentrations of 0, 5, 25 or 50 ppm (equal to 0, 0.43, 2.21 and 4.33 mg/kg bw per day for males, 0, 0.47, 2.38 and 4.96 mg/kg bw per day for females). The NOAEL for parental toxicity was 5 ppm (equal to 0.47 mg/kg bw per day) based on cholinergic clinical signs in lactating females and lowered body weight in females at 25 ppm (equal to 2.38 mg/kg bw per day). The NOAEL for offspring toxicity was 5 ppm (equal to 0.43 mg/kg bw per day) based on lower body weight in F1 and F2 offspring at 25 ppm (equal to 2.21 mg/kg bw per day). The NOAEL for reproductive toxicity in rats was also 5 ppm (equal to 0.43 mg/kg bw per day) based on lower mating index in the F1 generation at 25 ppm (equal to 2.21 mg/kg bw per day) (Salamon, 1987).

In a development toxicity study, pregnant rats were administered methidathion at doses of 0, 0.25, 1.0 or 2.5 mg/kg bw once daily by gavage. Animals were dosed during gestation days (GDs) 6 to 15. The NOAEL for maternal toxicity was 1.0 mg/kg bw per day based on cholinergic clinical signs, decreased body weight gain and reduced food consumption at 2.5 mg/kg bw per day. The NOAEL for embryo/fetal effects was 2.5 mg/kg bw per day, the highest dose tested (Maineiro, 1987).

In a development toxicity study in rabbits, methidathion was administered daily by gavage to pregnant rabbits from GD 6 until GD 18 at doses of 0, 0.5, 1.5 or 3.0 mg/kg bw The NOAEL for maternal toxicity and for embryo/fetal toxicity was 3.0 mg/kg bw per day, the highest dose tested (Mainiero, 1987).

In another developmental toxicity study, rabbits were administered methidathion by gavage at dose levels of 0, 2, 6 or 12 mg/kg bw per day from GD 7 to GD 19. The NOAEL for maternal toxicity was 6 mg/kg bw per day based on cholinergic clinical signs observed immediately after dosing at 12 mg/kg bw per day. The NOAEL for embryo/fetal effects was 12 mg/kg bw per day, the highest dose tested (Hummel et al., 1987).

The Meeting concluded that methidathion is not teratogenic.

An acute neurotoxicity study was conducted in rats administered a single dose of methidathion by gavage at 0, 1, 4, 8 or 16 mg/kg bw. The NOAEL for acute neurotoxicity was 1 mg/kg bw based on a decrease in brain and erythrocyte AChE at 4 mg/kg bw (Chang, 1994).

A single-dose study was conducted in rats to investigate the inhibition of AChE. Methidathion was administered by gavage at doses of 0, 0.5, 1, 2.5, 5 or 10 mg/kg bw. The NOAEL for AChE inhibition was 1.0 mg/kg bw based on a decrease in erythryocyte AChE in females at 2.5 mg/kg bw (Glaza, 1994).

In a subchronic neurotoxicity study in rats, methidathion was administered for 90 days at dietary concentrations of 0, 3, 10, 30 or 100 ppm (equal to 0, 0.18, 0.61, 1.86 and 6.36 mg/kg bw per day for males, 0, 0.20, 0.66, 2.01 and 7.19 mg/kg bw per day for females). The NOAEL was 3 ppm (equal to 0.18 mg/kg bw per day) based on the decrease in AChE activity in erythrocytes and cerebral cortex in males at 10 ppm (equal to 0.61 mg/kg bw per day) (Chow, 1995).

In a delayed neurotoxicity study in hens, there was no evidence of delayed neuropathy.

The Meeting concluded that methidathion is neurotoxic

No evidence of immunotoxicity was reported in routine toxicological studies with methidathion.

The Meeting concluded that methidathion is not immunotoxic

Toxicological data on metabolites and/or degradates

Summary overview of toxicological characterization of plant/livestock metabolites

Compound, codes and structure	**Rat ADME** Toxicity covered by toxicological properties of parent compound (content in rat biofluids >10% absorbed dose or 10% TRR)?	**Genotoxicity assessment** (data, QSAR, read-across [a])	**General toxicity**	**Health-based guidance values (HBGVs)**
Methidathion (GS-13005)	Parent	Not genotoxic (data)	Full data-set LD_{50}: 35 mg/kg bw [b]	Parent HBGVs ADI: 0–0.002 mg/kg bw ARfD: 0.01 mg/kg bw
Oxygen analogue of methidathion (GS-13007)	Not found in rats	Not genotoxic (QSAR and RA)	LD_{50}: 10 mg/kg bw [b] Around 4-fold greater acute toxicity in vivo	Parent HBGVs with 4-fold additional factor
Desmethyl-methidathion (GS-46572)	Yes > 10% in rat urine	Not genotoxic (QSAR and RA)	Covered by parent	Covered by parent ADI ARfD unnecessary
Cysteine conjugate of methidathion	No ≤ 10% in rat urine Downstream of GSH conjugate in ADME	Not genotoxic (QSAR and RA)	Covered by parent No common structure with that of AChE inhibitors	Covered by parent ADI ARfD unnecessary
RH (GS-12956) (2,3-dihydro-5-methoxy-1,3,4-thiadiazole-2-one)	No ≤ 10% in rat urine Upstream from RH-thiol	Not genotoxic (QSAR and RA)	Covered by parent LD_{50}: 750 mg/kg bw [b] No AChE inhibitory effect in vitro	Covered by parent ADI ARfD unnecessary
RH-thiol (GS-32978)	No Precursor of RH-sulfide	Not genotoxic (QSAR and RA)	Covered by parent No common structure with that of AChE inhibitors	Covered by parent ADI ARfD unnecessary

Compound, codes and structure	**Rat ADME** Toxicity covered by toxicological properties of parent compound (content in rat biofluids >10% absorbed dose or 10% TRR)?	**Genotoxicity assessment** (data, QSAR, read-across [a])	**General toxicity**	**Health-based guidance values (HBGVs)**
RH-sulfide (GS-28368)	Yes > 10% in urine	Not genotoxic (QSAR and RA)	Covered by parent LD_{50}: 1750 mg/kg bw [b] No AChE inhibitory effect in vitro	Covered by parent ADI ARfD unnecessary
RH-sulfoxide (GS-28370)	Yes > 10% in rat urine	Not genotoxic (QSAR and RA)	Covered by parent LD_{50}: 1110 mg/kg bw [b] No AChE inhibitory effect in vitro	Covered by parent ADI ARfD unnecessary
RH-sulfone (GS-28369)	Yes > 10% in rat urine	Not genotoxic (QSAR and RA)	Covered by parent LD_{50}: 535 mg/kg bw No AChE inhibitory effect in vitro	Covered by parent ADI ARfD unnecessary

QSAR: Quantitative structure–activity relationship; RA: Read across; TRR: Total radioactive residue; ADME: Absorption, distribution, metabolism and excretion; ADI: Acceptable daily intake; ARfD: Acute reference dose; LD_{50}: Median lethal dose

[a] Source of QSAR: de Amanqui, Harder & Hofer, 2020; [b] Source: Dupuis, Muecke & Esser, 1971;

The Meeting concluded that the toxicities of RH-thiol, RH-sulfide, RH-sulfoxide and RH-sulfone were covered by the ADI of the parent, methidathion.

The Meeting considered that it was possible that all the metabolites described above show similar hepatotoxicity to methidathion (a noncritical end-point of methidathion); this could not be excluded, since it was noted by the Meeting that they all share with the parent a methoxythiazole ring structure. Once the phosphate group is removed, they are predicted to retain similar toxicity, not related to AChE-related activity. Therefore the hepatotoxicity potential of these metabolites would be covered by the ADI for methidathion.

Microbiological data

There was no information available in the public domain and no experimental data were submitted which addressed the possible impact of methidathion residues on the human intestinal microbiome.

Human data

A human study had been evaluated by an earlier JMPR meeting. The current Meeting was made aware of information relating to the performance of this study that had become available since the review in 1997 (JMPR, 1998). No evidence of informed consent could be found and full protocol for the study was not available.

The present Meeting was unable to confirm that the study complied with the declaration of Helsinki and was ethically valid, even by its contemporary standards, and concluded that it would be inappropriate to use this study for assessment of methidathion.

Toxicological evaluation

The Meeting withdrew the previous ADI and established a new ADI for methidathion of 0–0.002 mg/kg bw based on the overall NOAEL of 0.16 mg/kg bw per day in 90-day and one-year oral studies in dogs. A safety factor of 100 was applied. The NOAEL was supported by NOAELs from a 90-day neurotoxicity study in rats, a 90-day toxicity study in rats and a two-year toxicity study in rats. The margin between the upper bound ADI and the LOAEL for liver tumours in male mice is 3350.

The Meeting re-affirmed the ARfD of 0.01 mg/kg bw based on the NOAEL of 1 mg/kg bw in the acute neurotoxicity study in rats, and using a safety factor of 100.

The Meeting concluded that the oxygen analogue of methidathion was covered by the ADI and ARfD values for methidathion when an additional four-fold potency factor is used.

The Meeting concluded that the existing database on methidathion was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

Levels relevant to risk assessment of methidathion

Species	Study	Effect	NOAEL	LOAEL
Mouse	22-month study of carcinogenicity [a]	Toxicity	10 ppm, equal to 1.4 mg/kg bw/day	50 ppm, equal to 6.7 mg/kg bw/day
		Carcinogenicity	10 ppm, equal to 1.4 mg/kg bw/day	50 ppm, equal to 6.7 mg/kg bw/day
Rat	90-day oral toxicity study [a]	Toxicity	2 ppm equal to 0.16 mg/kg bw/day	6 ppm equal to 0.48 mg/kg bw/day
	Two-year study of toxicity and carcinogenicity [a]	Toxicity	4 ppm, equal to 0.16 mg/kg bw/day	40 ppm, equal to 1.72 mg/kg bw/day
		Carcinogenicity	100 ppm, equal to 4.91 mg/kg bw/day [d]	-
	Two-generation study of reproductive toxicity [a, b]	Reproductive toxicity	5 ppm, equal to 0.43 mg/kg bw/day	25 ppm, equal to 2.21 mg/kg bw/day
		Parental toxicity	5 ppm, equal to 0.47 mg/kg bw/day	25 ppm, equal to 2.38 mg/kg bw/day
		Offspring toxicity	5 ppm, equal to 0.43 mg/kg bw/day	25 ppm, equal to 2.21 mg/kg bw/day
	Developmental toxicity study [c]	Maternal toxicity	1 mg/kg bw/day	2.5 mg/kg bw/day
		Embryo/fetal toxicity	2.5 mg/kg bw/day [d]	-
	Acute neurotoxicity [c]	Neurotoxicity	1 mg/kg bw	4 mg/kg bw
	90-day neurotoxicity	Neurotoxicity	3 ppm equal to 0.182 mg/kg bw/day	10 ppm equal to 0.608 mg/kg bw/day
Rabbit	Developmental toxicity study [c]	Maternal toxicity	3 mg/kg bw per day [d]	-
		Embryo/fetal toxicity	3 mg/kg bw per day [d]	-
Dog	90-day and one-year study of toxicity [a, b]	Toxicity	4 ppm equal to 0.16 mg/kg bw/day	40 ppm equal to 1.33 mg/kg bw/day

a Dietary administration. b Two studies combined c Gavage administration d Highest dose tested

*Acceptable daily intake (ADI)**

0 – 0.002 mg/kg bw

* *applies to methidathion, RH, RH-thiol, RH-sulfide, RH-sulfoxide, RH-sulfone, desmethyl-methidathion and the cysteine conjugate of methidathion, and also to the oxygen analogue of methidathion multiplied by four*

*Acute reference dose (ARfD)***

0.01 mg/kg bw

***Applies to methidathion, desmethyl-methidathion, and the oxygen analogue of methidathion (multiplied by four)*

Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human

Critical end-points for setting guidance values for exposure to methidathion

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid (T_{max} 2–6 h); and approximately 80% absorbed (rats);
Dermal absorption	No information.
Distribution	Extensive; highest concentrations in liver and kidney
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	Elimination was biphasic, with a rapid initial up to 24 hours followed by a slower terminal phase (72 hours at 0.25 mg/kg bw and 96 hours at 2.5 mg/kg bw)
Metabolism in animals	Cleavage of the heterocyclic moiety of methidathion after hydrolysis of the ether bond, conjugation with glutathione. CO_2 was considered to be the main metabolite.(rat)
Toxicologically significant compounds in animals and plants	Methidathion, RH, RH-thiol, RH-sulfide, RH-sulfoxide, RH-sulfone, desmethyl-methidathion, cysteine conjugate of methidathion (rat); Oxygen analogue of methidathion (plant)
Acute toxicity	
Rat, LD_{50}, oral	50–300 mg/kg bw
Rat, LD_{50}, dermal	>2000 mg/kg bw
Rat, LC_{50}, inhalation	0.105–0.195 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Not irritating
Guinea pig, dermal sensitization	Sensitizing (maximization)
Short-term studies of toxicity	
Target/critical effect	Inhibition of AChE in erythrocyte (mouse, rat, dog, rat) Hepatotoxicity (mouse, dog)
Lowest relevant oral NOAEL	0.16 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	No data
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Inhibition of AChE in erythrocyte and brain (mouse, rat) Hepatotoxicity, including tumours (mouse)
Lowest relevant NOAEL	0.16 mg/kg bw per day (rat)
Carcinogenicity	Carcinogenic in male mice [a]

Genotoxicity	Unlikely to be genotoxic
Reproductive toxicity	
Target/critical effect	Lower mating index (rat)
Lowest relevant reproductive NOAEL	0.43 mg/kg bw per day (rat)
Parent	0.47 mg/kg bw per day (rat)
Offspring	0.43 mg/kg bw per day (rat)
Developmental toxicity	
Developmental target/critical effect	Cholinergic clinical signs (rat, rabbit) Decreased body weight gain and feed consumption (rat)
Lowest relevant developmental NOAEL	2.5 mg/kg bw per day, highest dose tested (rat)
Neurotoxicity	
Acute neurotoxicity target/critical effect	Cholinesterase inhibition in the brain (rat)
Lowest relevant oral acute NOAEL	1 mg/kg bw (rat)
Subacute neurotoxicity target/critical effect	AChE inhibition in erythrocyte and the brain (rat)
Lowest relevant oral subacute NOAEL	0.18 mg/kg bw (rat)
Developmental neurotoxicity	No data
Delayed neurotoxicity	No delayed neuropathy (hen)
Immunotoxicity	Unlikely to be immunotoxic: no evidence from routine studies

Studies on toxicologically relevant metabolites

Acute toxicity	
Oxygen analogue of methidathion	Acute oral LD_{50}: 10 mg/kg bw
RH-sulfide	LD_{50}: 1750 mg/kg bw
RH-sulfoxide	LD_{50}: 1100 mg/kg bw
RH-sulfone	LD_{50}: 535 mg/kg bw
Microbiological data	No available data
Human data	No usable data available

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

Summary

	Value	Study	Safety factor
ADI [a]	0–0.002 mg/kg bw	90-day and one-year dog studies	100
ARfD [b]	0–0.01 mg/kg bw	Rat acute neurotoxicity study	100

[a] Applies to methidathion, RH, RH-thiol, RH-sulfoxide, RH-sulfide, RH-sulfone and desmethyl-methidathion, the cysteine conjugate of methidathion, and to the oxygen analogue of methidathion (multiplied by four)

[b] Applies to methidathion, desmethyl-methidathion, and to the oxygen analogue of methidathion (multiplied by four)

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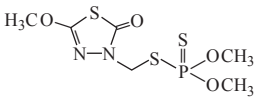
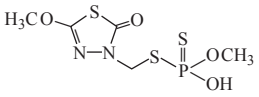
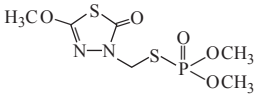
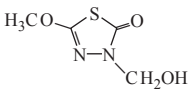
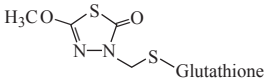
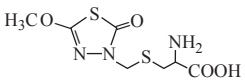
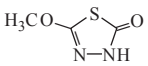
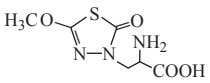
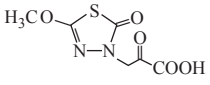
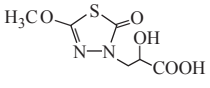
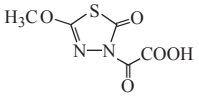
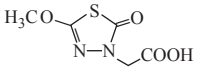
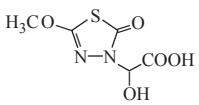
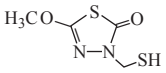
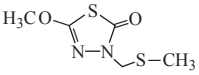
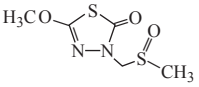
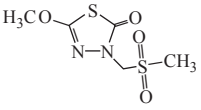
Appendix 1

Major metabolites of methidathion in plants, livestock and rats

Table 1. Major metabolites in plants, livestock and rats

Compound trivial name, systematic name and code	Structure	Compound found in:
Methidathion (GS-13005) *S*-2,3-dihydro-5-methoxy-2-oxo-1,3,4-thiadiazol-3-ylmethyl-*O,O*-dimethyl phosphorodithioate		(Parent compound)
Desmethyl-methidathion (GS-46572) *S*-2,3-dihydro-5-methoxy-2-oxo-1,3,4-thiadiazol-3-ylmethyl-*O*-methyl phosphorodithioate		Orange, tomato, rat
Oxygen analogue of methidathion (GS-13007) *S*-2,3-dihydro-5-methoxy-2-oxo-1,3,4-thiadiazol-3-ylmethyl-*O,O*-dimethyl phosphorothioate		Tomato, bean, alfalfa
GS-20685 2,3-dihydro-3-hydroxymethyl-5-methoxy-1,3,4-thiadiazol-2-one		Orange, rat
Glutathione conjugate of methidathion 2,3-dihydro-5-methoxy-2-oxo-1,3,4-thiadiazol-3-ylmethyl-thioglutathione		Orange, tomato, rat
Cysteine conjugate of methidathion 2-amino-3-(2,3-dihydro-5-methoxy-2-oxo-1,3,4-thiadiazol-3-ylmethylthioxy) propionic acid		Orange, tomato, rat
RH (GS-12956) 2,3-dihydro-5-methoxy-1,3,4-thiadiazol-2-one		Orange, bean, alfalfa, rat
RH-alanine conjugate 2-amino-3-(2,3-dihydro-5-methoxy-2-oxo-1,3,4-thiadiazol-3-ylmethyl) propionic acid		Orange
RH-keto acid conjugate 2-oxo-3-(2,3-dihydro-5-methoxy-2-oxo-1,3,4-thiadiazol-3-ylmethyl) propionic acid		Orange
RH-lactic acid conjugate 3-(2,3-dihydro-5-methoxy-2-oxo-1,3,4-thiadiazol-3-ylmethyl)-2-hydroxypropionic acid		Orange
RH-glyoxylic acid conjugate		Orange
RH-acetic acid conjugate 2-(2,3-dihydro-5-methoxy-2-oxo-1,3,4-thiadiazol-3-yl) acetic acid		Orange
RH-hydroxy acetic acid conjugate 2-(2,3-dihydro-5-methoxy-2-oxo-1,3,4-thiadiazol-3-yl)-2-hydroxyacetic acid		Orange

Compound trivial name, systematic name and code	Structure	Compound found in:
RH-thiol (GS32978) 2,3-dihydro-5-methoxy-4-sulfanyl-1,3,4-thiadiazol-2-one		Tomato, orange, bean, alfalfa, rat
RH-sulfide (GS 28368) 2,3-dihydro-5-methoxy-4-methylsulfanyl-1,3,4-thiadiazol-2-one		Tomato, bean, alfalfa, rat
RH-sulfoxide (GS 28370) 2,3-dihydro-5-methoxy-4-methylsulfinyl-1,3,4-thiadiazol-2-one		Tomato, bean, alfalfa, rat
RH-sulfone (GS 28369) 2,3-dihydro-5-methoxy-4-methylsulfonyl-1,3,4-thiadiazol-2-one		Tomato, bean, alfalfa, rat

Quintozene

First draft prepared by
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Explanation

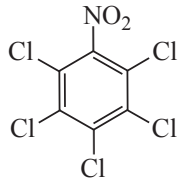
Quintozene is the International Organization for Standardization (ISO)-approved common name for 1,2,3,4,5-pentachloro-6-nitrobenzene (IUPAC) and is also known as pentachloronitrobenzene, Chemical Abstracts Service number 82-68-8.

Quintozene is a soil-applied fungicide and its fungicidal mode of action (MOA) is proposed to involve the inhibition of cellular peroxidation.

Quintozene was previously evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1969, 1973, 1977 and 1995. An acceptable daily intake (ADI) of 0–0.01 mg/kg bw was established at JMPR 1995 on the basis of a no-observed-adverse-effect level (NOAEL) of 1 mg/kg bw per day in a two-year rat study, a mechanistic 90-day study in rats and a two-generation rat study. The

establishment of an acute reference dose (ARfD) was not addressed. Quintozene was evaluated by the present Meeting within the periodic review program of the Codex Committee on Pesticide Residues (CCPR). The Meeting noted that a number of studies reviewed in 1995 were not made available for this review, but if these studies indicated critical aspects missing from the submitted database, reference would be made to the 1995 JMPR conclusions. In the past, manufactured quintozene was frequently contaminated with high levels (up to 11%) of hexachlorobenzene (HCB). The current specification for HCB in quintozene has a limit of 0.1% of the contaminant, therefore the present review on the toxicity of quintozene is predicated mainly on data from studies of quintozene containing less than 0.1% HCB.

Figure 1. Chemical structure of quintozene



All critical studies were conducted to internationally recognized guidelines (generally Organisation for Economic Co-operation and Development, OECD) and complied with and good laboratory practice (GLP) or were otherwise quality-audited, except where indicated. No additional information from a literature search was identified that complemented the toxicological information submitted for the current evaluation.

Evaluation for acceptable daily intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

(a) Oral route

The absorption and elimination characteristics of uniformly ^{14}C-labelled quintozene (PCNB) was studied in rats in a non-GLP study. Three male and three female Osborne-Mendell rats were administered [^{14}C] quintozene (purity and batch not indicated) in cottonseed oil at a dose of 5 mg/kg bw by oral gavage. Whole blood was taken from the orbital sinus at 0, 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96, 120 and 144 hours after administration. Urine and faeces were collected in 24 hour intervals until 144 hours. At 144 hours post treatment, all rats were exsanguinated under ether anaesthesia; liver, kidney and gastrointestinal (GI) wash were collected and the rat carcasses were placed in frozen storage. Total ^{14}C radioactivity was determined for each sample.

The average level of ^{14}C activity in whole blood reached a maximum of 0.62 µg equiv./mL at 12 hours post treatment. The half-life in whole blood was calculated to be 21.8 hours. The total ^{14}C activity in the urine ranged from 7.8% to 12.3% of administered dose (AD) in the males and 23.9% to 38.3% in the females. An apparent sex difference in urinary excretion of [^{14}C]quintozene was seen in the rats. The total ^{14}C activity in the faeces ranged from 56.6% to 90.9% of AD in the males and 37.9 to 76.0% in the females. The average ^{14}C activity in the faeces was 64% of AD for the males and 54% for the females. Thus, there was also an apparent sex difference in faecal excretion of ^{14}C activity. Measurable amounts of ^{14}C were found at 144 hours in all organs analyzed. The liver and kidney contained an average of 0.03% and 0.02% of AD, respectively, and the carcass and GI wash contained an average of 0.20% and 0.08% of AD , respectively. Total recovery of radioactivity from rats administered [^{14}C]quintozene ranged from 68.4% to 114.4% of AD with an average of 85%. According to the study author, the wide range of recoveries was perhaps due to the large number of manipulations on eye bleedings which were done outside the metabolism cages. All urine and faeces obtained during anaesthesia and bleedings were analysed in an effort to achieve quantitative recovery, but some excreta may have been lost during transfer of the animals from the metabolism cages to the anaesthesia jars (Adamovics & O'Grodnick, 1979; O'Grodnick, 1978a).

(b) Dermal route

No data on absorption, distribution and excretion following exposure via the dermal route was submitted.

1.2 Biotransformation

In the study described in section 1.1 (O'Grodnick, 1978a), metabolism was also studied in urine and faeces samples. The major metabolite in the hexane extractable fraction, before and after acid hydrolysis, was identified by gas chromatography (GC) as pentachloroaniline (PCA). There was an apparent sex difference in the amount of PCA found in the urine at most intervals. On direct extraction the male urine were seen to have 0.36, 0.21, 0.05, 0.04 and 0.01 ppm of PCA in the 24, 48, 72, 96, and 120 hour samples, respectively. Under the same conditions females had 0.83, 1.03, 0.28, 0.05 and 0.02 ppm at the same sampling times. Faecal samples were also analysed for quintozene metabolites at the 0–24, 24–48, 48–72, 72–96 and 96–120 hour post-treatment intervals. No detectable levels of pentachlorobenzene (PCB) or HCB were found in any of the samples taken. Measurable levels of quintozene were found in the 0–24 and 24–48 hour intervals. The major hexane extractable metabolite in the faeces was PCA. The levels of PCA in males were 2.8, 2.5, 0.4, 0.1 and 0. 1 ppm at 24, 47, 72, 96 and 120 hours respectively, post treatment. The levels of PCA in females were 5.0, 2.5, 0.33, 0.13 and < 0.06 ppm at the same sampling times. An apparent sex difference was seen in PCA extracted, but only at the 24 hour post treatment interval. Measurable levels of methylpentachlorophenyl sulphide (MPCPS; 0.21 and 0.19 ppm) were seen at the 24–48 hour interval. Large amounts of radioactivity were not extractable into hexane from the urine, before or after acid hydrolysis, suggesting the presence of other major metabolites in the urine including a conjugate of PCA (Adamovics & O'Grodnick, 1979; O'Grodnick, 1978a).

Characterization and identification

A non-GLP characterization and identification of [^{14}C]quintozene metabolites in rat urine and faeces was performed. The purpose of the study was to determine the nature of the ^{14}C radioactivity not extractable with hexane, from samples obtained in a previous study (described in Section 1.1). Samples of urine and faeces were adjusted to pH 13–14 using sodium hydroxide. The mixtures were then steam-distilled on a modified Bleidner apparatus for four hours using 2,2,4-trimethylpentane (*iso*-octane) as the organic phase. Bleidner distillation of the combined 0–24 hour faeces from male rats resulted in 22% of the radioactivity being extractable into *iso*-octane. The *iso*-octane layer contained PCA, MPCPS and methylpentachlorophenyl sulfone as evidenced by gas chromatography–mass spectrometry (GC-MS). The aqueous layer was then extracted with ethyl ethanoate. This extract contained pentachlorophenol (PCP) identical (shown by GC, GC-MS) to an authentic sample of PCP. A small amount of material, tentatively identified by GC-MS as a tetrachlorophenol, was also found in this extract.

The aqueous layers, after ethyl ethanoate extraction, still contained 24% of the original radioactivity present in the urine. No attempt was made to identify materials in this fraction because of its level of dilution. Bleidner distillation of combined 0–24 hour faeces from female rats resulted in 23% of the radioactivity being extracted into *iso*-octane. The *iso*-octane layer contained PCA as evidenced by thin layer chromatography (TLC) and mass spectrometric analysis. The aqueous layer was extracted with ethyl acetate at basic and neutral pH. The ethyl ethanoate extract at pH 13 and 7 contained 23% and 8%, respectively, of the radioactivity present in the faeces. Thin layer chromatography showed no migration of ^{14}C activity away from its origin, indicating the non-polar nature of the unknown compounds (Adamovics & O'Grodnick, 1979; O'Grodnick, 1978b).

Identification of polar metabolites of [^{14}C]quintozene

A non-GLP identification of polar metabolites of [^{14}C]quintozene after oral administration in rats was performed. Radiolabelled quintozene (purity and batch not indicated) was administered to Osborne-Mendell rats at a dose of 5 mg/kg bw in cotton seed oil. Urine and faeces were collected in the periods 0–24 and 24–48 hours post treatment. Urine and faeces were extracted with hexane to remove the organic-extractable metabolites which had been previously characterized. Amberlite XAD-2 column chromatography, normal and reverse phase Sep-Pak® cartridges, preparative high-performance liquid chromatography (HPLC) and TLC were used to isolate the major polar metabolites in urine and faeces (Adamovics & O'Grodnick, 1979; O'Grodnick, 1978c).

The urine samples displayed two major polar metabolites on TLC. One of these metabolites was assigned the structure pentachlorophenyl-*N*-acetylcysteine based on:

- chromatography in three TLC systems with authentic pentachlorophenyl-*N*-acetylcysteine,
- ninhydrin spray reagent,
- co-chromatography on a reverse phase HPLC column, and
- TLC co-chromatography of the methylated urine sample with pentachlorophenyl-*N*-acetylcysteine methyl ester.

Mass spectra of the material isolated from the urine matrix were obtained only at probe temperatures in excess of 350°C. Interpretation of the mass spectra indicated the presence of octachlorothianthrene. It is believed that this compound had resulted from the fragmentation of the pentachlorophenyl-*N*-acetylcysteine metabolite to yield a pentachlorothiophenolate moiety which dimerizes with a loss of two chlorine atoms to produce octachlorothianthrene. Pure pentachlorophenyl-*N*-acetylcysteine, synthesized in the laboratory, gives a mass spectrum consistent with its structure without dimerization taking place. Experimental evidence to support the hypothesis that octachlorothianthrene is not a true metabolite of quintozene rests on different HPLC retention times and different TLC R_f values for standard thianthrene and the urine metabolites.

The second major polar urinary metabolite of quintozene was apparently not a glucuronide based on:

- TLC in a two solvent systems,
- failure to yield a trimethylsilyl derivative on reaction with *bis*-trimethylsilyltrifluoro acetamide (BFSTA), and
- migration of the radioactivity after diazomethane treatment, which is indicative of a free phenolic or carboxylic acid moiety.

The exact structure of this compound is unknown.

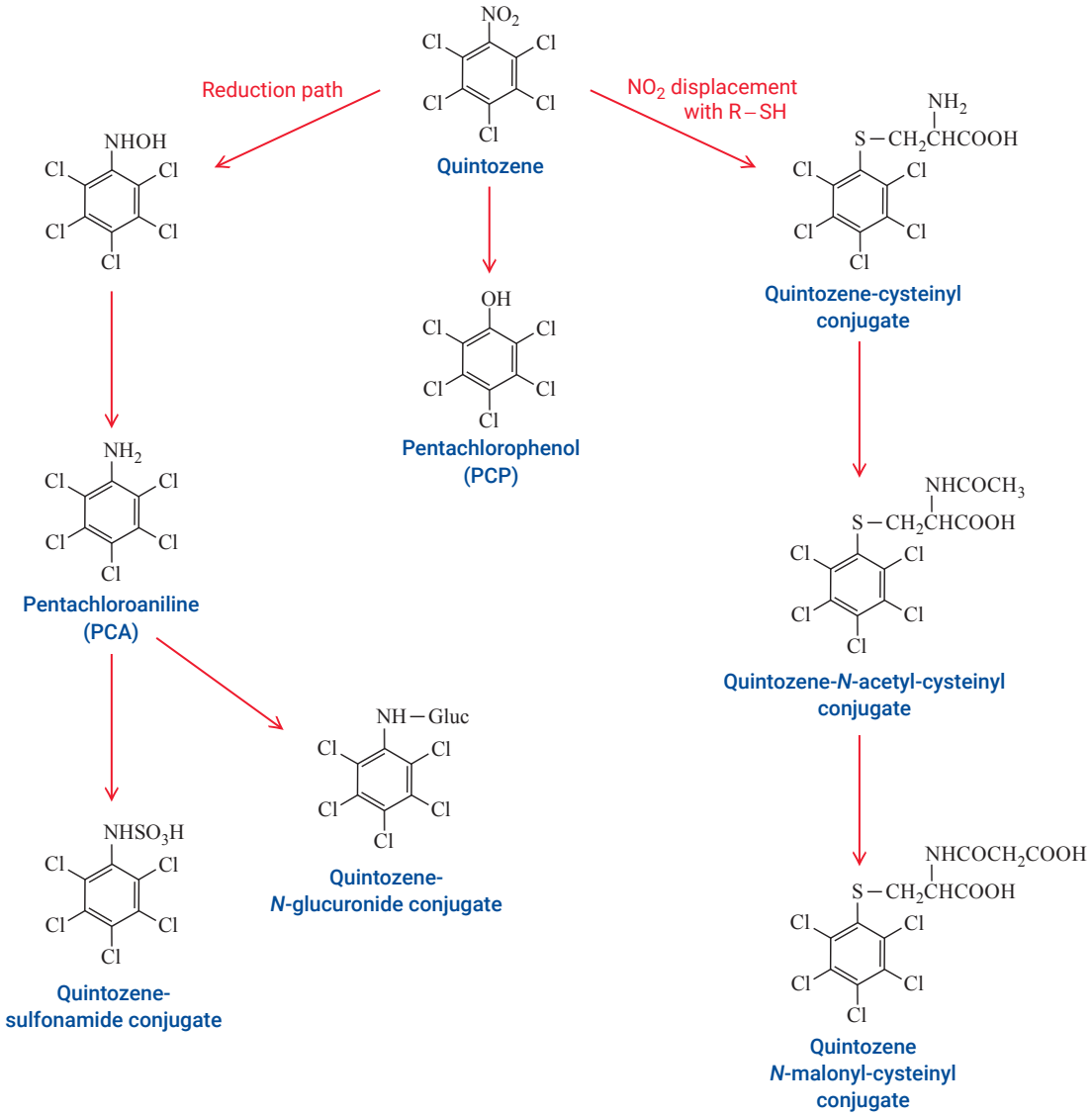
Hexane extracts were taken from samples of faeces and these were acid hydrolysed to liberate metabolites in their unconjugated form, neutralized and centrifuged. The supernatant contained 4.3%, 12.3%, 23% and 23% of the radioactivity originally present in the 0–24 hour male and female, and 24–48 hour male and female faeces, respectively. Amberlite XAD-2 column chromatography and ethyl acetate extraction followed by dry column chromatography indicated that two major metabolites of quintozene were found in the faeces after hexane extraction. One of these metabolites was moderately nonpolar and had migration characteristics similar to simple quintozene metabolites, for example pentachloroaniline. The other metabolite was polar and further identification was not pursued (O'Grodnick, 1979).

Pilot study to examine ultimate fate of quintozene

A non-GLP pilot study was performed to study the metabolic fate of quintozene. Ten Osborne-Mendell female rats were dosed by oral gavage with [^{14}C]quintozene (purity and batch not indicated) at 5 mg/kg bw. Urine and faeces were collected for assay at the following intervals: 0–24, 24–48 and 48–72 hours. No tissues were taken. The total radioactivity of the urine samples was determined using liquid scintillation counting; the faeces were not assayed. The primary purpose of this study was to identify PCC (*N*-acetyl-*S*-(pentachlorophenyl)cysteine) as a metabolite of quintozene in the rat.

The average recovery of radioactivity in the urine of the 10 female rats was 32.4% with a standard deviation of 11.7%. The major metabolite of quintozene in the urine is PCC; this is degraded to PCTP (pentachlorothiophenol) while stored in HPLC eluant. Subsequently, during the MS analysis, PCTP can easily dimerize to octachlorothianthrene (OCTA). The two remaining metabolites of quintozene in the urine of female rats are PCP and a PCA conjugate as previously reported (O'Grodnick, 1978c).

Figure 2. Proposed metabolic pathway of quintozene in animals [*leave 3/4 page for this; RP*]



(Redrawn from O'Grodnick, 1978a, b, c; 1979)

1.3 Effects on enzymes and other biochemical parameters

No data were available.

2. Toxicological studies

2.1 Acute toxicity

The results of acute oral, dermal and inhalation toxicity studies with quintozene are summarized in Table 1 below.

Table 1. Acute toxicity of quintozene

Species	Strain	Sex	Route	Purity (%)	Result	Reference
Rat	CD	M + F	Oral	99.42% (< 0.1% HCB)	LD_{50} > 5000 mg/kg bw	Warshawsky 1994a
Rat	HSD:(SD) BR	M + F	Oral	95%	LD_{50} > 5050 mg/kg bw	Kuhn, 1989a
Rabbit	New Zealand White	M + F	Dermal	99.42%	LD_{50} > 5000 mg/kg bw	Warshawsky 1994b
Rabbit	New Zealand White	M + F	Dermal	95%	LD_{50} > 2020 mg/kg bw	Kuhn, 1989b
Rat	Crl:CD® BR VAF/ Plus®	M + F	Inhalation	99.42% (< 0.1% HCB)	LC_{50} > 1.7 mg/L	Hilaski 1984
Rat	HSD:(SD) BR	M + F	Inhalation	95%	LC_{50} > 6.49 mg/L	Kuhn, 1989c

M: Male; F: Female; LD_{50} Median lethal dose; LC_{50} Median lethal concentration

(a) Lethal doses

Oral route

Quintozene (purity 99.42%, less than 0.1% HCB) was administered by gavage to groups of five male and five female CD rats at 0, 1300, 1700, 2000 or 5000 mg/kg bw in corn oil. One female of the 1700 mg/kg bw group died on study day 4; no other animals died during the study. Decreased defaecation was observed in most to some animals at 1300, 1700, 2000 and 5000 mg/kg bw dosage levels. Decreased activity was also observed in some animals at 1300, 1700 and 2000 mg/kg. Other clinical signs observed included liquid or soft stool and yellow staining of the anogenital region in 4–5 animals at 2000 and 5000 mg/kg bw. These signs were usually observed during study days 2–4 and generally resolved by day 5 with a few animals recovering by day 6. There were no changes or differences observed in the body weights of animals surviving to study termination. Based on the results obtained, the oral LD_{50} was greater than 5000 mg/kg bw for male and female rats (Warshawsky, 1994a).

Quintozene (purity 95%) was administered by gavage to five male and five female HSD: (SD) BR rats at 5050 mg/kg bw. Administration was as a 25.0% (w/v) solution of quintozene in 2.0% (w/v) carboxymethyl cellulose (CMC). No animals died during the study. Clinical signs observed included constricted pupils, diarrhoea, piloerection and polyuria. Gross necropsy did not reveal any abnormalities and no effects on body weight were seen. Based on the results of this study, the acute oral LD_{50} was greater than 5050 mg/kg bw (Kuhn, 1989a).

Dermal route

Quintozene (purity 99.42%; less than 0.1% HCB) was administered to five male and five female New Zealand White rabbits, as a single dermal dose lightly moistened with deionized water at a dose level of 5000 mg/kg bw. All animals survived until study termination, no clinical signs, effect on body weight or macroscopic changes were observed. The dermal LD_{50} was greater than 5000 mg/kg bw (Warshawsky, 1994b).

Quintozene (purity 95%) was administered to five male and five female New Zealand White rabbits, as a single dermal dose moistened with saline at a dose level of 2020 mg/kg bw. One male died on study day 6; gross necropsy showed the entire GI tract distended with a large amount of hair in the stomach. One male showed signs of diarrhoea, but no other clinical signs were noted. No effect on body weight or macroscopic changes was observed. The dermal LD_{50} was greater than 2020 mg/kg bw (Kuhn, 1989b).

Inhalation

Five male and five female SD-derived Crl:CD® BR VAF/Plus® rats were exposed to a four-hour, nose-only aerosol atmosphere of quintozene (purity 99.46%; less than 0.1% HCB) at concentration of 1.7 mg/L. This was the highest concentration attainable at conditions optimizing concentration and smallest particle diameter. The mass median aerodynamic diameter (MMAD) ranged from 3.2 µm to 3.6 µm. Clinical signs observed included decreased activity, increased salivation and rapid breathing. No deaths occurred during the study. No effects on body weight were observed. The significant findings noted at necropsy were one female animal with red discoloured nasal tissues and a dilated pelvis of the right kidney. Based on these results the LC_{50} for quintozene was considered to be greater than 1.7 mg/L (Hilaski, 1984).

Five male and five female HSD: (SD) BR rats were exposed to a four-hour whole-body aerosol atmosphere of quintozene (purity 95%) at a concentration of 6.49 mg/L. The MMAD ranged from 11.736 µm to 12.752 µm. Clinical signs observed included decreased activity, piloerection and ptosis. No deaths occurred during the study. No effects on body weight were apparent, nor were there any findings at necropsy examination. Based on these results, the LC_{50} for quintozene was considered to be greater than 6.49 mg/L (Kuhn, 1989c).

(b) Dermal irritation

In a dermal irritation study, quintozene (purity 99.46%; less than 0.1% HCB) was applied to the skin of six male New Zealand White rabbits. A dose of 0.5 g of test article was applied to one intact site on the back of each rabbit, under a 2.5 cm square gauze patch secured with strips of Demiform® tape. After four hours, bandaging and collar were removed and test sites were wiped with dry disposable paper towels. The test sites were evaluated for dermal irritation at 30–60 minutes, 24, 48 and 72 hours using the Draize method. All animals survived until study termination. No dermal irritation was observed in any animal during the study (Warshawsky, 1994c).

In a dermal irritation study with quintozene (purity 95%), the test article was applied to the skin of three male and three female New Zealand White rabbits. Each test site was treated with 500 mg of test material moistened with 0.2 mL of saline and then occluded for four hours. Observations for dermal irritation and defects were made at 1, 24, 48 and 72 hours and on day 7 after the occlusion period. Quintozene produced a maximum irritation score of 1.5 and was given a descriptive rating of slight irritant (Kuhn, 1989d).

(c) Ocular irritation

In an eye irritation study with quintozene (purity 99.42%; less than 0.1% HCB), the undiluted test article was placed into the cupped conjunctival sac of six male New Zealand White rabbits at a dose level of 0.0996 g per right (test) eye. The eyes remained unwashed. The eyes were observed and scored according to the Draize method at 1, 24, 48 and 72 hours after dosing. All animals exhibited conjunctival redness, chemosis and discharge with onset noted at the one-hour observation interval. Conjunctival swelling and discharge cleared in all animals by the 24-hour interval. Conjunctival redness cleared in two animals after 24 hours and after 48 hours in the remaining four animals. Based on the results obtained, quintozene was minimally irritating in this study (Washawsky, 1994d).

In a second eye irritation study, quintozene (purity 95%) was tested in male and female New Zealand White rabbits. Three males and three females were studied without washing of the eyes; three other females received an eye wash with deionized water for one minute beginning 30 seconds after treatment. Quintozene was rated as mildly irritating to non-washed eyes and minimally irritating to washed eyes (Kuhn, 1989e).

(d) Dermal sensitization

The potential of quintozene (purity 96%) in 75% and 50% (w/v) formulations in acetone to produce delayed contact hypersensitivity in Guinea pigs was evaluated using an adaptation of the method by Ritz & Buehler. Following a triple patch primary challenge, the incidence of a response (grade 1 or greater) in the test group, at levels of 5.0%, 2.5% and 0.5% was compared to that of the vehicle control group. The incidence and severity of these responses in the test group was greater than that produced

by the vehicle control group, indicating that sensitization had been induced and responses were elicited at all three levels. Following the double-patch rechallenge at levels of 5.0% and 0.5%, the incidence of grade 1 responses in the test groups was compared to the control group. Although a response of grade 1 was present among the control group at the 5.0% test level, the test animals continued to exhibit characteristics of hyper-reactivity at both rechallenge levels. This confirms the induction of sensitization and indicates that a no-effect level had not been achieved (Kreuzmann, 1988).

In a maximization study, quintozene (purity 96%; 0.026% HCB) was tested on 15 male and 15 female albino Guinea pigs. Test animals from Group I (10 per sex), each received three pairs of intradermal injections (adjuvant, a solution of test substance in cottonseed oil, and a 50:50 mixture of adjuvant and the test substance solution) followed one week later by a single topical application of the test substance in petrolatum. Ten additional animals (five per sex) served as a control group (Group II) and were treated at the same times and locations but with the vehicle. Two weeks after the topical application the test animals were challenged with a second topical application of the test substance in petrolatum at a virgin test site. Control animals were also given a topical application of the test substance in petrolatum. Since 90% of the animals receiving the test article exhibited scores greater than zero, quintozene was given a sensitization potency rating of extreme sensitizer (Kuhn, 2000).

In a third study, four groups of female albino Guinea pigs were treated with either the known sensitizing agent, 0.3% 2,4-dinitrovhlorobenzene (DNCB; four animals per group) or the test article, undiluted quintozene (10 animals per group) according to the Buehler method. No skin reactions were noted in either group of Guinea pigs dosed with the test article quintozene. Thus, under the conditions of this test, quintozene did not demonstrate any indication of being a sensitizing agent or a skin irritant (Wedig, 1988).

Finally, a modified Buehler assay was conducted on 10 male and 10 female short-haired albino Guinea pigs to determine if the test material, technical grade quintozene (purity 95%) produced a sensitizing reaction. Five males and five females were assigned to each of two groups. Group I animals remained untreated during the induction phase of the study and served as a naïve control group. Group II animals, the test group, were treated with 400 mg of the test material (selected from a previous screening) moistened with 0.3 mL of 95% ethanol. The animals were treated once weekly for three weeks. After a two-week rest period, all animals (Groups I and II) were challenged by application of 0.4 mL of a 75% (w/v) solution of the test material in acetone, at a virgin test site. The test material produced no irritation in animals of the naïve control group (Group I) after the single treatment at challenge. The test material likewise produced no irritation in animals of the test group (Group II) after the challenge, and therefore did not elicit a sensitizing reaction in Guinea pigs (Kuhn 1992).

Table 2. Summary of irritation and sensitization studies on quintozene

Species	Strain	Sex	Route	Purity (%)	Result	Reference
Rabbit	New Zealand White	M	Skin irritation	99.42% (< 0.1% HCB)	No dermal irritation	Warshawsky, 1994c
Rabbit	New Zealand White	M + F	Skin irritation	95%	Slight dermal irritation	Kuhn 1989d
Rabbit	New Zealand White	M	Eye irritation	99.42% (< 0.1% HCB)	Minimally irritating	Warshawsky, 1994d
Rabbit	New Zealand White	M + F	Eye irritation	95%	Minimally irritating	Kuhn, 1989e
Guinea pig	Hartley Albino	M + F	Skin sensitization (Buehler)	96%	Can induce sensitization	Kreuzmann, 1988
Guinea pig	Hartley Albino	M + F	Skin sensitization (M&K)	96% (0.026% HCB)	Sensitizing	Kuhn, 2000
Guinea pig	Hartley-derived American shorthair albino	F	Skin sensitization (Buehler)	95%	Not sensitizing	Wedig, 1988
Guinea pig	Hartley-derived American shorthair albino	M + F	Skin sensitization (Buehler)	95%	Not sensitizing	Kuhn, 1992

M: Male; F: Female;

2.2 Short-term studies of toxicity

(a) Oral administration

Mouse

In a 13-week study, B6C3F1 mice (10/sex per dose) were administered diets containing quintozene (purity greater than 99%; less than 0.07% HCB). Males were administered diets containing 0, 1250, 2500, 5000, 10 000 or 20 000 ppm quintozene. Females were administered diets containing 0, 2500, 5000, 10 000, 20 000 or 40 000 ppm quintozene. Animals were checked twice per day and any moribund animals were killed. Feed consumption was measured weekly by cage (five animals per cage), and individual animal weights were recorded weekly. At the end of the study, necropsy was performed on all animals. The following tissues were examined from control and high dose groups: gross lesions and tissue masses, mandibular lymph nodes, mammary gland, skin, salivary gland, sternebrae, thyroid/parathyroid, small intestine, colon, liver, prostate, testis, ovary, uterus, lungs, heart, oesophagus, stomach, brain, thymus, trachea, pancreas, spleen, kidneys, adrenals, urinary bladder, pituitary gland, spinal cord and eyes.

All the female mice in the top dose group (40 000 ppm) died within first two weeks of the study. Clinical signs observed were small body size and decreased body weight in 10/10 males at 20 000 ppm and 10/10 females at 20 000 and 40 000 ppm. The final mean body weight compared to controls of mice that received 10 000 or 20 000 ppm was 7% and 8% lower, respectively, for males, and 5% and 7% lower, respectively, for females. Data on feed consumption suggested that the dosed groups consumed more feed than the control group, however, scattering of feed (which could not be measured accurately) affects the interpretation of these results. Relative liver weight was increased in all dosed groups compared to controls. Lymphoid depletion of the spleen, mesenteric lymph node, or thymus was seen in 8/9 female mice examined in the 40 000 ppm group. No compound-related lesions were seen in male mice. A spectrum of inflammatory and proliferative lesions was seen in the lung of all dosed groups and was considered consistent with that associated with Sendai virus infection. The lung lesions found in both control and dosed animals included acute inflammation of bronchioles and alveolar epithelial hyperplasia. This study has limitations due to the limited investigations performed and the infections observed in the animals. Therefore, a robust NOAEL could not be established (NTP, 1987).

Table 3. Selected findings in the 13-week study in mice

Dose (ppm)	Mean body weight (g)			Final body weight rel. to controls (%)	Feed consumption (g/animal/day)		Relative liver weight (mg/g) [increase compared to controls]
	Initial	Final	Change		Week 4	Week 12	
Males							
0	22.8	29.9	+7.1		4.3	5.2	52.3
1250	22.6	30.8	+8.2	103.0	5.0	7.1	58.6 [+12%]
2500	23.2	30.2	+7.0	101.0	4.6	6.1	69.2 [+32%]
5000	22.5	30.0	+7.5	100.3	4.7	7.5	70.1 [+34%]
10 000	22.4	27.8	+5.3	92.6	5.2	8.3	63.1 [+21%]
20 000	22.3	27.5	+5.2	92.0	7.3	8.6	62.8 [+20%]
Females							
0	18.7	25.9	+7.2		4.4	4.7	52.2
2500	19.0	25.9	+6.9	100	4.9	5.7	60.7 [+16%]
5000	18.8	24.9	+6.1	96.1	5.3	7.8	66.6 [+28%]
10 000	19.0	24.7	+5.7	95.4	7.3	8.0	68.5 [+31%]
20 000	18.8	24.1	+5.3	93.1	8.3	9.2	65.0 [+25%]
40 000	18.8	-	-	-	-	-	-

Source: NTP, 1987

Rat

In a non-GLP preliminary dose range-finding study, quintozene (purity 95%; 0.037% HCB; 2.12% total tetrachloronitrobenzene, TCNB) was administered by gavage to groups of six male and female juvenile CD® IGS; CD(SD) rats at 0, 500 or 1000 mg/kg bw per day in CMC for seven consecutive days (PNDs 23–29 in males; PNDs 22–28 in females) to optimize the selection of the high dose level for a subsequent combined male and female pubertal assay in rats. No mortality or clinical signs were observed. A slightly depressed body weight gain was observed in females at 500 and 1000 mg/kg bw per day from PND22 until PND28 (Baxter, 2012a).

A 90-day study employed Sprague Dawley rats, initiation age 7–8 weeks. and body weights 243–294.3 g for males and 175.3–227 g for females. These were administered, orally by gavage, quintozene (purity 98%; Lot no. 05318-7D; 0.051% HCB) as a suspension in a vehicle of 0.2% Tween 80 and carrier of 2% CMC, for five days per week. Five groups consisting of 10 rats/sex per dose were used and dosed with 0, 5, 10, 100 or 1000 mg/kg bw per day at a dose volume of 5 mL/kg bw. Observations for clinical signs were performed daily. Body weights were recorded during the week prior to dosing, at dosing initiation, and weekly thereafter. Ophthalmological examinations were performed prior to dose initiation and at study termination in all dose groups. Serum chemistry and haematology studies were performed on all animals during week 7 and at termination. In addition, haematology studies were also performed before dosing. A full macroscopic examination was performed on all animals, and selected organs (kidney, liver, ovaries, testes) and tissues were collected and weighed. An adequate range of tissues was examined microscopically.

Six animals died prior to study termination: two control animals, two in the low-dose group, one at 10 and one at 100 mg/kg bw per day; histopathology confirmed these to be the result of dosing accidents. There was no treatment-related effect on clinical signs, ophthalmology, body weight, body weight gain or on food consumption. There were no significant differences found in haematology at the interim or terminal samplings. A treatment-related decrease in alanine transaminase (ALT) at week 7 and at terminal sampling was found for both sexes. The mean ALT of the high-dose males and females was significantly lower than controls at both week 7 and at terminal sampling. At 100 mg/kg bw per day, the mean ALT value for females at the week 7 interval was significantly decreased compared with controls and the mean ALT value for males at termination was significantly decreased compared to controls.

Table 4. Results from 90-day study in rat

		Sex and dietary dose (ppm)									
		Males					Females				
Parameter		**0**	**5**	**10**	**100**	**1000**	**0**	**5**	**10**	**100**	**1000**
		Clinical chemistry (IU/L)									
ALT:	Week 7	32.5	32.4	31.8	26.6	21.9**	29.3	27.2	26.9	23.3*	17.7**
	Terminal	39.3	36.8	34.4	30.2**	20.7**	36.8	44.7	35.3	29.4	20.7**
		Absolute organ weights (g)									
Kidney		3.93	3.96	4.27	4.27	4.43*	2.42	2.62	2.45	2.39	2.52
Liver		15.50	14.50	15.19	16.84	16.97	8.27	8.61	8.83	8.52	9.09
		Relative organ weights									
Kidney		0.0074	0.0076	0.0079	0.0079	0.0081	0.0077	0.0085	0.0077	0.0079	0.0083
Liver		0.029	0.028	0.026	0.031	0.031	0.026	0.028	0.028	0.028	0.030*
		Histopathology (incidences)									
Liver, centrilobular hypertrophy		0	0	0	0	6	0	0	0	0	2
Thyroid, hypertrophy hyperplasia follicular epithelium		2	2	3	3	6	0	0	0	0	5

ALT: alanine transaminase; Source: Keefe, 1992

Statistically significant: * $p < 0.05$; ** $p < 0.01$

No gross post-mortem or ophthalmologic observations associated with treatment were noted. However, there was a treatment-related increase in mean relative liver weights (10–15%) in both males and females, with increase in the high-dose females reaching statistical significance. Histopathological examination revealed treatment-related microscopic changes in liver and thyroid of high-dose male and female rats. These changes included hypertrophied hepatocytes in the centrilobular zone with dense, homogeneous, eosinophilic cytoplasm and an increased incidence and severity of hypertrophy and hyperplasia of the follicular epithelium in the thyroid of high-dose rats. The NOAEL was 71 mg/kg bw per day (corrected for five day per week dosing) based on histopathological findings in the thyroid (follicular cell hypertrophy/hyperplasia) at 710 mg/kg bw per day (corrected) (Keefe, 1992).

In a 13-week dose range-finding study, CD rats were exposed to quintozene (purity 96%; less than 0.5% HCB) in the diet at concentrations of 0, 50, 3000 or 6000 ppm (equal to 0, 3.07, 187 and 381 mg/kg bw per day for males, 0, 3.69, 223 and 455 mg/kg bw per day for females) with 15 rats/sex per dose. The animals were observed for moribundity and mortality twice daily throughout the study. Body weights were obtained prior to initiation of the study and weekly thereafter. Individual food consumption measurements were determined weekly. Ophthalmological examinations were performed on all rats in the four test groups prior to study initiation and at day 86. Clinical laboratory studies were conducted on 10 randomly selected animals/sex per group at week 13 for haematological and clinical chemistry measurements. Urine was collected during the fasting period. All animals received a complete post-mortem examination. The following organ weights were measured: adrenal, brain, heart, kidney, liver, ovary, testes. Representative samples of protocol-designated organs (n = ca 40) and tissues were processed for microscopic examination.

No animals died during the study and no treatment-related clinical signs were observed. After 13 week of treatment slightly lower (but statistically significant) mean body weights were evident for both sexes from the 6000 ppm group (−8 and −6% for males and females respectively) and for females from the 3000 ppm group (−5%) compared to the controls. Differences in overall body weight gain compared to the controls were −15% and −16% for the 6000 ppm males and females, and −11% and −14% for the 3000 ppm males and females, respectively. Slight differences in food consumption were noted at week 1 in the 6000 ppm group for both sexes; these were considered the result of a transient palatability effect. No treatment-related effects were seen in the ophthalmoscopic examination, in haematology or urinalysis.

Table 5. Results from 13-week range-finding study in the rat

	Sex and dietary dose (ppm)							
	Males				**Females**			
Dose (ppm)	**0**	**50**	**3000**	**6000**	**0**	**50**	**3000**	**6000**
	Clinical chemistry							
ALT (IU/L)	29	31	18**	15**	24	25	15**	12**
Total protein (g/dL)	6.6	6.7	6.7	6.8	6.4	7.0*	7.2**	7.1*
Globulin (g/dL)	3.2	3.2	3.3	3.3	2.8	3.1	3.3*	3.2*
	Organ weights (relative to body weight) [% difference from controls]							
Liver	3.34	3.50	3.96* [19%]	4.14** [24%]	3.67	3.53	4.08	4.41** [20%]
Kidney	7.42	8.12	9.52** [28%]	9.16** [23%]	8.31	7.81*	8.78	9.04** [9%]
	Histopathology (individuals affected of 15)							
Hepatocellular hypertrophy:	0	0	0	7	0	0	0	8
trace	0	0	0	6	0	0	0	5
mild	0	0	0	1	0	0	0	3

ALT: Alanine transaminase; Source: McGee, 1988

Statistically significant: * $p < 0.05$; ** $p < 0.01$

A decrease in ALT was seen in both sexes at 3000 and 6000 ppm, as well as a slight increase in serum total protein and/or globulin in females. Mean relative liver weights were increased (statistically significant) at 6000 ppm in both sexes and in males at 3000 ppm. Trace to mild hepatocellular hypertrophy was evident at 6000 ppm in both sexes. No other treatment-related findings in macroscopic or microscopic pathology were present. As this was a dose range-finding study with limited scope of investigation, no NOAEL was determined (McGee, 1988).

In a 90-day mechanistic study, male CD rats were administered quintozene in the diet (purity 99%; Lot no. 103Q121) at 0, 20 or 6000 ppm (equal to 0, 1.1 and 333 mg/kg bw per day) . Each group consisted of 75 male rats; at dosing initiation rats were 12 weeks old, with body weights 311–407 g. Fifteen rats per group were sacrificed on each of study days 7, 14, 30 and 90. Following the 90-day treatment, the remaining rats (15 per group) were maintained untreated on a basal diet for at least 90 additional days. The objective of the study was to determine if the administration of quintozene affects levels of thyroid hormone in a 90-day rat study followed by a 90-day recovery period. The animals were observed for moribundity and mortality twice daily throughout the study. Body weights were obtained prior to initiation of the study and weekly thereafter. Individual food consumption measurements were determined weekly. Blood samples were collected from 15 animals/sex per group at days 7, 14,30, 90, and 180 (or 183) of the study. Fifteen male rats per group were sacrificed on each of study days 7, 14, 30, 90, and 180. All animals received a complete post-mortem examination. The following organs were weighed: pituitary, liver, thyroid/parathyroid. Representative samples of protocol-designated organs and tissues were processed for microscopic examination (pituitary and thyroid/parathyroid for all animals; liver for all animals at 90 days and after recovery sacrifices only).

All animals survived until the designated study termination. No treatment-related clinical signs were observed. Body weight gain was depressed during the dosing period at 6000 ppm; the reduction in weight gain occurred primarily in week 1 of the study (−6% compared to controls). Similarly, food consumption was reduced in week 1 of the study at 6000 ppm. Triiodothyronine (T3) and thyroxine (T4) values were lower and thyroid-stimulating hormone (TSH) levels higher than the control values at 6000 ppm at all intervals, with statistical significance being achieved at most intervals. All of these changes were initially observed at study day 7 and persisted throughout the 90-day dosing period. Reverse T3 (rT3)was lower than controls (statistically significant) at 6000 ppm, but only at study day 90. There were no test article-related changes in any of these values at 20 ppm. At the end of the 90-day recovery period, T3, T4 and TSH values had returned to normal and were comparable to control values.

Table 6. Results from the 90-day mechanistic study rats

		Dietary dose of quintozene (ppm)		
Parameter	**Day of study**	**0**	**20**	**6000**
Thyroid hormone measurements				
Triiodothyronine, T3 (mg/dL)	Day 7	85	79	63**
	Day 14	95	97	77**
	Day 30	87	92	79
	Day 90	114	114	91**
	Day 180	93	91	92
Thyroxine, T4 (µg/dL)	Day 7	3.1	3.9**	1.6**
	Day 14	3.7	4.2	1.7**
	Day 30	4.9	5.1	2.3**
	Day 90	5.5	6.1	2.6**
	Day 180	2.7	3.2	2.9
Thyroid-stimulating hormone, TSH (mg/mL)	Day 7	3.2	3.3	4.2
	Day 14	3.6	3.5	4.8*
	Day 30	2.5	2.6	5.8**
	Day 90	2.4	2.0	4.3**
	Day 180	1.9	2.0	2.3

Parameter	Day of study	Dietary dose of quintozene (ppm) 0	20	6000
Reverse triiodothyronine, rT3 (ng/dL)	Day 90	4.6	4.7	3.3**
Absolute and relative organ weights				
Liver (g)	Day 7	13.35 ± 1.632	13.17 ± 1.543	12.81 ± 1.484
	Day 14	14.33 ± 2.321	13.52 ± 1.824	14.43 ± 1.768
	Day 30	15.65 ± 1.612	15.72 ± 1.676	16.34 ± 1.603
	Day 90	17.74 ± 2.272	17.59 ± 1.549	19.22 ± 2.253
	Day 180	20.42 ± 3.255	21.06 ± 3.098	21.46 ± 3.424
Liver/bw (%)	Day 7	3.56 ± 0.334	3.58 ± 0.264	3.66 ± 0.393
	Day 14	3.59 ± 0.352	3.44 ± 0.281	3.89* ± 0.306
	Day 30	3.52 ± 0.301	3.44 ± 0.292	3.95** ± 0.243
	Day 90	3.31 ± 0.375	3.30 ± 0.290	3.92** ± 0.317
	Day 180	3.52 ± 0.404	3.43 ± 0.257	3.59 ± 0.454
Thyroid (mg)	Day 7	23 ± 2.9	21 ± 2.3	22 ± 2.6
	Day 14	26 ± 5.2	24 ± 3.2	26 ± 4.4
	Day 30	29 ± 2.4	28 ± 3.7	25** ± 3.9
	Day 90	33 ± 3.1	30 ± 4.9	30 ± 7.6
	Day 180	26 ± 6.7	27 ± 4.7	28 ± 5.0
Thyroid/bw (% × 10^3)	Day 7	6.14 ± 0.837	5.78 ± 0.60	6.16 ± 0.732
	Day 14	6.69 ± 1.354	6.13 ± 0.849	6.98 ± 1.445
	Day 30	6.64 ± 0.583	6.24 ± 0.801	6.09 ± 0.866
	Day 90	6.09 ± 0.722	5.60 ± 0.882	6.07 ± 1.432
	Day 180	4.57 ± 1.017	4.51 ± 0.812	4.75 ± 0.856
Histopathology				
Hepatocellular hypertrophy	Day 90			
	trace	0	14	0
	mild	0	0	15
Thyroid hypertrophy	Day 14			
	trace	0	0	15
	Day 30			
	mild	0	0	15
	Day 90			
	mild	0	15	0
	moderate	0	0	15

Statistically significant: * $p < 0.05$; ** $p < 0.01$; Source: Goldenthal, 1993

No treatment-related macroscopic pathology changes were observed. Liver weights were increased and thyroid weights decreased starting at study day 30 and continuing to day 90. At the end of the recovery period, liver and thyroid weights at 6000 ppm were comparable to controls. Histopathology showed hepatocellular hypertrophy at both 20 and 6000 ppm at study day 90; these changes were not evident in rats examined after the 90-day recovery period. Follicular epithelial hypertrophy of the thyroid was observed in rats of the 6000 ppm group examined at study days 14, 30 and 90. After 90 days of study, this thyroid change was also seen in the 20 ppm group. No treatment-related thyroid changes were seen in rats examined after the 90-day recovery period. The LOAEL was 20 ppm (equal to 1.1 mg/kg bw per day) based on thyroid follicular hypertrophy (Goldenthal, 1993).

Dog

In a 28-day pilot study, beagle dogs (three/sex per dose) were administered via the diet quintozene (purity 96%; less than 0.1% HCB) at dose levels of 0, 40, 2000 or 4000 ppm (equal to 1.20, 70.7 and 114.5 mg/kg bw per day for males, 1.23, 58.1 and 124.5 mg/kg bw per day for females). Mortality, moribundity and overt toxicity were examined twice daily with detailed observations at least once each week. Body weight was determined pretest and weekly thereafter; food consumption was determined weekly. Physical examinations were performed on all dogs pretest and prior to termination. Haematology, clinical chemistry and urinalysis were performed pre test and at four weeks of study. All animals received a complete post-mortem examination. The following organs were weighed: adrenal, brain, heart, kidney, liver, ovary, pituitary, spleen, testis, thyroid/parathyroid. No organs or tissues were examined microscopically.

There were no signs of overt toxicity noted during the four-week study period. No treatment-related effects were seen on body weight or body weight gain, food consumption, nor from detailed physical examinations, haematological determinations, urinalysis or macroscopy. With regard to serum biochemistry data at four weeks, marked reductions were recorded in the 2000 and 4000 ppm groups in ALT for both males (−93% and −97% respectively) and females (−95% and −97% respectively). Animals in these two groups also showed statistically significant increases in the liver to body weight ratios (30% and 33% in males, 26% and 36% in females at 2000 and 6000 ppm respectively). Although absolute liver weights were also increased by similar margins, the differences from controls were not statistically significant. Thyroid/parathyroid weights relative to body weight were increased at the mid dose in males (38%, statistically significant) and in females (25%, not significant). The biological significance of these findings is unknown without the results of a microscopic examination, therefore no NOAEL was proposed (Johnson, 1989).

In a one-year toxicity study, beagle dogs (six/sex per dose) were administered quintozene (purity 96%; less than 0.1% HCB) via the diet at dose levels of 0, 15, 150 or 1500 ppm (equal to 0, 0.40, 4.31 and 40.05 mg/kg bw per day for males, 0, 0.44, 4.22 and 41.48 mg/kg bw per day for females). Animals were observed for mortality, moribundity and clinical signs at least twice a day; detailed clinical observations were conducted at least once a week. Body weights were determined pretest and weekly for the first 14 weeks followed by once every four weeks thereafter. Food consumption was measured weekly for the first 14 weeks, once every four weeks thereafter, then at week 52. Ophthalmoscopic examinations were performed pretest and during the last week of study. Clinical pathology was conducted pre test, and after 6 and 12 months of study. After 52 weeks, all animals received a complete post-mortem examination, organ weights were measured and microscopic examinations performed.

One male from the 15 ppm group was sacrificed in extremis during study week 28. This dog had appeared thin, displaying eye effects (e.g. conjunctivitis, constricted pupil, discharge) short rapid laboured breathing, slight wheezing, scabs, a cyst (left hind paw) and enlarged lymph nodes. All other dogs survived until scheduled sacrifice. No treatment-related clinical signs were observed. Mean weekly body weights and food consumption were comparable throughout the study for both male and female treatment groups compared to the controls. No treatment-related effects were observed during ophthalmoscopic examination. With the exception of a decrease in haemoglobin (Hb) at 15 ppm in males, (not considered treatment-related) there were no treatment-related haematological changes at any dose level. Alkaline phosphatase (ALP) levels were increased at 6 and 12 months in males and females from the 1500 ppm group. These increases were statistically significant at both intervals for males and at the six-month interval for females. An increase in cholesterol at 1500 ppm was observed at both intervals, but was only statistically significant in males. In both males and females, there were decreases in ALT levels at 150 ppm, and marked decreases at the 1500 ppm dosage level values at both intervals. There were no other treatment-related biochemical findings at any dose. There were no teatment-related urological changes or macroscopic changes at any dose level. Liver weights were increased in males and females in the high-dose group. These animals also had a slight enlargement of hepatocytes with granular cytoplasm in the centrilobular region. In the high-dose females, an increase in relative kidney weight was observed, however there were no treatment-related microscopic changes in the kidney. The NOAEL for this study was 150 ppm (equal to 4.22 mg/kg bw per day) based on increased liver weight, increased ALP activity and cholesterol, and liver histopathology (hepatocellular hypertrophy) at 1500 ppm (equal to 40.05 mg/kg bw per day) (Goldenthal, 1990).

Table 7. Results from one-year study in the dog

		Sex and dietary dose (ppm)							
		Males				Females			
Dose level (ppm)		**0**	**15**	**150**	**1500**	**0**	**15**	**150**	**1500**
					Clinical chemistry				
ALP (IU/L)	6 months	56	52	46	75*	62	58	51	97*
	12 months	52	36	47	81*	60	61	60	82
Cholesterol	6 months	165	169	187	226*	199	174	185	247
(mg/dL)	12 months	160	160	170	226**	209	208	214	230
					Organ weights				
Liver, absolute (g)		305.79	317.93	325.52	362.02	244.09	262.28	291.13	338.96**
Liver, relative to bw (%) [difference from control]		2.28	2.34	2.56	2.87* [26%]	2.38	2.47	2.65	3.09** [30%]
Kidney, absolute (g)		61.78	54.10	62.05	61.48	47.11	50.02	46.84	55.61
Kidney, relative to bw (%×10)		4.65	4.02	4.90	4.85	4.28	4.69	4.26	5.02**
Thyroid, absolute (g)		1.61	1.74	2.80	1.63	1.23	1.13	1.56	1.39
Thyroid, relative to bw (%×10)		11.99	12.91	21.65	12.64	11.48	10.43	14.05	12.89
					Histopathology (individuals affected of 6)				
Liver hypertrophy		0	0	0	6	0	0	0	6
Thyroid: C-cell hyperplasia;									
	trace	3	1	1	1	2	1	2	2
	mild	1	1	0	1	3	1	1	1
Kidney: Hyaline casts:trace		0	1	1	0	1	0	0	1
Inflammation		2	1	0	1	1	1	1	0
Mineralization		5	6	6	6	6	6	6	6

ALP: Alkaline phosphatase; bw: Body weight; Source: Goldenthal, 1990

Statistically significant: * $p < 0.05$; ** $p < 0.01$;

In a non-GLP, two-year toxicity study, beagle dogs (3/sex per dose) were administered quintozene (purity 98.8%) at dose levels of 0, 500, 1000 or 5000 ppm (equivalent to 0, 37.5, 75 and 375 mg/kg bw per day) via the diet for two years. Except for five dogs administered the highest dose, which had to be killed because of their moribund condition, all other dogs survived until the end of the study. In the groups which received 5000 ppm, pronounced, disorders of food-intake and considerable substance-related body weight losses, up to high-grade emaciation, were observed. The animals in this group displayed a poor general condition in contrast to those in the other dose groups. Quintozene in higher doses provoked conjunctivitis, mono- or binocular cloudiness and ulcerations of the cornea. Moreover, within this test group significant haematological adverse effects were observed with the following diagnostic findings: moderate to pronounced anaemia, leukocytosis with neutrophilia and lymphocytopenia, also an accelerated erythrocyte sedimentation rate. No pathologic indications arose in the groups receiving 500 ppm, but a tendency to the same haematological adverse effects as seen in the high-dose dogs was observable in the mid-dose groups which were fed 1000 ppm quintozene. Occasionally an excretion of protein and bilirubin occurred in the urine of high-dose animals. In all animals of the high-dose group the test substance caused “profound hepatic damages [sic]”. The liver parenchyma was reduced in five out of six dogs by partly more than the half. The parenchyma was replaced partly by a proliferation of connective tissue and partly by pullulation of the bile duct/ductus biliferus. Formation of pseudolobules (dogsoccurred31 and 43), fibrosis, (dog 45) and dense leukocytic infiltration (dogs 44 and 45) were observed. The sinusoids were consistently strongly dilated, hepatocyte trabeculae strongly constricted with dilated sinusoids. These serious symptoms of liver damage were directly related to the dose, as in the group receiving 1000 ppm as well that at 500 ppm the first alteration of single liver cells and also liver cell groups/formations changes could be observed. Often this involved foam cell formation and necrobiosis.

After treatment at the mid and low doses, liver alterations were found to have occurred within two years; this could be interpreted as the preliminary phases of the serious liver damage that was seen in dogs receiving the highest dose treatment. At the low dose of 500 ppm, liver effects included: hepatocyte trabeculae strongly constricted with dilated sinusoids; foam cells/lipid-laden macrophages with core debris, here and there single cell necrosis; periodic acid–Schiff (PAS) staining showing tissues relatively rich in glycogen uniformly distributed; Kupffer cell siderosis. Effects were seen at all dose levels tested, so no NOAEL was derived due to "profound hepatic damages [sic]" at the low dose of 500 ppm (equivalent to 37.5 mg/kg bw per day). It is noted that the original study report was in German and the English translation provided was not complete. In addition, findings on individual animals were not provided and details of the hepatic damage found were limited. Therefore, the reliability of the study is considered to be low and it was not used further (Scholz & Brunk, 1968).

(b) Dermal application

In a seven-day dose range-finding dermal toxicity study, Crl: CD BR rats (two/sex per dose) were treated with quintozene (purity 98%; 0.051% HCB, 0.61% 2,3,5,6-TCNB, 0.40% 2,3,4,5-TCNB) moistened with reverse osmosis water at dose levels of 0, 250, 500 or 1000 mg/kg bw per day. The test material was administered dermally for five days per week for one week using a moistened porous gauze dressing which was secured by non-irritating tape and wrapped with Coban™. Daily contact exposure was for at least six hours. The residual material was removed with reverse osmosis water and a paper towel. The animals were examined for viability twice daily and dermal responses were evaluated prior to dosing on days 0, 1, 4 and 7. Clinical observations were performed daily. Body weights were recorded pre test, on days 0 and 7. Food consumption was recorded during the test period. After day 7 all rats were sacrificed and subjected to gross necropsy.

All animals survived until terminal sacrifice and no treatment-related clinical signs were observed. Repeated topical administration of quintozene did not elicit a dermal response in any of the test animals. All animals showed normal weight gain and food consumption. There were no adverse effects observed at post-mortem examination. Based on this study, it was considered that the highest dose level to be tested in a subsequent 21-day dermal rat study should be 1000 mg/kg bw per day (Trimmer, 1992a).

In a 21-day dermal study, CD rats (6/sex per dose) were exposed once daily for seven days per week to quintozene (purity 98.72%) moistened with deionized water at dose levels of 0, 30, 300 or 1000 mg/kg bw per day. The doses were evenly distributed over the test site which was wrapped with gauze bandaging and secured with nonirritating tape. Each exposure period was approximately six hours. After each exposure the bandaging materials were removed and the test site washed with tepid tap water. Rats were observed for moribundity, mortality and clinical signs twice daily throughout the study. The animals were observed for dermal irritation once daily prior to dosing in accordance with the Draize method. Body weights were obtained pretest and weekly thereafter; food consumption was determined weekly. Blood samples were obtained after an overnight fasting period for haematological and clinical chemistry investigations; urine was collected during the fasting period. All animals received a complete post-mortem examination. Kidney, liver and testis were weighed. Representative samples of liver, kidney and skin were processed for microscopic examination.

All animals survived until terminal sacrifice and no treatment-related clinical signs were observed. No dermal irritation was observed in any rat during the study period. No treatment-related effect on body weight or food consumption was seen, nor were there effects on haematology, urinalysis, organs weights or histopathology. A statistically significant decrease in ALT compared to the controls was seen at the highest dose tested in males (−50%) and females (−31%). Bearing in mind the limited histopathological investigation undertaken, no NOAEL could be proposed. (Goldenthal, 1992).

In a 21-day dermal toxicity study, Crl:CD BR (Sprague Dawley) rats (five/sex per dose) were treated with quintozene (purity 98%; 0.051% HCB, 0.61% 2,3,5,6-TCNB, 0.40% 2,3,4,5-TCNB) applied to clipped, unabraded skin under a porous dressing, for six hours per day, five days a week for three weeks. One group served as a control, receiving reverse osmosis water only. Other groups received 100, 300 and 1000 mg/kg bw per day quintozene. Clinical observations were made daily as to the nature, onset, severity and duration of toxicological signs. Dermal irritation was assessed on days 0, 1, 4, 7, 11, 18, 21 and 22. Body weight and food consumption measurements were made weekly. Serum chemistry and haematology studies were performed on all surviving animals at study termination on day 22.

A full macroscopic examination was performed on all animals and selected organs, which were also weighed (kidney, liver, testes/ovaries). A limited range of tissues from the control and high-dose animals were examined microscopically (liver, ovaries/testes, kidneys, skin, gross lesions), while the thyroid from all dose groups was examined.

All animals survived until terminal sacrifice and no treatment-related clinical signs were observed. Repeated topical administration of quintozene did not elicit a dermal response in any of the test animals. All animals showed normal body weight gain and food consumption and no treatment-related effects on haematological parameters were observed. In males a decrease in ALT (−25%; not statistically significant) was observed at the highest dose. No were effects were seen on organ weights nor during macroscopic examination. Treatment-related microscopic changes were observed in the thyroid gland of four out of five male rats in the high-dose group; these consisted of hypertrophy of the follicular epithelium. In three of the affected males the follicles were dilated with a pale colloid. A similar change was not observed at lower doses in males, or in females at any dose level. Given the limited histopathological examination no NOAEL could be proposed (Trimmer, 1992b).

(c) Exposure by inhalation

No short-term inhalation toxicity studies were submitted.

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

In a 103-week toxicity and carcinogenicity study, B6C3F1 mice (50/sex per dose) were administered for 103 weeks, diets containing quintozene (purity greater than 99%; 0.07% HCB)at doses of 0, 2500 or 5000 ppm (equivalent to 0, 400 and 1000 mg/kg bw per day for males, 0, 600 and 1400 mg/kg bw per day for females). These doses were selected based on an earlier 13-week study (NTP, 1987). Clinical observations were made twice a day; animals were weighed once a week for the first twelve weeks followed by monthly thereafter. During necropsy all organs and tissues were examined for grossly visible lesions. Examination of tissues was performed according to the inverse pyramid design (McConnell, 1983a, b). Complete histopathological examinations were performed on high-dose and control animals and on all animals that died early in the study, including those receiving lower doses. In addition, histopathological examinations were performed on all gross lesions and tissues/organs from animals in the lower-dose groups when chemically related neoplastic or non-neoplastic effects were identified in the high-dose animals. If mortality in a high-dose group exceeded that in the control group by 15%, a complete histopathological examinations was performed on all the animals in the next highest dose group, in addition to those in the high-dose group. The quality assessment report and slides were submitted to the Pathology Working Group chairperson, who reviewed all target tissues and those about which there was a disagreement between the laboratory and quality assessment pathologists.

Survival after week 86 among the high-dose females was significantly less than for controls. No other differences in survival were observed in any groups of either sex. No treatment-related clinical signs were reported. Final mean body weights of low-dose and high-dose male mice were 96% and 90% those of the controls. The mean body weights of high-dose female mice were more than 10% lower than those of the controls after week 20, and 21% lower by week 104. Mean body weights of low-dose females were more than 10% lower than those of the controls after week 88. The data suggest that the dosed groups consumed more food at certain time points than did the corresponding controls, but some food was scattered and it is not possible to determine if dosed groups actually ate more food than did the controls. Based on these data, the average amount of quintozene consumed per day was calculated to be equal to 400 mg/kg bw and 1000 mg/kg bw for low dose and high dose male mice, and the comparable values to be 600 mg/kg bw and 1400 mg/kg bw for low- and high-dose females. Gross and microscopic pathological findings in female mice were limited to those considered to be secondary to bacterial infection. Ovarian abscesses, characteristic of bacterial infection, were observed in all groups of female mice (control, 12/49 or 24%; low dose, 22/50 or 44%; high dose, 29/50 or 58%). The incidences in dosed groups were significantly greater than ($p < 0.05$) in the controls. Six abscesses were cultured, and the bacteriologic findings indicated *Klebsiella* in five of the six samples. Plasma cell hyperplasia of the mediastinal lymph node was observed at an increased incidence ($p < 0.01$) in high-dose female

mice (control, 1/44 or 2%; low dose, 4/47 or 9%; high dose, 9/45 or 20%). In the liver haematopoiesis was observed at increased incidences (significant at $p < 0.01$) in dosed female mice (control, 9/50 or 18%; low dose, 21/50 or 42%; high dose, 23/50 or 46%); this effect was not seen in male mice (control, 0/49; low dose, 2/50 or 4%; high dose, 0/50). In the spleen haematopoiesis was observed at increased incidences (significant at $p < 0.05$) in dosed female mice (control 14/50 or 28%; low dose, 23/48 or 48%; high dose, 27/50 or 54%). It was concluded that there was no evidence from this study of carcinogenicity in either male or female B6C3F1 mice. Infection was considered to have decreased survival of the female mice and thus reduced the overall sensitivity of the study. Therefore, no robust NOAEL could be proposed. It is noted that even in the face of infection, no evidence of carcinogenicity was reported (NTP, 1987).

Table 8. Survival and body weight in mice receiving quintozene (group size n = 50)

	Sex and dietary dose (ppm)					
	Males			Females		
Time point	**0**	**2500**	**5000**	**0**	**2500**	**5000**
			Survival (%)			
Week 84	92	92	84	76	68	60
Week 88	92	92	82	74	52	50
Week 104	68	62	64	60	40	28
			Body weight (g)			
Week 80	41.3	41.3	38.0	46.3	42.5	38.0
Week 84	39.9	39.9	37.4	45.9	42.2	37.1
Week 88	40.2	40.1	38.1	46.2	41.5	37.9
Week 92	40.5	39.9	36.1	48.4	41.4	36.6

Source: NTP, 1987

Rat

In a non-GLP two-year carcinogenicity study, Wistar rats (50/sex per dose) were administered quintozene (purity 98.2/98.3%; 2.7% HCB, 0.64% 2,3,5,6-TCNB/2,3,4,6-TCNB, 1.1% 2,3,4,5-TCNB, less than 0.5% 1,2,4,5-tetrachlorobenzoquinone) via the diet at dose levels of 0, 100, 400 or 1200 ppm (equivalent to 0, 5, 20 and 60 mg/kg bw per day). Observations were made of general appearance, behaviour, mortality, growth and food intake. Haematological investigations were carried out in weeks 52 and 104. Clinical measurements in blood and urine were made at termination. At week 104 all surviving rats were killed by decapitation, underwent a gross examination and eight different organs were weighed (heart, kidneys, liver, spleen, brain, gonads, thyroid, adrenals). Extensive histopathological examinations were carried out on 20 males and females per dose group. The histological examination of the remaining 30 animals per sex and dose group was restricted to the liver and to gross visible tumours, or lesions suspected of being tumours.

There were no treatment-related clinical signs, effects on mortality, body weight or food consumption. Some slight, though statistically significant, differences in Hb concentration, packed cell volume, erythrocyte count and total leucocyte count occurred only in the intermediate groups or at one of the two stages. These differences were therefore considered to be incidental findings. Quintozene had no adverse effect on clinical chemistry or urinalysis parameters. At the highest dose level, the relative weights of the liver and kidneys were increased in both sexes. Relative liver weight was increased by 17% in high-dose males and by 30% in high-dose females. At 1200 ppm relative kidney weight was increased in males by 14% and in females by 12% compared to controls. The relative weight of ovaries was decreased by 22% compared to controls at 1200 ppm. The relative weights of the testes were slightly higher in all quintozene-treated groups compared to controls, but the differences did not show any indication of a dose-related response. Histopathological effects were found in the liver in all treatment groups. The liver findings in the low-dose group consisted of a slight enlargement of centrilobular liver cells occasionally accompanied by a fine vacuolar fatty metamorphosis of centrilobular liver cells and a slightly increased incidence of isolated cell necrosis in the centrilobular area. The liver lesions in

rats of the intermediate- and high-dose groups were characterized by a more frequent and pronounced enlargement of hepatocytes which in advanced stages exhibited distinct hyaline intracytoplasmic bodies (referred to in the literature as Mallory bodies) and an increased incidence of centrilobular isolated cell necrosis. The observed liver cell alterations were, however, not accompanied by centrilobular or periportal fibrosis, architectural abnormalities or other indications of a progressive liver disease. The most common neoplasms were localized lymphoreticular malignancies derived from pulmonary peribronchial lymphoid tissue and systemic neoplastic disease of the haematopoietic tissue, adenomas of the pituitary, phaeochromocytomas of the adrenal gland and tumours of the mammary gland. No relationship could be demonstrated between treatment and the frequency or onset of the different tumours.

No indication for a tumourigenic effect was found in this rat study. Therefore, the carcinogenicity NOAEL was 1200 ppm (equivalent to 60 mg/kg bw per day), the highest dose tested. The chronic toxicity NOAEL was 100 ppm (equivalent to 5 mg/kg bw per day) based on liver histopathology (enlarged centrilobular hepatocytes with Mallory bodies and single cell necrosis) at 400 ppm (equivalent to 20 mg/kg bw per day) (Sinkeldam et al., 1974).

Table 9. Results from the two-year study in the rat: histopathological findings in the liver

	Sex and dietary dose (ppm)							
	Males				Females			
Parameter	**0**	**100**	**400**	**1200**	**0**	**100**	**400**	**1200**
Number of animals examined	20	20	19	20	20	20	20	20
Enlarged centrilobular hepatocytes, at times showing large hyaline intracytoplasmic bodies								
total	0	11***	19***	18***	0	12***	18***	20***
slight	0	7	8	4	0	10	10	1
moderate	0	4	8	8	0	2	5	11
marked	0	0	3	6	0	0	3	8
Fatty metamorphosis of hepatocytes, mainly centrolobular								
total	3	4	12**	13**	3	9 ($p=0.08$)	12** ($p=0.008$)	9
slight	3	4	12	11	3	9	11	8
marked	0	0	0	2	0	0	1	1
Single cell necrosis	1	1	5 ($p=0.18$)	9	0	2	6 ($p=0.02$)	9

Statistically significant using Fisher Exact two-tailed test: ** $p < 0.01$, *** $p < 0.001$; Source: Sinkeldam et al., 1974

In a two-year chronic toxicity and carcinogenicity study, Crl;CDBR (Sprague Dawley) rats were administered quintozene (purity 98%; batch 05318; 0.61% 2,3,5,6-TCNB, 0.40% 2,3,4,5-TCNB, 0.061% HCB) five days per week for a approximately two years, orally by gavage. The vehicle was 1% (w/v) CMC and 0.1% (w/v) Tween 80 in reverse osmosis water. Five groups consisting of 60 rats/sex per dose were used. The control group received just the vehicle. Other groups received quintozene at a dose of 0, 5, 50, 500 or 1000 mg/kg bw per day. Observations were made weekly for the duration of the study as to the nature, onset, severity and duration of toxicological signs. Additionally, cageside observations were performed daily Monday to Friday during the study (beginning on test day 106). Body weights were recorded prior to dosing, at dosing initiation (day 0), weekly for the first 13 weeks, then every four weeks thereafter. Food consumption was measured weekly for the first 13 weeks, and every four weeks thereafter. Serum chemistry, haematology and urinalysis studies were performed on the first ten survivors from each sex and group at 6, 12, and 18 months, and prior to study termination. Ophthalmoscopic observations were performed pre test (all test groups) and prior to study termination on all female animals and males in the control, 500 and 1000 mg/kg bw per day groups. A full macroscopic examination was performed on all animals, selected organs and tissues were collected and weighed. A range of tissues was examined microscopically.

There were no treatment-related effects on survival or clinical signs, however survival was low (25% or less at termination). Treatment-related decreases in mean body weight compared to controls

were observed in males given 1000 mg/kg bw per day (up to 9%), and in females given 1000 mg/kg bw per day (up to 12%) and 500 mg/kg bw per day (up to 10%). There were no significant differences between treated and control animals' mean food consumption, nor was any difference noted in ophthalmoscopy, haematology or urinalysis parameters which were considered related to treatment with quintozene.

At the high dose in males the mean liver weight was significantly increased ($p \leq 0.05$) compared with controls, absolute weight by 29% and relative weight by 32%. This finding was considered treatment-related. There were no statistically significant differences between organ weights of treated and control females. Histopathological effects were observed in liver and thyroid gland at the two higher dose levels, including centrilobular hepatocellular hypertrophy and thyroid follicular cell hypertrophy/ hyperplasia.

Table 10. Selected non-neoplastic and neoplastic lesions found in two-year study in rats

	Sex and dietary dose (ppm)									
	Males					Females				
	0	**5**	**50**	**500**	**1000**	**0**	**5**	**50**	**500**	**1000**
	Liver (number affected [% affected])									
Number of animals examined	60	60	60	60	60	60	60	60	60	60
Centrilobular hepatocellular. hypertrophy	2	2	9 ($p = 0.053$)	19**	26**	1	1	1	10**	22**
Hepatocellular adenoma	3 [5%]	2 [3.3%]	2 [3.3%]	0	4 [6.7%]	0	1 [1.7%]	0	1 [1.7%]	0
Hepatocellular carcinoma	0	0	2 [3.3%]	1 [1.7%]	2 [3.3%]	0	0	0	0	0
	Thyroid (number affected [% affected])									
Number of animals examined	60	60	59	60	60	59	59	59	59	60
Follicular hypertrophy/ hyperplasia, follicular epithelium	2	2	2	6 ($p = 0.27$)	12**	1	0	2	5	10**
Follicular focal hyperplasia	1	0	1	2	4	0	0	0	0	2
Follicular adenoma	0	0	2 [3.3%]	4* [6.7%]	1 [1.7%]	0	0	0	0	1 [1.7%]
Follicular adenocarcinoma	0	0	1 [1.7%]	1 [1.7%]	0	0	0	0	0	0

Statistically significant: * $p < 0.05$; ** $p < 0.01$; Source: Plutnick, 1993

An increase in thyroid follicular adenoma and adenocarcinoma was seen in males at 500 mg/kg bw per day, but not at the higher dose level of 1000 mg/kg bw per day. An increase in hepatocellular adenoma and carcinoma was observed, however without a clear dose–response relationship. Treatment with quintozene produced no evidence of carcinogenicity in male or female rats in this study. Therefore, the NOAEL for carcinogenicity was 1000 mg/kg bw per day, the highest dose tested. The chronic toxicity NOAEL was 50 mg/kg bw per day based on decreased body weight and histopathological changes in the thyroid gland at 500 mg/kg bw per day (Hildebrandt, 1993; Plutnick, 1993).

In a two-year chronic toxicity and carcinogenicity study, CD rats (60/sex per dose) were administered quintozene (purity 99.4%; batch 804Q011, less than 0.1% HCB) via the diet. The dose levels were 0, 20, 3000 and 6000 ppm (equal to 0, 0.91, 141 and 303.2 mg/kg bw per day for males, 0, 1.14, 179.19 and 369.9 mg/kg bw per day for females). Mortality and moribundity were observed twice daily; detailed observations were conducted at least once a week. Body weights were measured pretest, then body weights and food consumption were obtained weekly for the first 16 weeks and once every four weeks thereafter. Ophthalmoscopic examination was conducted on all animals pretest and at 12 and 24 months. Haematology and clinical chemistry examinations were conducted on 10 animals/

sex per group at 6, 12, 18 and 24 months. Interim necropsy was performed on 10 rats/sex per dose at 12 months. Terminal necropsy was performed on 50 animals/sex per group after 24 months. All animals received a complete post-mortem examination, organ weights were determined and histopathological examination conducted.

Neither mortality nor clinical signs of toxicity showed treatment-related effects following exposure to quintozene. Body weights were decreased in males (by 10.5%) and females (by 11.5%) at the 6000 ppm dosage level, but at the 3000 ppm dosage level in males only during just the last six months of study. Food consumption was reduced for both males and females at the 6000 ppm. No treatment-related ophthalmological abnormalities were detected. Cholesterol values were increased in females at 6000 ppm for all intervals of analysis. There was no other notable treatment-related biochemical or haematological changes at any dosage level.

At necropsy a few males administered 3000 and 6000 ppm showed enlargement of the thyroid. An increased incidence of accentuated lobulations of the liver and an increased incidence of tan/yellow foci in the lungs were seen in males and females at the 3000 and 6000 ppm dosage levels. These changes correlated with microscopic findings. Absolute and relative liver and thyroid weights were increased at the 3000 and 6000 ppm dosage levels. Microscopically, treatment-related changes were present in the liver, thyroid and lungs of males and females at the 3000 and 6000 ppm dosage levels. The liver displayed hepatocellular hypertrophy. In the thyroid there was hypertrophy and hyperplasia of the follicular epithelium in many males and females at 3000 and 6000 ppm. In addition, there was an increased incidence of colloid cysts in males at 6000 ppm, and an increased incidence of follicular adenomas in males and females at 3000 and 6000 ppm. Historical control data (HCD) indicate that the average for follicular adenoma is 2.88% in males (range 0–11%) and 0.30% in females (range 0–3.2%); the average for follicular carcinoma is 1.73% in males (range 0–9.4%) and 0.60% in females (range 0–3.2%). In the present study with quintozene the incidence of follicular adenoma and carcinoma was above the HCD mean.

Table 11. Selected results from two-year chronic toxicity and carcinogenicity study in rats

	Sex and dietary dose (ppm)							
	Males				**Females**			
Parameter	**0**	**20**	**3000**	**6000**	**0**	**20**	**3000**	**6000**
	Organ weights							
Absolute liver weight (g)	22.47	24.13	25.25	25.88*	18.13	19.75	20.16	19.15
Relative liver weight (%)	3.45	3.37	4.13*	4.30**	4.04	4.23	4.58	4.86**
Absolute thyroid weight (mg)	47	53	59**	68**	38	42	46	50**
Relative thyroid weight (% × 10^3)	7.16	7.72	9.64**	11.28**	8.26	9.20	10.28*	11.88**
	Histopathology (number affected [% affected])							
Number of rats examined	49	49	48	49	50	49	50	47 (thyroid 46)
Hepatocellular hypertrophy	0	0	13	30	0	0	19	29
Thyroid, colloid cyst	2	0	2	8	1	0	2	1
Thyroid hypertrophy	3	2	20	33	0	0	18	23
Thyroid hyperplasia	2	2	7	8 ($p = 0.095$)	0	0	6 ($p = 0.027$)	8
Thyroid follicular adenoma	0	0	6 ($p = 0.027$)	5 [10.2%] ($p = 0.057$)	1	0	2	4 [8.7%]
Thyroid follicular carcinoma	0	1	0	2 [4.08%]	1	0	0	1 [2.2%]

Statistically significant: * $p < 0.05$; ** $p < 0.01$; Source: Goldenthal, 1991

Studies investigating the postulated underlying MOA for the thyroid tumours are described below in 2.6(e) Mechanistic studies.

Based on the results of this study the NOAEL for chronic toxicity was 20 ppm (equal to 0.91 mg/kg bw per day) based on histopathological findings in the thyroid. At the highest dose level of

6000 ppm in both sexes and at 3000 ppm in males, thyroid follicular cell adenomas and carcinomas were seen which could be secondary to liver enzyme induction followed by thyroid hormone perturbation, and thus would represent a nongenotoxic mechanism. The NOAEL for carcinogenicity was 20 ppm (equal to 0.91 mg/kg bw per day), based on thyroid follicular cell adenomas and carcinomas in males at 3000 ppm (equal to 141 mg/kg bw per day) (Goldenthal, 1991).

2.4 Genotoxicity

Quintozene was included in the National Toxicology Program (1987) which described a negative bacterial reverse mutation assay, a negative mammalian cell gene mutation assay, a negative sister chromatid exchange assay and a positive chromosome aberration assay.

For the current evaluation, an additional in vivo micronucleus assay in mice was submitted, which was negative. In this study exposure of bone marrow was sufficiently demonstrated.

In the in vitro data package, the chromosome aberration assay was positive. However, an in vivo micronucleus assay was also available which showed quintozene does not exhibit clastogenic or aneugenic potential in vivo. Overall, it is concluded that quintozene raises no concern for genotoxicity in vivo.

Table 12. Overview of genotoxicity with quintozene[a,b]

End-point	Test object	Concentration/ dose	Purity of quintozene	Results	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* strains TA98, TA100, TA1535, T1537[c]	0–6667 µg/plate	Purity 99%; 0.07% HCB	Negative	NTP, 1987
Gene mutation	Mouse lymphoma cells L5178Y	0–10 µg/mL	Purity 99%; 0.07% HCB	Negative	NTP, 1987
Sister chromatid exchange	Chinese hamster ovary cells	0–7.5 µg/mL (−S9) 0–75 µg/mL (+S9)	Purity 99%; 0.07% HCB	Negative	NTP, 1987
Chromosome aberration	Chinese hamster ovary cells	0–75 µg/mL	Purity 99%; 0.07% HCB	Positive (+ or – S9)	NTP, 1987
In vivo					
Micronucleus test	Male and female ICR mice (5–15/sex per dose)	0, 230, 460 or 920 mg/kg bw	Purity 99%; 0.07% HCB	Negative[d]	Guidi & Krsmanovic, 2000

S9: 9000 × *g* supernatant fraction from rat liver homogenate;

[a] Positive and negative (solvent) controls were included in all studies, except the Ames test which lacked a positive control;

[b] Statements of adherence to quality assurance and GLP were included unless indicated otherwise;

[c] Study did not contain a strain to detect cross-linking mutagens (e.g. TA102 or E.coli WP2 uvrA).

[d] Dose administration via intraperitoneal injection; exposure of bone marrow was demonstrated by a decrease in the ratio of polychromatic erythrocytes to total erythrocytes; in this study no mortality occurred, clinical signs observed included lethargy and piloerection at 460 and 920 mg/kg bw.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

A two-generation study was conducted in rats with quintozene. Groups of 26 males and 26 female COBS® CD® rats were administered quintozene (purity 99.4%; Lot no. 805-Q020; 0.08% HCB) at dietary concentrations of 0, 20, 3000 or 6000 ppm, beginning at 55 days of age for 81 days prior to an initial mating. In the F0 generation these doses were equal to 0, 1.1, 168 and 350 mg/kg bw per day for males, 0, 1.4, 221 and 459 mg/kg bw per day for females. During gestation these doses were equal to 0, 1, 155–161 and 324–328 mg/kg bw per day. During lactation they were equal to 0, 3, 454–461 and

795–817 mg/kg bw per day. For the F1 generation these doses were equal to 0, 1.4, 200 and 443 mg/kg bw per day for males, 0, 1.6, 240 and 556 mg/kg bw per day for females. During gestation these doses were equal to 0, 1, 152–166 and 337–347 mg/kg bw per day, and during lactation 0, 2.5–2.8, 412–433 and 753–761 mg/kg bw per day. The F0 parents were mated twice to produce the F1a and F1b litters. From the F1b litters, 26 male and 26 female rats from each group were selected to become F1 parents. These animals were treated beginning at 22 days of age for a minimum of 90 days prior to initial mating. The F1 parents were mated again to produce the F2a and F2b litters. All animals were observed twice a day for mortality and overt toxicity. Detailed clinical observations were recorded once every week. Body weights and food consumption were recorded weekly. Maternal body weights and food consumption were recorded at specified intervals during gestation and lactation. Reproductive and litter parameters that were assessed during lactation included fertility indices, events at parturition, gestation length, litter size, number of viable and stillborn pups, offspring survival and growth. Litter size was reduced to eight pups (equal sex distribution whenever possible) on lactation day (LD) 4. The culled pups were examined externally. All parental animals, F1b and F2b weanlings were subjected to gross examinatioin at necropsy.

At the high dose of 6000 ppm decreased parental body weights and food consumption in comparison to the control values occurred in both generations. The decrease in body weight at the high dose in males and females was around 6–8% for F0 animals and 25% for the F1 animals compared to the controls. During gestation, body weight in F0 adult females was decreased by around 6% compared to controls, and body weight gain by 9%. In F1 females during gestation body weight was decreased by 15% and body weight gain by 14%. During lactation, body weight loss was seen in F0 females for the F1a and F1b mating during lactation over days 4–7 and 7–14. In the F1 females during gestation for the F2 mating, body weight decreased by 18%, but no effect on body weight gain was observed over the whole study, days 0–21.

Table 13. Body weight and weight gain in female rats during gestation and lactation

		Dietary dose			
Parameter		**0 ppm**	**20 ppm**	**3000 ppm**	**6000 ppm**
Females F0, gestation F1a mating					
Body weight (g)	day 0	271	272	264	254**
	day 7	296	295	290	279**
	day 14	317	316	313	301**
	day 20	382	378	368*	355**
Body weight gain (g)	days 0–7	25	22	26	25
	days 7–14	21	22	23	22
	days 14–20	65	62	55	54**
	days 0–20	110	106	104	102*
Females F0, lactation F1a mating					
Body weight (g)	day 0	291	297	284	272**
	day 4	313	311	306	283**
	day 7	311	315	311	280**
	day 14	328	329	319	274**
	day 21	318	310	314	281**
Body weight gain (g)	days 0–4	22	14	22	10**
	days 4–7	−2	4	5	–3
	days 7–14	17	14	9	–6**
	days 14–21	−11	−19	−5	7**
	days 0–21	27	14*	30	8**

Parameter		Dietary dose			
		0 ppm	20 ppm	3000 ppm	6000 ppm
Females F1, gestation F2a mating					
Body weight (g)	day 0	278	276	264*	231**
	day 7	297	287	286	231**
	day 14	322	316	316	275**
	day 20	382	378	373	323**
Body weight gain (g)	days 0–7	19	11	22	0*
	days 7–14	25	29	30	44**
	days 14–20	61	62	57	48**
	days 0–20	104	101	109	91*
Females F1, lactation F2a mating					
Body weight (g)	day 0	302	304	293	252**
	day 4	318	313	314	261**
	day 7	316	316	318	264**
	day 14	314	322	320	259**
	day 21	315	309	316	265**
Body weight gain (g)	days 0–4	16	9	21	9
	days 4–7	−2	3	4	3
	days 7–14	−2	5	2	–5
	days 14–21	1	−13	−4	6
	days 0–21	13	5	23	13

Statistically significant: * $p < 0.05$; ** $p < 0.01$; Source: Schardein, 1991

No adverse treatment-related differences in male or female fertility or copulatory indices, the gestation index, the mean copulatory interval or the mean gestation length were noted. Offspring survival (mean number of stillborn pups, mean number of live pups at birth and LD 0, sex ratio) was not affected by treatment with quintozene. Offspring body weight was decreased in the F1 (12–40% compared to controls) and F2 litters (ca 30% compared to controls) at the high dose. At the mid-dose, offspring body weights in F1a, F1b females and F2b litters were lower by around 8% compared to controls, a less pronounced effect than in the high-dose offspring. A dose-related increase in the incidence of offspring that appeared small was observed among the F1a and F1b litters at the mid- and high-dose levels, and in the F2a and F2b, but in this case at the high-dose only.

Histopathological examination showed macroscopic tan or yellow foci in the lungs of the F1 adults, particularly in the high-dose groups. Microscopically these foci were found to represent aggregates of alveolar macrophages, perivascular/peribronchial lymphoid cell infiltration and/or interstitial pneumonia. According to the authors, these changes were often seen in respiratory viral infections and on several occasions were seen to be exacerbated in treated groups of rats, particularly females. The microscopic picture of the changes, their minimal severity and occurrence in just a few rats suggested that the pulmonary changes were a stress-related exacerbation of the naturally occurring disease. Selected results are shown below in Tables 14–16.

Table 14. Selected results for the offspring in the two-generation study in rats (number individuals affected [% of offspring affected])

		Dose group (ppm)			
Generation	**Effect**	**0**	**20**	**3000**	**6000**
F1a	Small in size (LD 21)	3 [1.9%] 2 litters	1 [0.6%] 1 litter	32 [16.6%] 4 litters	165 [82.5%] 22 litters
F1b	Small in size (LD 21)	2 [1.6%] 2 litters	4 [2.9%] 4 litters	30 [18.9%] 7 litters	170 [95.5%] 22 litters
F2a	Small in size (LD 21)	19 [14.1%] 4 litters	21 [11.7%] 5 litters	13 [7.5%] 5 litters	145 [81.9%] 18 litters
F2b	Small in size (LD 21)	1 [0.9%] 1litter	28 [21.9%] 8 litters	18 [15.8%] 4 litters	142 [80.7%] 19 litters
	Live pup numbers (m + f)/number of litters with live pups				
		Live pups on day 0			
F1a	274/20	283/22	311/25	319/25	274/20
F1b	202 /18	232/18	276/21	301/23	202 /18
F2a	225/17	300/24	282/22	274/25	225/17
F2b	209/16	212/20	197/16	290/24	209/16
		Live pups on day 21			
F1a	160	166	193	200	F1a
F1b	127	138	159	178	F1b
F2a	135	180	173	177	F2a
F2b	118	128	114	176	F2b

LD: Lactation day; Source: Schardein, 1991

Table 15. Pup body weights in the two-generation study in rats (g)

	Dose group (ppm)							
Period:	**Males**				**Females**			
group	**0**	**20**	**3000**	**6000**	**0**	**20**	**3000**	**6000**
Day 0								
F1a	6.1	6.4	6.2	5.9	5.8	6.0	6.0	5.6
F1b	6.5	6.5	6.4	5.9	6.2	6.2	6.2	5.6*
F2a	6.0	6.3	5.9	5.9	5.6	6.0	5.6	5.6
F2b	6.2	6.3	5.9	5.5*	6.0	5.7	5.7	5.3
Day 7								
F1a	15.3	16.1	14.9	12.0*	14.7	15.5	14.7	12.2*
F1b	16.6	16.8	16.5	13.3*	16.3	16.2	16.1	12.7*
F2a	13.7	14.6	14.5	12.3	13.2	13.7	13.6	12.1
F2b	16.0	15.5	14.5	12.1*	15.1	13.9	14.1	11.0*

Period:	Dose group (ppm)							
	Males				Females			
group	0	20	3000	6000	0	20	3000	6000
Day 21								
F1a	51.1	52.6	46.7*	31.5*	49.0	50.7	45.1*	30.7*
F1b	54.6	54.8	50.9	32.5*	53.4	52.4	49.0*	31.4*
F2a	43.1	45.2	42.6	28.9*	40.7	43.2	40.1	28.6*
F2b	51.5	51.3	47.0*	33.1*	48.7	44.9	44.8*	32.6*
Week 5								
F1	151	152	143	103*	130	128	120*	89*
Week 20								
F1	531	539	521	438*	287	328	282	259

Statistically significant: * $p < 0.05$; Source: Schardein (1991)

Table 16. Selected lung pathology results for the adult animals in the two-generation study in rats

		Dose group (ppm)							
		Males				Females			
		0	20	3000	6000	0	20	3000	6000
		Macroscopic observations; lungs							
F0 ($n=26$)	Lung foci, tan, mild	0	0	1	1	0	0	1	3
F1 ($n=26$)	Lung foci, tan, mild	0	0	0	3	0	0	2	6
		Microscopic observations; lungs							
F0	Alveolar macrophages:								
	trace	0	0	1	1	0	0	0	1
	Perivascular lymphoid infiltration:								
	mild	0	0	1	0	0	0	1	1
F1	Alveolar macrophages								
	trace	0	0	0	1	0	0	1	4
	mild	0	0	0	1	0	0	1	1
	Perivascular lymphoid infiltration:								
	(mild in males, trace in females)	0	0	0	2	0	0	0	1

Source: Schardein, 1991

The NOAEL for parental toxicity was 3000 ppm (equal to 168 mg/kg bw per day) based on decreased body weight and food consumption. The NOAEL for reproductive toxicity was 6000 ppm (equal to 350 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 20 ppm (equal to 1.1 mg/kg bw per day) based on increased incidence of undersized pups at 3000 ppm (equal to 168 mg/kg bw per day) (Schardein, 1991).

A two-generation study was conducted in Crl:CD BR (Sprague Dawley) rats with quintozene. For this study, four groups consisting of 35 rats/sex per group were used. Quintozene (purity 98%; Batch 05318-7D; 0.051% HCB) was administered by oral gavage at dose levels of 0, 10, 100 or 1000 mg/kg bw per day. One group was used as a carrier control which received 1% (w/v) CMC containing 0.1% (w/v) Tween 80 in reverse osmosis water. A dose volume of 10 mL/kg bw was administered to all groups. F0 males and females received test material daily for at least ten weeks prior to mating and during the mating period. Additionally, F0 female animals received test material during gestation, lactation and until the weaning of the Fl offspring on postpartum day (PPD) 21. Parental Fl males were dosed from postnatal day (PND) 21 for at least 10 weeks prior to mating, and then through the mating period for F2 litters, until euthanized following delivery of the last litter they had sired. Parental F1

females were dosed from PND 21 for at least 10 weeks prior to mating and then during mating, gestation, lactation, and until they were euthanized following weaning of the F2 progeny on PPD 21. Clinical in-life observations, body weight, and food consumption were recorded for all F0 and F1 parental animals at least weekly during the premating and mating periods (food consumption was not measured during mating due to cohabitation), and for females on gestation days (GDs) 0, 7, 14, and 21 and on PPDs 0, 1, 4, 7, 10, 14, and 21. Following birth, the offspring were counted, sexed, and examined externally daily from PND 0 to PND 21. Offspring were weighed on PNDs 0, 1, 4, 7, 14, and 21. Parental F0 and F1 males were euthanized after the birth of the last litter they had sired, while females were euthanized following weaning of their litters. A gross necropsy was performed on all adult animals, selected F1 and F2 neonates, and on all animals that died during the study. A full macroscopic examination was performed on these animals and selected organs and tissues collected. Selected reproductive organs and tissues were weighed from parental F0 and F1 animals used for mating. A range of tissues was examined microscopically.

There were no treatment-related clinical signs observed in the F0 and F1 parental generations. Unscheduled mortality in the F0 parental generation was limited to one control male, one low-dose male, and one high-dose female. Unscheduled mortality during the F1 parental generation was limited to two control males, two mid-dose males, and one high-dose female. All deaths were considered to be incidental and not related to treatment. There were no differences in mean body weight, mean body weight change or mean food consumption between the treated and control animals of either sex during the premating/mating, gestation or postpartum periods of either the F0 or F1 parental animals. There were no gross post-mortem findings in the F0 or F1 adult animals that could be related to treatment with quintozene. Additionally, there were no meaningful differences in mean absolute or relative organ weights in either sex between treated and control animals. Absolute ovary weight was increased in F1 parental females in the high-dose group, however their relative weight was not increased compared to the control group. Treatment-related microscopic changes included an increased incidence of minimal or moderate hepatocellular hypertrophy in the parental F0 and F1 high-dose males and parental F0 high-dose females. Histologically the hepatocellular hypertrophy observed consisted of an increased prominence of enlarged hepatocytes with pale and finely granular cytoplasm, predominantly in the centrilobular zone of the hepatic lobules. Additionally, there was a treatment-related effect in the thyroid gland which consisted of an increased incidence of hypertrophy and hyperplasia of the follicular epithelium in mid- and high-dose males and the high-dose females of both generations. The histomorphological change in the thyroid consisted of enlarged follicular epithelial cells (hypertrophy) and an increased amount of follicular epithelium (hyperplasia) in some of the affected follicles. The affected follicles were often distended, with a pale and homogeneous eosinophilic colloid or clumps of dark colloid. There were no treatment-related microscopic changes observed in the reproductive tract of any of the quintozene-treated male or female rats.

There were no statistically significant differences from their respective controls in either the F0 or the F1 generation with regard to any of the following indices: male mating, male fertility, female fertility, female fecundity and female gestational index. The mean days of gestation of the treated F0 and F1 groups and control groups were essentially equivalent. There were no apparent differences in mean litter size or mean number of live offspring between treated and control groups in either the Fl or F2 generation. Similarly, there were no statistically significant differences in the mean sex ratio of the treated offspring compared with controls in either generation. There were several small, but statistically significant, decreases observed sporadically in the survival indices of the Fl treated offspring compared with controls. However, these differences were small (less than 5%), did not show any consistent response over the weaning period, and there was no clear dose–response relationship. Therefore, none of the differences in offspring survival indices were considered biologically or toxicologically important. There were no statistically significant differences in the survival indices or lactation index of the F2 treated offspring compared with the F2 controls. There were no treatment-related clinical findings observed in the offspring of any group of either generation. The majority of offspring in all groups were free of observable abnormalities from PND 0–21. Some offspring across all groups were observed without milk in their stomachs during the study. Lacerations were observed at a slightly increased incidence in the F2 high-dose offspring compared with controls. The significance of this finding is unknown. There were no apparent differences in mean offspring body weight between the treated and control offspring of either sex.

In general, there were no gross post-mortem findings in the Fl or F2 offspring judged to be related to treatment with quintozene. The majority of animals were free of observable abnormalities at the scheduled terminal sacrifice on PND 21. One F0 parental, low-dose dam had a retained fetus with multiple malformations including micrognathia, anophthalmia, microcephaly, adactylies, rudimentary tail, small genital tubercle, hypertrophy of the ventricle, and a short innominate artery. Additionally, one F2 mid-dose animal was euthanized on PND 37 with hydrocephaly. The significance of these findings is unknown, but these isolated occurrences of malformations were not considered treatment- related.

The NOAEL for parental toxicity was 10 mg/kg bw per day based on histopathological findings in the thyroid of male rats. The NOAEL for offspring and reproductive toxicity was 1000 mg/kg bw per day, the highest dose tested (Philips, 1994).

Table 17. Selected histopathological findings in F0 and F1 adult rats in the two-generation study

		Dose group (mg/kg bw per day)							
		Males				Females			
		0	10	100	1000	0	10	100	1000
Number of animals per group examined		35	34	35	35	35	35	35	34
Histopathological findings									
F0	Liver hepatocellular hypertrophy	2	0	2	22	0	0	0	4
	Thyroid, follicular epithelium hypertrophy/hyperplasia	5	3	11	26	7	4	7	22
F1	Liver hepatocellular hypertrophy	2	1	4	22	0	0	0	0
	Thyroid, follicular epithelium hypertrophy/hyperplasia	5	0	12	32	5	4	5	19

Source: Philips, 1994

(b) Developmental toxicity

Rat

In a range-finding study, mated female Charles River COBS® CD® rats were assigned to one control and five treatment groups of five animals each to determine dosage levels of quintozene (purity 96%; batch not indicated; less than 0.5% HCB) for a developmental toxicity study. Doses of 50, 125, 250, 500 or 1000 mg/kg bw per day were administered orally by gavage as a single daily dose on GDs 6–15. The control group received the vehicle only, 0.2% high viscosity CMC, according to the same regimen. Uterine examinations were performed on all females on GD 20. All animals survived until scheduled sacrifice. None of the groups administered quintozene showed treatment-related effects with respect to clinical signs, necropsy findings or maternal body weight gains. In addition, no developmental toxicity was observed in this range-finding study (Keller, 1988a).

In the main study, mated female Charles River COBS® CD® rats were assigned to one control and three treatment groups of 25 animals each. Dosage groups received quintozene (purity 96%; Batch MDD 080687; 0.025% HCB) at 30, 600 or 1200 mg/kg bw per day, administered via gavage once daily on GDs 6–15. Caesarean sections were performed on all surviving females on GDs 20 and the fetuses were removed for teratological evaluation. All animals survived until scheduled sacrifice and no treatment-related clinical signs were observed. A slight reduction in maternal body weight gain (–8% compared to controls) was seen over the entire gestation period (GDs 0–20) in the high dose group compared to the control group. Body weight gains during the first days of dosing (GDs 6–9) were 8, 12, 7 and 8 grams for the control, 30, 600 and 1200 mg/kg bw per day groups respectively. A slight but statistically significant decrease (−10%) in food consumption was seen during GDs 6–9 in the high-dose group compared to controls. There were no differences found in the mean number of viable fetuses, post-implantation loss, implantations, corpora lutea, fetal body weight or in sex ratio. No treatment-related effects were found concerning fetal malformations or variations. The maternal and embryo/fetal NOAEL in this study was 1200 mg/kg bw per day, the highest dose tested (Keller, 1988b).

In a developmental toxicity study, quintozene (purity 98.5%; Batch #05168-6D) was administered orally via gavage to mated Crl:CD™(SD)BR rats on GDs 6–15 at doses of 0 (1% aqueous CMC), 250,

750 or 1500 mg/kg bw per day. Viability checks were made twice daily. The rats were also observed for clinical signs of test substance effect, abortion and/or viability several times each day during the dosage period (days 6–15 of) and for clinical signs, abortion and natural delivery during the post dosage period (GDs 16–20). Body weight observations for the rats were recorded at least once weekly prior to mating. Body weight and feed consumption observations were recorded for the rats on day 0 and days 6–20 of presumed gestation. All rats were sacrificed by carbon dioxide asphyxiation on day 20 of presumed gestation.

One non-pregnant animal in the high-dose group was found dead on day 15 of presumed gestation. This death was secondary to an intubation accident; necropsy revealed adhesions between the diaphragm and lung and congestion of the lungs with what appeared to be the test suspension. No other animals died during the study. No treatment-related clinical signs were observed. Maternal body weight gain was decreased in the mid- and high-dose groups considering GDs 6–7 and 6–9. Average maternal body weights for the entire dosing period and for the post-dosage period did not differ among the four groups. A non-significant decrease in food consumption was seen in the mid- and high-dose groups during the first days of dosing. Maternal food consumption parameters did not notably differ among the four groups during the remainder of the dosage period and were similar when calculated for the entire dosage period. No gross lesions attributable to the test substance were revealed by necropsy of the dams and no treatment-related findings were seen concerning liver weight. No dose-dependent or statistically significant differences occurred among the four groups in the average numbers of corpora lutea, implantations, live litter sizes, resorptions or number of dams with resorptions. There were no dose-dependent, statistically significant differences among the four groups in the averages for fetal sex ratios, body weights or dead/resorbed conceptuses. Gross external examination revealed no treatment-related malformations or variations. No treatment-related soft tissue alterations or skeletal malformations were seen. Analyses of the average number of fetal ossification sites per litter revealed significant increases in the 750 and 1500 mg/kg bw per day dosage groups for the average number of thoracic ribs per fetus, a common reversible variation in this rat strain, however there was no clear dose–response relationship. In 26 studies performed at the test facility from 1985 to1987 ossification site averages based on 573 control group litters (total of 4852 fetuses) were 13.02 pairs of ribs per fetus (range = 13.00 to 13.09), 13.02 thoracic vertebrae (range = 13.00 to 13.13) and 5.97 lumbar vertebrae (range = 5.85 to 6.00). Ossification site averages for the hyoid, cervical, sacral and caudal vertebrae, sternum (manubrium, sternal centres and xiphoid), forepaws (carpals, metacarpals and phalanges) and hind paws (tarsals and phalanges) did not demonstrate dosage-dependent, biologically important, statistically significant differences among the four dosage groups.

Table 18. Selected findings in the rat developmental toxicity study

	Dose (mg/kg bw per day)			
	0	**250**	**750**	**1500**
Maternal food consumption (g per day)				
Days 6–9	24.5	24.7	23.0	23.2
Days 16–20	30.7	31.4	31.4	32.5
Maternal body weight (g)				
Day 6	279	280	279	279
Day 9	295	295	288.9	289.2
Day16	355	354	349	353
Day20	424	426	418	426
Maternal body weight gain (g)				
Days 6–7	+3.6	+4.5	+1.0*	+0.9*
Day 6–9	+16.0	+15.3	+10.0*	+10.2*
Day 6–20	+144.5	+146.3	+139.6	+147.5

	Dose (mg/kg bw per day)			
	0	**250**	**750**	**1500**
Caesarean section data				
Live fetuses	349	301	366	356
Litter data				
Fetal weight (g)	3.8	3.7	3.6	3.8
Ossification sites per litter :				
– ribs	13.03	13.13	13.17**	13.15**
– thoracic vertebrae	13.05	13.16	13.23*	13.21**
– lumbar vertebrae	5.94	5.84	5.77**	5.79**

Significantly different from the vehicle control group: * $p \leq 0.05$; ** $p \leq 0.01$; Source: Hoberman 1989a

The maternal NOAEL in this study was 250 mg/kg bw per day based on decreased body weight gain and food consumption at 750 mg/kg bw per day. The embryo/fetal NOAEL was 1500 mg/kg bw per day, the highest dose tested (Hoberman, 1989a).

Rabbit

In a range-finding study, inseminated New Zealand White rabbits were assigned to one control and five treatment group of five animals each. Doses of quintozene (purity 96%; batch not stated; less than 0.5% HCB) at 25, 100, 200, 400 or 800 mg/kg bw per day were administered once daily by oral gavage on GDs7–19. Maternal toxicity (mortality, weight loss) in excess of that acceptable for a developmental toxicity study was present in the 400 and 800 mg/kg bw per day dosage groups. No dose-related developmental toxicity was evident in any of the treatment groups (Keller, 1988c).

In the main developmental toxicity study, inseminated New Zealand White rabbits were assigned to two control and five treatment group of 12–16 animals each. Doses of quintozene (purity 96%; Batch MDD 080687; less than 0.03% HCB) at 6.25, 12.5, 125(A), 125(B) or 250 mg/kg bw per day were given once daily by gavage from GDs 7–19. Two control groups received the vehicle only, 0.2% high viscosity CMC, according to the same regimen. Unanticipated maternal toxicity (which resulted in a reduced sample size) was observed at doses of 125 and 250 mg/kg per day. Consequently, additional treatment groups and a concurrent control group were subsequently initiated in order to gain more data for the 125 mg/kg per day dosage level and evaluate a additional dosage level of 6.25 mg/kg per day. Caesarean sections were performed on all surviving females on GD 29 and the fetuses were removed for teratological evaluation.

One, two and five rabbits died in the 12.5, 125(A) and 250 mg/kg bw per day groups between GDs 19 and 28. The two rabbits that died in the 125(A) mg/kg bw per day group and one of the rabbits in the 250 mg/kg bw per day group aborted prior to death. At necropsy, mucoid material was present in the intestinal tract of the 125(A) mg/kg bw per day does and the cause of death for these two does was mucoid enteritis. Pneumonia was the cause of death for three of the five does in the 250 mg/kg bw per day group. Although the causes of death were not obviously related to treatment in the 125(A) and 250 mg/kg bw per day groups, the dose-responsive nature of the deaths suggests that test article administration at these levels may have contributed to the susceptibility of the does to infection. The cause of death could not be determined for the doe from the12.5 mg/kg bw per day group or for the other two 250 mg/kg bw per day does that died. A higher number of abortions was observed in the 125(A) and 250 mg/kg bw per day groups; anogenital staining was observed at increased levels in the original dose groups (12.5, 125(A) and 250 mg/kg bw per day).

Body weight losses were noted during the treatment period (GDs 7–19) for the does in the 125(A), 125(B) and 250 mg/kg bw per day groups. Similarly, a marked weight loss, both total and adjusted (minus gravid uterus weight), occurred during the entire gestation period (GDs 0–29) for the 250 mg/kg per day group. There was a decrease in food consumption during the treatment period for the rabbits in the 125(B) and 250 mg/kg bw per day groups in comparison to that of the concurrent control

group. However, no similar decrease relative to the appropriate control value was noted for the does in the 125 mg/kg bw per day group (A) during this interval. Food consumption by the rabbits receiving 250 mg/kg bw per day was reduced compared to the control value (control group A) throughout the entire gestation period.

Table 19. Selected findings in the developmental study in rabbits

	Dose group (mg/kg bw per day)						
Parameter	**0 Control A**	**0 Control B**	**6.25**	**12.5**	**125 (A)**	**125 (B)**	**250**
Antemortem observations							
Animals on study	16	16	16	16	16	12	16
Animals that were gravid	15	15	13	16	13	11	15
Died	0	0	0	1	2	0	5
Aborted	2	0	0	2	5[a]	1	5[b]
Anogenital area stained	3	2	3	8	9	3	10
Maternal body weight (g)							
GD 7	4595	4440	4263	4518	4620	4290	4558
GD 13	4586	4523	4372	4630	4564	4312	4409
GD 20	4539	4591	4408	4609	4428	4248	4034
GD 29	4547	4518	4404	4510	4489	4273	3929
GD 29 adjusted[c]	4231	4160	3917	4152	4101	3844	3693
Body weight gain (g)							
GDs 7–13	−9	83	109	112	−56	22	−149
GDs 7–19	−55	151	145	91	−192	−42	−523
Maternal and fetal observations at caesarean section							
Pre-implantation loss (%)	49.1	52.0	39.2	47.5	40.9	49.7	50.0
Postimplantation loss (%)	7.0	24.8	10.6	12.5	12.7	5.6	30.6
Mean fetal body weight (g)	41.4	44.7	42.6	39.1	40.3	47.2	28.4*

GD: Gestation day; Source: Keller, 1988d

[a] Includes 2 females that died following abortion; [b] Includes female that died following abortion

[c] Body weight minus gravid uterus weight

* Statistically significant from control, $p < 0.05$

A statistically significant reduction in mean fetal body weight (−31%) was noted for the 250 mg/kg bw per day group compared to control A values. There were no statistically significant differences in mean number of viable fetuses, post-implantation loss, number of implantations or corpora lutea. No treatment-related or statistically significant differences in the incidence of fetal malformations or variations were observed. The maternal NOAEL was 12.5 mg/kg bw per day based on decreased body weight gain at 125 mg/kg bw per day. The embryo/fetal NOAEL was 125 mg/kg bw per day based on decreased fetal weight at 250 mg/kg bw per day. It is noted that this study is of low reliability, due to the infections found in the animals (Keller, 1988d).

In a developmental toxicity study, presumed-pregnant rabbits (Hra:(NZW)SPF; 20 animals per group) were administered quintozene (purity 98.5%; Batch No. 05168-6D) at 0 (vehicle only, 1% CMC), 100, 300 or 900 mg/kg bw per day, once daily by gavage from GDs 6–18. Does that aborted or prematurely delivered a litter were necropsied on the day the event occurred. On day GD 29 all other rabbits were sacrificed, Caesarean sectioned, and examined for gross lesions. The intact uterus of each rabbit was removed and subsequently examined for number and placement of implantations, early and late resorptions, live and dead fetuses. Each ovary was examined for the number of corpora lutea. Fetuses were weighed and examined for sex and gross external, soft tissue and skeletal alterations.

No deaths occurred during the study. Two high-dose does aborted on GDs 24 and 25, and one high-dose doe prematurely delivered its litter on GDs 28. In the high-dose group there was an increased number of does with abnormal faeces (absent, dried, soft or liquid). Decreased motor activity was seen in one high-dose animal on the day before it prematurely delivered its litter. No other clinical signs were noted. Maternal body weight gain was suppressed in all dosed groups during treatment (GDs 6–19). Increased body weight gain occurred for the high-dose group during the post dosage period. In the mid- and high-dose groups food consumption was decreased during GDs 6–19 by 10% and 15% respectively. The pregnancy incidences were comparable in all dosage groups in this study. Increased incidences of resorption occurred in all groups given quintozene compared with the controls, the number achieving statistical significance at the top dose. There were no dosage-dependent or statistically significant differences among the groups for corpora lutea, implantations, live litter sizes, or number of does with live fetuses. There was no treatment-related effect on sex ratio or fetal body weight. No fetal gross external, soft tissue or skeletal alterations that occurred in this study were considered to be effects of administration of the test substance to the does,even at dosages as high as 900 mg/kg bw per day. The maternal NOAEL was 300 mg/kg bw per day, based on decreased body weight gain and increased abortions at the LOAEL of 900 mg/kg bw per day. The embryo/fetal NOAEL was 900 mg/kg bw per day, the highest dose tested. Selected findings from this study (Hoberman, 1989b) are shown below in Table 20.

Table 20. Selected findings in the developmental study in rabbits

	Dose (mg/kg bw per day)			
Parameter	**0**	**100**	**300**	**900**
Number of rabbits pregnant	19	17	14	19
Food consumption (g/kg bw per day; days 6–19	42.7	42.7	38.8	36.0
Food consumption (g/kg bw per day; days 19–29	31.5	31.8	27.2	35.0
Maternal body weight (kg)				
– Day 6	3.69	3.73	3.69	3.73
– Day 9	3.73	3.75	3.72	3.75
– Day 19	3.93	3.91	3.86	3.79
– Day 29	4.05	4.00	3.94	4.10 [a]
Maternal body weight gain (kg)				
– Days 6–9	0.04	0.02	0.02	0.02
– Days 9–12	0.06	0.06	0.05	0.03
– Days 6–19	0.24	0.18	0.16	0.06
– Days 6–29	0.36	0.28	0.24	0.37 [a]
Fetal weight (g)	45.4	44.4	43.7	44.8
Live fetuses (*n*)	140	95	111	115
Abortions (*n*)	0	0	0	2
Does with any resorptions [b] (*n*)	2	5	4	9**
Average resorptions	0.2	0.6	0.6	0.7

n: Number counted; Source: Hoberman 1989b

[a] Number assessed = 17 as two animals aborted and one animal delivered prematurely;

[b] Historical control data: mean number of resorptions per doe 0.6, range 0–1.5 (based on 42 studies performed 1985–1987)

** Statistically significant compared with control at $p < 0.01$

2.6 Special studies

(a) Neurotoxicity

No specific studies addressing neurotoxicity were submitted. No indications of neurotoxicity were reported in the routine toxicology studies.

(b) Immunotoxicity

No specific studies addressing immunotoxicity were submitted. No indications of immunotoxicity were reported in the routine toxicology studies. Increased levels of viral or bacterial infections were reported in some studies, with a greater incidence in quintozene-treated groups. However, there was no consistent pattern of infection across studies and no evidence of specific effects on the immune system.

(c) Studies on metabolites

No specific studies on metabolites were submitted.

(d) Studies on endocrine disruption

Table 21. Overview of endocrine disruption studies with quintozene

Type of study	Test object	Dose	Batch and purity	Results	Reference
Androgen receptor binding	Rat prostate cytosol	Concentration range $10^{-3.5}$ to 10^{-10} M	Batch 107030L052; purity 95.6%, 0.037% HCB	Negative	Matthews 2012
Steroidogenesis	H295R cells	Concentration range 0.0001–20 µM	Batch 107030L052; purity 95.6%, 0.037% HCB	Equivocal increase (< 40%) in estradiol No effect on testosterone	Akhurst 2012
Aromatase	Human CYP19+P450 reductase Supersomes™	Concentration range 10^{-4} to 10^{-10} M	Batch 107030L052; purity 95.6%, 0.037% HCB	Negative	Foster 2012
Hershberger	Castrated male Crl:CD(SD) rats (10/group)	100, 300, 1000 mg/kg bw/day for 10 days via gavage	Batch 107030L052; purity 95.6%, 0.037% HCB	Negative	Donington 2012

In a pubertal development and thyroid function study in juvenile males and female rats, juvenile Sprague Dawley rats (15/sex per group) were dosed once daily via gavage with either 0, 500 or 1000 mg/kg bw per day of quintozene technical (purity 95.6%; Lot no. 107030L052; 0.037% HCB) in 1% sodium CMC. The males were dosed on PNDs 23–53 and females were dosed on PNDs 22–54. On the last day of treatment all animals were euthanized and necropsied two hours post-dose. Parameters evaluated were: viability, clinical observations, body weight, food consumption, age and body weight at preputial separation/vaginal opening, vaginal cytology, hormone and clinical chemistry analysis (at termination), organ weights, macroscopic observations and microscopic pathology.

In the males no mortality was observed and there were no effects on clinical signs, body weight or food consumption. Increased liver weight compared with controls was observed at 500 mg/kg bw per day (8%), and 1000 mg/kg bw per day (10%), and minimally increased inflammatory foci with or without single cell necrosis was slightly higher than for controls at 1000 mg/kg bw per day. A statistically significant decrease in ALT activity was observed, of 38% at 500 mg/kg bw per day and 52% at 1000 mg/kg bw per day. A decrease in T4 was observed at 500 mg/kg bw per day and above. Decreases were such that many samples were below the lower limit of detection and any degree of difference could not be accurately determined. However, results were not associated with any treatment-related effects on TSH, thyroid organ weight or histopathology findings. There were no effects on the weights

of androgen-sensitive organs, testosterone levels or preputial separation data, nor treatment-related microscopic findings in reproductive organs.

In females, no mortality was observed and there were no effects on clinical signs, body weight or food consumption. Statistically significant increases in liver and kidney weights were not considered treatment-related as they were small and without microscopic correlates. The level of T4 was depressed by 70% at 500 mg/kg bw per day and by 72% at 1000 mg/kg bw per day compared with controls, and the level of TSH increased by 53% at 500 mg/kg bw per day and by 59% at 1000 mg/kg bw per day. No correlates with thyroid organ weight or microscopic observations were however found. According to the study report, the observed changes in thyroid hormones and increased liver weight may be associated with enhanced excretion of T4, mediated by hepatic enzyme induction. There were no treatment-related effects on the weights of estrogen-sensitive organs, estrous cyclicity or vaginal opening data, and no treatment-related microscopic findings in reproductive organs.

Based on these data, administration of quintozene to peripubertal rats at dose levels of 500 or 1000 mg/kg bw per day resulted in effects indicative of systemic/liver target organ toxicity. There were no findings suggesting potential interaction with the estrogen, androgen or steroidogenesis pathways or the hypothalamic–pituitary–gonadal (HPG) axis. The data suggest a possible indirect thyroid pathway interaction, probably mediated by hepatic enzyme induction, but no adverse effects on the thyroid, based on the absence of exposure-related thyroid weight changes or histopathological findings (Baxter, 2012b).

In a weight of evidence evaluation from the US EPA Endocrine disruption screening program (EDSP) the results for quintozene in the Tier 1 battery of tests are discussed; the battery consists of 11 screening assays to evaluate endocrine MOA. Analysis of the whole database showed that quintozene is not estrogenic, anti-estrogenic, androgenic or anti-androgenic, does not interact with E or A hormonal pathways, does not disrupt the HPG axis or disrupt steroidogenesis in mammals or fish. The male and female pubertal assays show that quintozene disrupts thyroid hormones in rats. Previous studies indicate that this is a consequence of liver enzyme induction and increased metabolism of T4, although chronic dosing may lead to thyroid follicular hyperplasia in rats. There were no thyroid-related effects in amphibians. Further Tier 2 testing in mammals was not considered justified as Tier 2 tests on quintozene in rats already exist. Reproduction studies demonstrate that quintozene has no effects on reproduction, fertility or development. Existing studies have already described the effects on the rat HPT axis. New Tier 2 tests on quintozene in mammals are not justified because the weight of evidence available from the Tier 1 tests and other scientifically available information indicates no potential hazard via interaction with the E, A or steroidogenesis disruption MOAs. In addition, many of the additional end-points that would be included in a new Tier 2 test have already been studied in the pubertal assays (serum testosterone, estrous cyclicity). The conclusion that there were no endocrine effects in fish or amphibians also precludes conducting Tier 2 tests on these groups. (Odum & Croudace, 2013).

In a publication by Ashby et al. (2005), the (anti-)estrogen activity of quintozene was investigated following a finding that quintozene inhibits the formation of foci in E2-stimulated MCF-7 cells in vitro. The study showed that quintozene did not bind significantly to hERα or hERβ or rat uterine ER and was devoid of anti-estrogenic activity in the yeast hER assay; quintozene did not delay the onset of puberty in the female pubertal rat assay. In the uterotrophic assay, quintozene enhanced the activity of estradiol, but did not affect the activity of diethylstilbestrol which suggests the effect on estradiol might be related to its metabolism. However, quintozene was found not to be hepatotoxic and did not alter cytochrome P450 or estradiol sulfotransferase.

(e) Mechanistic studies

The biliary excretion of T4 and thyroid uptake of iodine in quintozene-treated rats was studied. For this, groups of five male CD rats were dosed orally via the diet for ten days with 0, 20 or 6000 ppm of quintozene (99% purity; Lot no. 23503-158) (equivalent to 0, 2, 600 mg/kg bw per day). The positive control groups were treated with Aroclor 1254 for three days prior to euthanasia. The study design is shown in Table 22:

Table 22. Study design for the biliary excretion of thyroxine and thyroid uptake of iodine in rats

Group	Diet dosage level (ppm)	Days 7–10	Day 11
1	0 (control)	NA	Bile duct cannulation
2	0 (control)	IP corn oil	Bile duct cannulation
3	0 (positive control)	IP Aroclor 1254	Bile duct cannulation
4	20	NA	Bile duct cannulation
5	6000	NA	Bile duct cannulation
6	0 (control)	NA	Euthanasia/thyroid excision
7	0 (control)	IP corn oil	Euthanasia/thyroid excision
8	0 (positive control)	IP Aroclor 1254	Euthanasia/thyroid excision
9	20	NA	Euthanasia/thyroid excision
10	6000	NA	Euthanasia/thyroid excision

Source: Kossor, 1996

A radiolabelled tracer, [^{125}I]thyroxine was injected via a femoral cannula just prior to bile sample collections. Bile samples were collected via the femoral cannula for three 15-minute periods followed by three 30-minute periods. A blood sample was collected from each rat in Groups 1–5 just prior to the [^{125}I]thyroxine injection and at the midpoint of each bile collection period. Following sample collections the animals in Groups 1–5 were anaesthetized and exsanguinated and the liver from each animal was excised and weighed. Animals in Groups 6–10 were fed the diet for 10 days and then given a single intravenous does of ^{125}I. Approximately 8.5 hours after the intravenous dose, the animals were euthanized and their thyroids collected. Blood and bile samples were not collected from animals in Groups 6–10, but the thyroid from each of these animals was excised, weighed and analysed for ^{125}I content after euthanasia.

Each rat was observed twice daily for mortality and morbidity. Individual body weights were recorded prior to dosing with radiolabel material. Individual food consumption measurements were recorded daily. Analysis for thyroxine concentration was performed on bile samples and on serum processed from the blood samples. Bile flow rates were determined and biliary T4 excretion rates were calculated. Tissue iodine uptake was determined in aliquots of digested thyroid tissue.

No mortality, clinical signs or effects on body weight or food consumption were observed. The mean serum T4 concentration in control rats was 3.39 ± 0.61 µg/dL. Rats given 20 ppm of quintozene or control diet plus intraperitoneal injections of corn oil had serum T4 concentrations of 3.36 ± 0.57 or 3.22 ± 1.03 µg/dL, respectively, which were comparable in magnitude to those of control rats. The mean serum T4 concentration in rats treated with 6000 ppm of quintozene was 1.80 ± 0.69 µg/dL which was significantly less than that of control rats ($p \leq 0.05$). The mean serum thyroxine concentration in rats treated with Aroclor 1254 was 1.12 ± 0.63 µg/dL which was significantly less than that of rats treated intraperitoneally with vehicle ($p \leq 0.05$).

Biliary T4 excretion (percent of administered [^{125}I]) was linear with respect to time for up to 135 minutes for all treatment groups. After 135 minutes, 6.297 ± 0.367% of the T4 dose was excreted in bile by the control group. Comparable extents of T4 excretion were observed in rats treated with either 20 ppm of quintozene (5.916 ± 0.899%) or intraperitoneal vehicle (6.022 ± 1.284%). The biliary T4 excretion rates in rats treated with 6000 ppm of quintozene (8.046 ± 0.914%) were significantly greater than those of control rats over the course of bile collection ($p \leq 0.05$). Biliary T4 excretion rates in rats treated with Aroclor 1254 (14.67 ± 2.234%) were significantly greater than those of rats given vehicle intraperitoneally ($p \leq 0.05$).

After an initial 30 minutes distribution phase following [^{125}I]thyroxine administration, consistent rates of biliary T4 clearance were observed throughout the period of bile collection for all treatment groups evaluated. In control rats the T4 clearance rate was 113.0 ± 14.7 µL/hour per g liver (range, 91.8 to 135.9). Biliary T4 clearance in rats treated with 20 ppm of quintozene was 92.5 ± 16.0 µL/hour per g liver (range, 66.8 to 119.3) which was comparable in magnitude to that of the controls. However, biliary T4 clearance in rats treated with 6000 ppm of quintozene

was 184.6 ± 29.1 µL/hour per g liver (range, 128.1 to 235.7) which was significantly greater ($p \leq 0.05$) than that of the controls from 30 to 135 minutes following [^{125}I]thyroxine administration. Biliary T4 clearance in rats treated with Aroclor 1254 was 451.9 ± 179.2 µL/hour per g liver (range, 209.5 to 941.5) which was significantly greater ($p \leq 0.05$) than that of rats treated intraperitoneally with corn oil (98.2 ± 28.1 µL/hour per g liver (range, 59.6 to 162.0).

No significant differences in thyroid weight were observed. At 8.5 hours after administration of ^{125}I to control rats, 10.61% ± 2.58% of the dose was quantified in the thyroid gland. Alternatively, thyroid uptake of ^{125}I in rats treated with 6000 ppm of quintozene (4.95% ± 1.55 %) was significantly less ($p \leq 0.05$) than that of control rats. Thyroid ^{125}I uptake in rats treated with 20 ppm of quintozene (10.84% ± 2.22%) was comparable in magnitude to that of rats treated intraperitoneally with vehicle alone (10.71% ± 1.31%).

In conclusion, quintozene treatment at 6000 ppm was shown to produce both an increase in hepatobiliary clearance of T4 and a decreased thyroidal iodine uptake (Kossor, 1996).

In another study of biliary excretion of T4 and thyroid uptake of iodine, male Sprague Dawley rats (18 per group) received quintozene (purity 99.8; Lot no. 23010 112) via the diet for seven days at dose levels of 0, 20 or 6000 ppm (equal to 0, 1.65 and 416.4 mg/kg bw per day). Nine positive control animals received three daily intraperitoneal injections of benzo[*a*]pyrene as a pretreatment for determination of biliary excretion of T4. Nine positive control animals received three daily intraperitoneal injections of Aroclor 1254 as a pretreatment for determination of iodine uptake in the thyroid. For the determination of biliary excretion of T4, animals were bile duct-cannulated and received a single intravenous dose of 1 µg/kg [^{125}I]thyroxine in the caudal tail vein. Bile samples were collected at 15 minutes and thereafter at intervals of 30 minutes for a total period of four hours. Total T4-derived radioactivity in blood and bile samples was determined. For the determination of thyroid uptake of iodine, the animals received a single intraperitoneal dose of 10 µg/kg ^{125}I. After three hours the animals were sacrificed, their thyroid glands excised and radioactivity in the thyroid gland determined.

Regarding biliary excretion of [^{125}I]thyoxine-derived radioactivity, after four hours the mean cumulative percentage of dose excreted was significantly higher in the positive control group than the negative control values ($p < 0.05$). There appeared to be a slight increase in biliary excretion of thyroid equivalents in rats treated with 6000 ppm quintozene, however this increase was not statistically significant. A similar pattern was observed in mean bile : blood ratios, as well as the mean biliary clearance of thyroxine-derived radioactivity. No apparent difference was noted in the rate of bile excretion, as evidenced by weight of bile excreted per time interval. Regarding thyroid uptake of ^{125}I, the 6000 ppm group exhibited a significantly lower percentage of dose in thyroid ($p < 0.01$). There were no apparent differences in thyroid weights. The data demonstrate that animals exposed to seven days of quintozene in the diet at a concentration of 6000 ppm, showed a significantly lower percentage of administered dose of iodine in the thyroid than any of the remaining treatment groups. The positive control agent, Aroclor 1254, did not produce a significant effect on thyroid uptake of labelled iodine compared with negative control animals. The reduction in thyroid uptake in rats treated with 6000 ppm quintozene may partially explain the previous finding that rats treated for seven days with 6000 ppm quintozene displayed reduced levels of serum T4 (Shae 1996).

The weight of evidence suggests the thyroid tumours following chronic administration of quintozene had developed by a non-genotoxic mechanism, possibly associated with chronic hormonal imbalances. A definitive MOA case for the human non-relevance was therefore not demonstrated.

3. Observations in humans

Human sensitization study

Fifty human subjects received one 6.4 mm moistened cotton square dipped in a 75% wettable powder formulation of quintozene (purity unspecified) on the palmar surface of the forearm. The squares were then covered with foil, which was taped into place. Exposure was for 48 hours. No irritation was observed when the dressings were removed. When the test was repeated two weeks later, four individuals showed positive reactions, comprising erythema, itching, and (in three) oedema and small vesicle formation. Of the individuals who showed no reaction at the time the patch was removed, nine developed a delayed

reaction eight hours to several days after testing (Finnegan et al., 1958). These results indicate the skin sensitization seen in Guinea pigs is relevant to humans.

Medical surveillance on manufacturing plant personnel

Workers who were still employed and had been exposed during the production of quintozene for two and a half years (one person), seven years (one person), eight and a half years (one person), or 11 years (10 people) had undergone twice-yearly health checks, including monitoring of height, weight, vision, blood pressure, X-ray (once per year only), haematological parameters (erythrocyte count, Hb, Ht, total leukocyte count), an evaluation of subjective and objective symptoms, life style, family history, and individual past history, throughout the employment period. After 1989, hearing tests, blood chemistry (serum aspartate transaminase and ALT, GGTP, ALP, partial thromboplastin time, total cholesterol, and blood glucose), urinalysis (BUN, uric acid, glucose, protein, and occult blood) and electrocardiography were added. No compound-related effects were observed. A further group of nine individuals, with exposure for three months (one person), one year (one person), two years (one person), three years (one person), four years (one person), seven years (one person) and 10 years (three people), who were engaged in the manufacture of quintozene dust formulations were subjected to similar examinations and showed no compound-related abnormalities. No estimate of exposure or information on protective measures used (for example clothing, gloves or masks) was provided (Yamazaki, 1994).

4. Microbial aspects

There was no information available in the public domain, and no experimental data were submitted that addressed the possible impact of quintozene residues on the human intestinal microbiome.

Comments

Biochemical aspects

Following the dosing of rats with radiolabelled quintozene at 5 mg/kg bw radioactivity levels in blood reached a maximum 12 hours post treatment, with a half-life of 21.8 hours. Radiolabel was mainly excreted via the faeces. An apparent sex difference was seen in urinary and faecal excretion: the total ^{14}C activity in urine ranged from 7.8% to 12.3% in males and 23.9% to 38.3% in females, while total ^{14}C activity in faeces ranged from 56.6% to 90.0% in males, and 37.9% to 76.0% in females. Biotransformation studies showed two general metabolic pathways. One involved the reduction of the nitro group to an amino group and subsequent formation of secondary metabolites, the second consisted of the replacement of the nitro group with a sulfur-containing group. No individual metabolite occurred in urine at levels above 10% of the administered dose (AD).

Toxicological data

The acute oral median lethal dose (LD_{50}) of quintozene in the rat was above 5000 mg/kg bw (Warshawsky, 1994a; Kuhn, 1989a). The acute dermal LD_{50} in rabbits was above 5000 mg/kg bw (Warshawsky, 1994b). The acute inhalation median lethal concentration (LC_{50}) in rat was above 6.49 mg/L (Kuhn, 1989c). In rabbits quintozene was slightly irritating to the skin (Kuhn, 1989d) and minimally irritating to the eye (Warshawsky, 1994d; Kuhn, 1989e), and it was a skin sensitizer in Guinea pigs (Kuhn, 2000).

In repeat-dose oral toxicity studies in rats and dogs the main effects were lower body weight, increased weight and histopathological changes in the liver, and histopathological changes in the thyroid.

In a 90-day rat study, quintozene was administered orally via gavage for five days a week at doses of 0, 5, 10, 100 or 1000 mg/kg bw per day. The NOAEL was 71 mg/kg bw per day (corrected for five day per week dosing) based on histopathological findings in the thyroid (follicular cell hypertrophy/hyperplasia) at 710 mg/kg bw per day (corrected value) (Keefe, 1992).

In a one-year dog study, quintozene was administered at dietary concentrations of 0, 15, 150 or 1500 ppm (equal to 0, 0.40, 4.31 and 40.05 mg/kg bw per day for males, 0, 0.44, 4.22 and 41.48 mg/kg bw

per day for females). The NOAEL was 150 ppm (equal to 4.22 mg/kg bw per day) based on increased liver weight, increased alkaline phosphatase (ALP) activity and cholesterol, and liver histopathology (hepatocellular/hypertrophy) at 1500 ppm (equal to 40.05 mg/kg bw per day) (Goldenthal, 1990).

In a 103-week toxicity and carcinogenicity study in mice, quintozene was administered at dietary concentrations of 0, 2500 or 5000 ppm (equal to 0, 400 and 1000 mg/kg bw per day for males, 0, 600 and 1400 mg/kg bw per day for females). No neoplastic lesions related to treatment were observed, however survival in females was decreased to as low as 28% due to infection, which reduced the overall sensitivity of the study. Due to these infections, no chronic toxicity NOAEL could be identified. However, despite the infections, no evidence of carcinogenicity was reported (NTP, 1987).

In a two-year toxicity and carcinogenicity study in rats, quintozene was administered at dietary concentrations of 0, 100, 400 or 1200 ppm (equivalent to 0, 5, 20 and 60 mg/kg bw per day). The chronic toxicity NOAEL was 100 ppm (equivalent to 5 mg/kg bw per day) based on liver histopathology (enlargement of hepatocytes, hyaline intracytoplasmic bodies and centrilobular single cell necrosis) at 400 ppm (equal to 20 mg/kg bw per day). The NOAEL for carcinogenicity was 1200 ppm (equivalent to 60 mg/kg bw per day), the highest dose tested (Sinkeldam et al., 1974).

In a two-year toxicity and carcinogenicity study in rats, quintozene was administered by oral gavage at dose levels of 0, 5, 50, 500 or 1000 mg/kg bw per day. The NOAEL for chronic toxicity was 50 mg/kg bw per day based on decreased body weight and follicular cell hypertrophy/hyperplasia in the thyroid gland. The NOAEL for carcinogenicity was 1000 mg/kg bw per day, the highest dose tested (Plutnick, 1993).

In a two-year toxicity and carcinogenicity study in rats, quintozene was administered at dietary concentrations of 0, 20, 3000 or 6000 ppm (equal to 0, 0.91, 141 and 303 mg/kg bw per day for males, 0, 1.14, 179 and 370 mg/kg bw per day for females). The NOAEL for chronic toxicity was 20 ppm (equal to 0.91 mg/kg bw per day) based on histopathological findings of follicular cell hypertrophy and hyperplasia in the thyroid. The NOAEL for carcinogenicity was 20 ppm (equal to 0.91 mg/kg bw per day) based on thyroid follicular cell adenomas and carcinomas in males at 3000 ppm (equal to 141 mg/kg bw per day) (Goldenthal, 1991).

In a mechanistic study in male rats focusing on the thyroid, quintozene was administered at dietary concentrations of 0, 20 or 6000 ppm (equal to 0, 1.1 and 333 mg/kg bw per day) for up to 90 days, with an additional recovery group continuing for 90 days without treatment. At 6000 ppm serum triiodothyronine (T3) and thyroxine (T4) were decreased and thyroid-stimulating hormone (TSH) was increased: during the recovery period these values returned to normal. Hepatocellular hypertrophy was seen at 20 and 6000 ppm. A NOAEL was not identified as thyroid hypertrophy was seen at 20 ppm (equal to less than 1.1 mg/kg bw per day) (Goldenthal, 1993).

A mechanistic study in rats, focusing on biliary excretion of T4 and thyroid uptake of iodine, showed that quintozene produced both an increase in hepatobiliary clearance of T4 and a decrease in thyroidal iodine uptake (Kossor, 1996). The weight of evidence suggests that the thyroid tumours following chronic administration of quintozene developed by a nongenotoxic mechanism, possibly associated with chronic hormonal imbalances. A definitive case for human non-relevance of the MOA was not demonstrated.

The Meeting concluded that quintozene is carcinogenic in rats, but not in mice.

Quintozene was tested for genotoxicity in an adequate range of in vitro and in vivo assays. It gave a positive response in the in vitro chromosome aberration assay, but was negative in the in vivo micronucleus assay (Guidi & Krsmanovic, 2000; NTP, 1987).

The Meeting concluded that quintozene is unlikely to be genotoxic in vivo.

In view of the lack of genotoxicity in vivo, the absence of carcinogenicity in mice and the fact that the thyroid tumours in rats were increased at the two highest doses tested in only one of the three two-year studies, and that the mechanistic study indicated the MOA to be thyroid hormone disruption, the Meeting concluded that quintozene is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation reproductive toxicity study in rats, quintozene was administered at dietary concentrations of 0, 20, 3000 or 6000 ppm (equal to 0, 1.1, 168 and 350 mg/kg bw per day for males,

0, 1.4, 221 and 459 mg/kg bw per day for females). The NOAEL for parental toxicity was 3000 ppm (equal to 168 mg/kg bw per day) based on decreased body weight and food consumption. The NOAEL for reproductive toxicity was 6000 ppm (equal to 350 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 20 ppm (equal to 1.1 mg/kg bw per day) based on an increased incidence of undersized pups (Schardein, 1991).

In a two-generation reproductive toxicity study in rats, quintozene was administered by oral gavage at dose levels of 0, 10, 100 or 1000 mg/kg bw per day. The parental NOAEL was 10 mg/kg bw per day based on follicular cell hypertrophy and hyperplasia in the thyroid of male rats. The NOAELs for offspring and reproductive toxicity were both 1000 mg/kg bw per day, the highest dose tested (Philips, 1994).

In a developmental toxicity study in rats, quintozene was administered by oral gavage at 0, 30, 600 or 1200 mg/kg bw per day from gestation day (GD) 6–15. The maternal and embryo/fetal NOAELs were both 1200 mg/kg bw per day, the highest dose tested (Keller, 1998b).

In a developmental toxicity study in rats, quintozene was administered by oral gavage at doses of 0, 250, 750 or 1500 mg/kg bw per day from GD 6–15. The maternal NOAEL was 250 mg/kg bw per day based on decreased body weight gain and feed consumption at 750 mg/kg bw per day. The embryo/fetal NOAEL was 1500 mg/kg bw per day, the highest dose tested (Hoberman, 1989a).

In a developmental toxicity study in rabbits, quintozene was administered by oral gavage at doses of 0, 100, 300 and 900 mg/kg bw per day from GD 6–18. The maternal NOAEL was 300 mg/kg bw per day based on decreased body weight gain and increased abortions at 900 mg/kg bw per day. The embryo/fetal NOAEL was 900 mg/kg bw per day, the highest dose tested (Hoberman, 1989b).

The Meeting concluded that quintozene is not teratogenic.

No specific study on neurotoxicity was submitted, but no evidence of neurotoxicity was reported in routine toxicological studies with quintozene.

The Meeting concluded that quintozene is unlikely to be neurotoxic.

No specific data were provided regarding immunotoxicity. In routine toxicity studies there was evidence of viral and bacterial infections being slightly more prevalent in animals treated with quintozene. However there was no evidence of direct effects on the immune system.

The Meeting concluded that quintozene is unlikely to be immunotoxic.

Studies on estrogen, androgen and steroidogenesis activity were provided and these showed negative results (Akhurst, 2012; Donington, 2012; Foster, 2012; Matthews, 2012).

Toxicological data on metabolites and/or degradates

No data was available. If needed for assessment of residue metabolites, the threshold of toxicological concern (TTC) value for potentially genotoxic substances (0.0025 µg/kg bw per day) should be applied.

Microbiological data

There was no information available in the public domain, and no experimental data were provided that addressed the possible impact of quintozene residues on the human intestinal microbiome.

Human data

Evidence of skin sensitization was reported in a human patch test. In reports on manufacturing plant personnel, no adverse health effects were noted. No clinical cases or poisoning incidents had been recorded.

The Meeting concluded that the existing database on quintozene was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

Toxicological evaluation

The Meeting re-affirmed the ADI of 0–0.01 mg/kg bw established by the 1995 Meeting. The Meeting based the ADI on the NOAEL of 1.1 mg/kg bw per day seen in the two-generation study in rats, and 0.91 mg/kg bw per day from the two-year rat study (noting the large dose spacing to the LOAEL of 141 mg/kg bw per day). The Meeting noted that in the case of the LOAEL of 1.1 mg/kg bw per day in the mechanistic 90-day rat study, the LOAEL was based on minimal changes which were found not to progress in the two-year rat study. A safety factor of 100 was used. This gives a margin of 14 000 to the lowest dose causing tumours in rats.

The Meeting concluded that it was not necessary to establish an ARfD for quintozene in view of its low acute oral toxicity and the absence of developmental toxicity and any other toxicological effects that would be likely to be elicited by a single dose.

Levels relevant to risk assessment of quintozene

Species	Study	Effect	NOAEL	LOAEL
Rat	Two-year studies of toxicity and carcinogenicity [a]	Toxicity	20 ppm, equal to 0.91 mg/kg bw/day	3000 ppm, equal to 141 mg/kg bw/day
		Carcinogenicity	20 ppm, equal to 0.91 mg/kg bw/day	3000 ppm, equal to 141 mg/kg bw/day
	Two-generation study of reproductive toxicity [a]	Reproductive toxicity	6000 ppm, equal to 350 mg/kg bw/day [c]	-
		Parental toxicity	3000 ppm, equal to 168 mg/kg bw/day	6000 ppm, equal to 350 mg/kg bw/day
		Offspring toxicity	20 ppm, equal to 1.1 mg/kg bw/day	3000 ppm, equal to 168 mg/kg bw/day
	Two-generation study of reproductive toxicity [b]	Reproductive toxicity	10 mg/kg bw/day	100 mg/kg bw/day
		Parental toxicity	1000 mg/kg bw/day [c]	-
		Offspring toxicity	1000 mg/kg bw/day [c]	-
	Developmental toxicity study [b]	Maternal toxicity	250 mg/kg bw/day	750 mg/kg bw/day
		Embryo/fetal toxicity	1500 mg/kg bw/day [c]	-
Rabbit	Developmental toxicity study [b]	Maternal toxicity	300 mg/kg bw/day	900 mg/kg bw/day
		Embryo/fetal toxicity	900 mg/kg bw/day [c]	-
Dog	One-year study of toxicity [a]	Toxicity	150 ppm, equal to 4.22 mg/kg bw/day	1500 ppm, equal to 40.05 mg/kg bw/day

[a] Dietary administration

[b] Gavage administration

[c] Highest dose tested

Acceptable daily intake (ADI) for quintozene

0–0.01 mg/kg bw

Acute reference dose (ARfD) for quintozene

Not necessary

Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure.

Critical end-points for setting guidance values for exposure to quintozene

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	T_{max}: 12 hours; < 40%
Dermal absorption	No data available
Distribution	Limited data available
Potential for accumulation	No indication of accumulation
Rate and extent of excretion	Predominantly in faeces (38–91%), less in urine (7.8–38%); sex difference in faecal and urinary excretion
Metabolism in animals	Reduction of nitro group; replacement of nitro group with thiol group.
Toxicologically significant compounds in animals and plants	Quintozene
Acute toxicity	
Rat, LD_{50}, oral	> 5000 mg/kg bw
Rat, LD_{50}, dermal	> 5000 mg/kg bw
Rat, LC_{50}, inhalation	> 6.49 mg/L
Rabbit, dermal irritation	Slightly irritating
Rabbit, ocular irritation	Minimally irritating
Guinea-pig, dermal sensitization	Sensitizing (Buehler; maximization test)
Short-term studies of toxicity	
Target/critical effect	Body weight, liver, thyroid histopathology
Lowest relevant oral NOAEL	< 1.1 mg/kg bw per day, lowest dose tested (rat)
Lowest relevant dermal NOAEL	No adequate data
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Liver and thyroid (rat)
Lowest relevant NOAEL	0.91 mg/kg bw per day (rat)
Carcinogenicity	Carcinogenic in rats [a]
Genotoxicity	Unlikely to be genotoxic in vivo
Reproductive toxicity	
Target/critical effect	Parental: thyroid histopathology Offspring: pups small in size
Lowest relevant parental NOAEL	10 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	1.1 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	350 mg/kg bw per day, the highest dose tested (rat)
Developmental toxicity	
Target/critical effect	Body weight and food consumption (rat)
Lowest relevant maternal NOAEL	250 mg/kg bw per day (rat)
Lowest relevant embryo/fetal NOAEL	900 mg/kg bw per day, highest dose tested (rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	No data; no evidence of neurotoxicity from routine studies
Subchronic neurotoxicity NOAEL	No data; no evidence of neurotoxicity from routine studies
Developmental neurotoxicity NOAEL	No data

Other toxicological studies	
Immunotoxicity	No data; no evidence of immunotoxicity from routine studies
Studies on toxicologically relevant metabolites	No data
Microbiological data	No data
Human data	No clinical cases or poisoning incidents had been recorded Evidence of skin sensitization

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

Summary

	Value	Study	Safety factor
ADI	0–0.01 mg/kg bw [a]	Two-year study of toxicity and carcinogenicity (rat); two-generation reproductive toxicity (rat)	100
ARfD	Not necessary		

[a] Applies to quintozene

Appendix 1:

Studies with high levels of hexachlorobenzene (HCB)

In a non-GLP two-year toxicity study, beagle dogs (four/sex per dose) were exposed to quintozene (purity 98.2%; HCB 1.4%) via the diet (moistened) at dose levels of 0, 5, 30, 180 or 1080 ppm (equivalent to 0.375, 2.25, 13.5 and 81 mg/kg bw per day). Food consumption was determined daily and body weights weekly. Haematological studies and urinalysis were carried out on all dogs at the start of the test, then at 3, 6, 12, 18 and 24 months. Clinical chemistry was assessed on all dogs at start of test and prior to sacrifice, and on control and 1080 ppm dogs at 3, 6, 12 and 18 months. One dog of each sex in each group was sacrificed for histopathological study at 12 months. Organ weights were obtained at sacrifice for heart, spleen, kidneys, liver and testes. Tissues submitted for histopathological study were: brain, lung, hurt, aorta, liver, spleen, kidney, stomach, ileum, jejunum, large intestine, urinary bladder, bone marrow (sternal and long bone), pituitary, thyroid, pancreas, adrenal, gonad, lymph node, and eye.

All dogs survived to scheduled sacrifice. No treatment-related findings were observed on body weight or food consumption. Regarding haematology, no apparent differences were seen during the first year of the study. At the 18-month time point, a statistically significant decrease in Ht was seen in males at 30 and 180 ppm, but not at the high dose of 1080 ppm. At 24 months there were no significant differences in Ht, although two males (one in the 30 ppm group and one in the 180 ppm group) had lower values accompanied by terminal weight losses. These two male animals also had lower Hb values at 24 months. No treatment-related findings were observed in urinalysis. Clinical chemistry results showed significantly higher serum glutamate oxaloacetate transaminase (SGOT) values in females at 1080 ppm at three months. At 12 months significantly elevated serum ALP levels were seen at 1080 ppm in combined data for males and females. At 18 and 24 months higher trends for serum ALP were present in 1080 ppm dogs but these did not achieve statistical significance. Organ weight results showed an increased liver weight at 1080 ppm. Histopathological findings in the dogs sacrificed at one year showed no significant treatment-related effects. However, in dogs sacrificed at two years, cholestatic hepatosis with secondary bile nephrosis was present in dogs receiving 180 and 1080 ppm. This was judged of minimal degree in 180 ppm dogs and of moderate degree in 1080 ppm dogs. While these observations were relatable to the test material, they were considered to be a reversible phenomenon by the pathologist. The NOAEL in this study was 30 ppm (equivalent to 2.25 mg/kg bw per day) based on histopathological findings (Borzelleca & Larson, 1968).

Table A1. Results from two-year study in the dog using quintozene containing 1.4% HCB

		Sex and dietary dose (ppm)									
		Males					Females				
Parameter	**Time point**	**0**	**5**	**30**	**180**	**1080**	**0**	**5**	**30**	**180**	**1080**
						Haematology					
Ht (%)	78 weeks	41	37	36	37	42	40	41	40	42	40
	104 weeks	44	40	38	35	39	45	41	41	40	39
Hb (g/100mL)	104 weeks	13.5	12.9	11.8	11.1	13.8	13.8	13.1	12.9	12.5	11.8
						Clinical chemistry					
SGOT (K units)	3 months	25	–	–	–	32	33	–	–	–	46
	12 months	21	21	31	29	26	19	22	29	17	25
	24 months	34	34	33	40	38	36	39	35	29	25
SAP (B–L units)	12 months	1.3	2.3	1.9	1.0	2.5	1.3	1.4	2.1	1.2	1.8
	24 months	1.0	1.3	1.2	1.4	4.9	1.2	1.3	1.0	1.5	2.2

		Sex and dietary dose (ppm)									
		Males					Females				
Parameter	Time point	0	5	30	180	1080	0	5	30	180	1080
Organ weights											
Absolute liver weight (g)	12 months	290.0	289.3	276.8	361.5	346.8	276.8	306.5	245.2	302.2	353.8
	24 months	342.4 ±11.3	310.9 ±34.2	262.3* ±19.7	268.0* ±16.9	431.8 ±68.5	307.7 ±16.1	266.9 ±75.5	257.5 ±45.1	281.0 ±29.9	385.8 ±24.5
Relative liver weight (g/kg)	12 months	30.45	34.43	33.22	36.15	36.41	30.73	32.18	29.42	34.45	42.10*
	24 months	32.11 ±3.3	33.37 ±1.8	28.75 ±3.9	29.18 ±3.9	44.62[a] ±10.9	34.41 ±4.0	28.52 ±1.4	28.32 ±6.2	30.84 ±2.9	47.89[a] ±14.0
Histopathology											
Excess bilirubin in liver cord cells	24 months	0	0	0	0	3	0	0	0	0	3
Liver swollen cells	24 months	0	0	0	0	3	0	0	0	0	3
Liver, cholestasis	24 months	0	0	0	3 (min)	3 (mod)	0	0	0	1 (min)	3 (2 min, 1 mod)
Kidney, bile nephrosis	24 months	0	0	0	3 (min)	3 (1 min, 2 mod)	0	0	0	1 (min)	2 (mod)

SAP: Serum alkaline phosphatase (measured in Bessey–Lowry units); Source: Borzelleca & Larson, 1968

SGOT: Serum glutamate oxaloacetate transaminase (measured in Karmen units);

Ht: Haematocrit; Hb: Haemoglobin min: Minimal; mod: Moderate;

[a] Differs significantly from control when data for males and females are combined; * Statistically significant at $p < 0.05$

In a non-GLP, 80-week carcinogenicity study, SPF Swiss random strain mice (100/sex per dose) were administered quintozene (purity 98.2/98.3%; 2.7% HCB) via the diet at dose levels of 0, 100, 400 or 1200 ppm (equivalent to 0, 15, 60 and 180 mg/kg bw per day). Observations were made of general appearance, behaviour, survival and growth. Haematological investigations were carried out on 10 males and 10 females from each group at weeks 55 and 75. At week 80, all surviving animals were autopsied, underwent gross examination and their livers and kidneys weighed.

General appearance, behaviour and survival were not affected by quintozene. Body weights were slightly decreased at 1200 ppm in both sexes. The differences were statistically significant only in males from week 47 onwards (5–10% decrease compared to controls). There were no treatment-related findings in haematology. The relative weights of the liver were increased at 400 and 1200 ppm in both sexes. In males, relative liver weight was increased by 10% at 400 ppm and by 40% at 1200 ppm; in females the increase in relative liver weight was by 11% at 400 ppm and 54% at 1200 ppm. The relative weights of the kidneys were increased only at 1200 ppm in females (by 21% compared to controls). Subcutaneous fibrosarcomas were found in females only. Their incidence appeared to be of greater statistical significance compared to controls in the 1200 ppm group (incidence in females of 0, 2, 2 and 11 in the 0, 100, 400 and 1200 ppm groups, respectively). The study author did not find it justifiable to consider quintozene responsible for the high incidence of subcutaneous fibrosarcomas in females at the top dose based on the following considerations:

- no treatment-related differences in incidence of lung tumours and lymphocytic leukemias,
- no increased incidence in mesenchymal tumours in organs other than the subcutis; it is unlikely that only the subcutaneous mesenchymal tissue is adversely involved in a feeding study,
- there was no distinct dose–response relationship,
- subcutaneous fibrosarcomas were not found at all in males,
- an increased incidence of subcutaneous fibrosarcomas was observed neither in mice nor in rats fed diets containing higher levels of quintozene for periods of 18 and 24 months (Innes et al., 1969; Finnegan et al., 1958).

No other possible treatment-related neoplasms were found (van der Heijden & Til, 1974).

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Tetraniliprole (addendum)

First draft prepared by
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Explanation

Tetraniliprole is the ISO-approved common name for 1-(3-chloropyridin-2-yl)-*N*-[4-cyano-2-methyl-6-(methylcarbamoyl)phenyl]-3-{[5-(trifluoromethyl)-*2H*-tetrazol-2-yl]methyl}-*1H*-pyrazole-5-carboxamide, with the CAS number 1229654-66-3.

Tetraniliprole is an anthranilic diamide-class insecticide. The proposed pesticidal mode of action (MOA) for tetraniliprole is by activation of ryanodine receptor channels, leading to internal calcium store depletion that impairs regulation of muscle contraction. Mammalian ryanodine receptors are substantially less sensitive to the effects of anthranilic diamides than are insect ryanodine receptors.

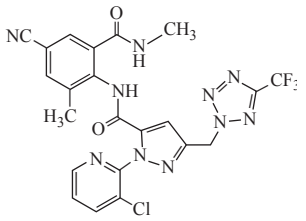
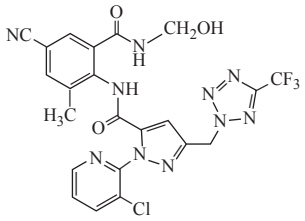
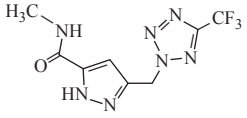
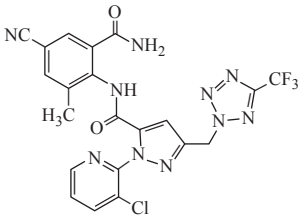
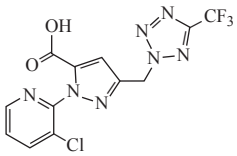
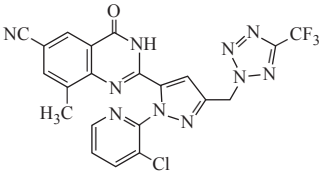
Tetraniliprole was evaluated for the first time by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 2021 (JMPR, 2022), when an acceptable daily intake (ADI) of 0–2 mg/kg body weight (bw) was established for tetraniliprole, BCS-CZ91631 and BCS-CQ63359. An acute reference dose (ARfD) was considered to be unnecessary. Tetraniliprole was evaluated at the current Meeting for maximum residue limits (MRLs), at the request of the Codex Committee on Pesticide Residues (CCPR).

As the toxicology and exposure evaluations were not performed in the same year, further information was requested on metabolites of toxicological concern. In order to predict the toxicity of five metabolites not addressed in the JMPR 2021 evaluations the sponsor provided in silico data on general toxicity and genotoxicity for tetraniliprole-hydroxy-N-methyl, tetraniliprole-pyrazole-5-N-methyl-amide, tetraniliprole-desmethyl-amide, tetraniliprole-pyridinyl-pyrazole-5-carboxylic acid and tetraniliprole-quinazolinone.

Evaluation for acceptable daily intake

As the current evaluation is based on quantitative structure–activity relationships (QSARs) and read-across, the chemical structures of tetraniliprole and metabolites of concern identified by the FAO evaluator are shown in Table 1.

Table 1. Chemical structures of tetraniliprole and associated metabolites

Compound name code	Structure	Compound name code	Structure
Tetraniliprole		Tetraniliprole-hydroxy-N-methyl BCS-CZ91629	
Tetraniliprole-pyrazole-5-N-methyl-amide BCS-CZ84317		Tetraniliprole-desmethyl-amide BCS-CN42374	
Tetraniliprole-pyridinyl-pyrazole-5-carboxylic acid BCS-CL73217		Tetraniliprole-quinazolinone BCS-CZ73507	

1. Toxicological studies on metabolites

In silico predictions of the toxicity of metabolites tetraniliprole-hydroxy-N-methyl, tetraniliprole-pyrazole-5-N-methyl-amide, tetraniliprole-desmethyl-amide, tetraniliprole-pyridinyl-pyrazole-5-carboxylic acid and tetraniliprole-quinazolinone were submitted to the present Meeting. The predictions were generated using Derek Nexus and the Organisation for Economic Co-operation and Development (OECD) QSAR Toolbox and were prepared in accordance with guidance on the establishment of the residue definition for dietary risk assessment (EFSA, 2016).

Tetraniliprole-hydroxy-N-methyl was considered equivocal for mutagenicity, a flag which was not covered by the parent. The metabolite occurs in the rat metabolism studies at 3.91%–10.06% of the administered dose (AD) in males and 2.88%–8.02% of AD in females. According to the cumulative excretion-balance and recovery data on [*pyrazole-carboxamide*-^{14}C]tetraniliprole, biliary and urinary excretion comprised only 45% of AD in males and 29% in females (Bongartz & Miebach 2016). Tetraniliprole-hydroxy-N-methyl and its downstream metabolite, tetraniliprole-hydroxy-N-methyl-hydroxypyridyl-glucuronide, when taken together, occurred at 5.87% and 3.76% of AD in males and females, respectively, in the same dose group. As the summing of the sequential metabolites/conjugates would be equivalent to 13% of AD in males and females, respectively, their toxicities are considered to be covered by that of the parent.

Tetraniliprole-pyrazole-5-N-methyl-amide, tetraniliprole-desmethyl-amide, tetraniliprole-pyridinyl-pyrazole-5-carboxylic acid and tetraniliprole-quinazolinone were all concluded to be nongenotoxic on the basis of DEREK modelling. The DEREK modelling produced flags that were equivocal for a positive AMES assay; however, these flags were also identified in the DEREK modelling for the parent and were not reflected in the parent compound's in vitro database. Of these metabolites, tetraniliprole-desmethyl-amide was considered sufficiently structurally similar to tetraniliprole that it would be considered no more toxic than the parent. However, tetraniliprole-pyrazole-5-N-methyl-amide, tetraniliprole-pyridinyl-pyrazole-5-carboxylic acid and tetraniliprole-quinazolinone were insufficiently similar and therefore no conclusions could be made as to their toxicity relative to tetraniliprole, their parent.

2. Microbial aspects

There was no information available in the public domain and no experimental data were submitted that addressed the possible impact of tetraniliprole residues on the human intestinal microbiome.

Comments

Toxicological data on metabolites and/or degradates

In silico predictions of the genotoxicity of tetraniliprole-hydroxy-N-methyl (BCS-CZ91629; goat and hen), tetraniliprole-pyrazole-5-N-methyl-amide (BCS-CZ84317; goat and hen), tetraniliprole-desmethyl-amide (BCS-CN42374; goat), tetraniliprole-pyridinyl-pyrazole-5-carboxylic acid (BCS-CL73217; goat) and tetraniliprole-quinazolinone (BCS-CZ73507; goat) were provided to the present Meeting. The predictions were prepared using a QSAR program.

Tetraniliprole-desmethyl-amide (BCS-CN42374)

Based on its structural similarity to the parent, tetraniliprole-desmethyl-amide was predicted to be no more toxic than the parent.

Tetraniliprole-hydroxy-N-methyl (BCS-CZ91629)

This metabolite was considered to be covered by the parent as it is a major metabolite in the rat.

Tetraniliprole-pyrazole-5-N-methyl-amide (BCS-CZ84317), tetraniliprole-pyridinyl-pyrazole-5-carboxylic acid (BCS-CL73217) and tetraniliprole-quinazolinone (BCS-CZ73507)

These metabolites did not show any alerts for genotoxicity on QSAR analysis. However, they were considered insufficiently similar to the parent to read across their toxicity and hence the Cramer class III threshold of toxicological concern (TTC) of 1.5 µg/kg bw per day should be applied.

The Meeting concluded that the metabolite tetraniliprole-desmethyl-amide (BCS-CN42374) was unlikely to be more toxic than the parent on the basis of in silico data and its structural similarity to tetraniliprole. Hence the reference values of the parent are also applicable to tetraniliprole-desmethyl-amide.

Tetraniliprole-hydroxy-N-methyl (BCS-CZ91629) is a major metabolite in the rat and was therefore considered to be covered by the reference values of the parent.

For tetraniliprole-pyrazole-5-N-methyl-amide (BCS-CZ84317), tetraniliprole-pyridinyl-pyrazole-5-carboxylic acid (BCS-CL73217) and tetraniliprole-quinazolinone (BCS-CZ73507), the TTC value for Cramer class III of 1.5 µg/kg bw per day should be applied.

Microbiological data

There was no information available in the public domain and no experimental data were submitted that addressed the possible impact of tetraniliprole residues on the human intestinal microbiome.

Toxicological evaluation (addendum)

The Meeting concluded that the ADI of 0–2 mg/kg bw established in 2021 applies to tetraniliprole, BCS-CQ63359, BCS-CZ91631, BCS-CZ91629 and BCS-CN42374. The 2021 conclusion that an ARfD was not necessary for tetraniliprole also applies to these metabolites.

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Triflumuron (toxicological evaluation only)

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Explanation

Triflumuron is the ISO-approved common name for 2-chloro-*N*-{[4-(trifluoromethoxy)phenyl] carbamoyl}benzamide (IUPAC), for which the Chemical Abstract Service number is 64628-44-0.

Triflumuron is a synthetic insecticide from the active ingredient group of chitin biosynthesis inhibitors (chitin inhibitors) type 15. Triflumuron acts primarily as a feeding poison for biting and sucking pests. It disturbs the chitin biosynthesis of insects, particularly in immature life stages. It is used in a wide range of crops, including apple, pear, cabbage, citrus, cotton, potato and tea. It is also used as a veterinary drug.

Triflumuron was previously evaluated by JMPR in 2019 (JMPR, 2020), when an ADI for triflumuron of 0–0.008 mg/kg body weight (bw) was established, based on the NOAEL of 20 ppm (equal to 0.82 mg/kg bw per day) based on haematological effects and an increase in spleen weight seen at the LOAEL of 200 ppm (equal to 8.45 mg/kg bw per day), observed in the two-year carcinogenicity study in rats and using a safety factor of 100. The Meeting in 2019 concluded that it was unnecessary to establish an acute reference dose (ARfD).

Triflumuron is being evaluated by the current Meeting in support of an FAO panel review of triflumuron to conclude on residue definitions for dietary risk assessment for plant and animal commodities.

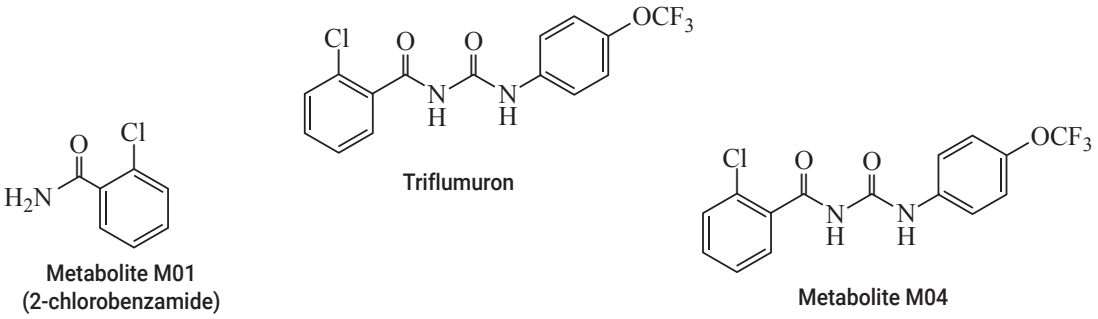
The new information made available on triflumuron comprised: an Ames test for triflumuron metabolite M01, an in vitro micronucleus test in human lymphocytes for metabolite M01, and an in silico genotoxicity assessment of triflumuron and its crop metabolite M04.

All critical studies contained statements of compliance with GLP and were conducted in accordance with relevant national or international test guidelines, unless otherwise specified. No additional information from a literature search was identified that complemented the toxicological information submitted for the current assessment.

The plant metabolites of triflumuron, M01 (2-chlorobenzamide) and M04 (1-(2-chloro-3-hydroxybenzoyl)-3-[4-trifluoromethoxyphenyl]urea) had not been detected in rat metabolism studies. No toxicological data were provided to assess the toxicity of the triflumuron plant metabolite M01 or M04. Therefore at JMPR 2019, the Meeting concluded that for dietary exposure assessment the genotoxic TTC value was appropriate for M01 and M04.

Evaluation for acceptable daily intake

Figure 1. Structure of triflumuron and its metabolites M01 and M04



1. Toxicological studies on metabolites

1.1 Genotoxicity assessment of metabolite M01

Table 1. Genotoxicity of plant metabolite M01

End-point	Test object	Concentration	Purity	Results	Reference
		In vitro			
Bacterial reverse mutation	*Salmonella typhimurium* strains TA 98, TA 100, TA 102 TA 1535 and TA 1537	Pre-experiment and Experiment 1 0, 3, 10, 33, 100, 333, 1000, 2500, and 5000 µg/plate Experiment 2 0, 33, 100, 333, 1000, 2500 and 5000 µg/plate All with and without metabolic activation in DMSO [a]	97.6%	Negative	Chang, 2020
Micronucleus test	Human lymphocytes	Experiment 1 4-hour exposure, 10.4–1594 µg/mL Experiment II 20-hour exposure, 124–1594 µg/mL All with and without metabolic activation in DMSO [b]	97.6%	Negative	Naumann, 2020

[a] No precipitation observed; DMSO: dimethylsulfoxide;

[b] Precipitation was observed at the end of the treatment at 1594 µg/mL

1.2 In silico genotoxicity assessment of metabolite M04

In silico genotoxicity assessment of triflumuron and its metabolite M04 was conducted to predict the toxicity of metabolite M04 using a combination of in silico models and read-across methods. The in silico assessment was performed using DEREK Nexus, Leadscope and Toxtree.

All alerts contained within DEREK Nexus were used. Leadscope was used to predict mutagenicity and chromosome damage. Toxtree was used to generate Cramer classifications, to make genotoxicity predictions for Ames mutagenicity, in vivo micronucleus clastogenicity and carcinogenicity predictions. Secondly, the OECD QSAR Toolbox (v4.4) was used to assess the similarity of triflumuron with metabolite M04. In addition to toxicity profiling, functional group profiling was carried out using the empirical profiler Organic functional groups (v3.8) (Pellizzaro & Beevers, 2020).

The predictions from DEREK Nexus, Toxtree and Leadscope are summarized in Table 2. There were no alerts indicated for most of the end-points for triflumuron and metabolite M04. DEREK Nexus activated an alert for sensitization in the case of M04, but not for triflumuron. However, this end-point is not of a concern for dietary risk assessment. The Toxtree end-points investigated gave identical an response for triflumuron and metabolite M04.

Table 2. Summary of in silico alerts triggered in DEREK Nexus, Leadscope, and Toxtree

Package	End-point		Triflumuron	Metabolite M04
Experimental data	Ames		Negative	Not tested
	In vitro CA in human lymphocytes		Negative	Not tested
	In vitro HPRT		Negative	Not tested
	In vivo MNT		Negative	Not tested
DEREK Nexus [a]	Mutagenicity in vitro in bacterium		Inactive [a]	Inactive [a]
	Hepatotoxicity in mammal		Plausible (alert 619)	Plausible (alert 619)
	Mitochondrial dysfunction in mammal		Equivocal (alert RP111)	Equivocal (alert RP111)
	Skin sensitization in mammal		Non-sensitizer	Equivocal (alert 439)
Toxtree	Cramer class		III (high)	III (high)
	Ames mutagenicity		No alerts	No alerts
	In vivo mouse micronucleus		SA34	SA34
	Genotoxic carcinogenicity		No alerts	No alerts
	Nongenotoxic carcinogenicity		SA31a	SA31 [b]
Leadscope	Clastogenicity in vitro	Chrom Abb CHL v2	Positive	Positive
		Chrom Abb CHL v2	Negative	Positive
	Clastogenicity in vivo	Chrom Abb. Comp v2 (A7P)	Negative	Negative [c]
		Mouse micronucleus v2	Positive	Negative
	Gene mutation	HGPRT Mut v1	Negative	Negative
		Mouse lymphoma act v2	Negative	Negative
		Mouse lymphoma unact v2	Negative	Negative
		Bacterial Mut v2	Negative	Negative

[a] No misclassified or unclassified features

[b] End-points not included did not return any alerts

[c] Outside of the applicability domain for that particular model

Grey background indicates a difference in the prediction between triflumuron and M04.

Alert 619: *para*-aminophenol or derivative ; Alert RP111: *para*-aminophenol or derivative

Alert 439: Substituted phenol; SA34: hacceptor-path 3-hacceptor; SA31a: Halogenated benzene

Triflumuron and metabolite M04 were classified as Cramer class III based on the Toxtree prediction. The predictivity of structural alert SA34 within the Toxtree in vivo micronucleus test was shown by the developers of the model to be of poor reliability (34% true positives against the training set; Benigni et al., 2009). Therefore, the structural alert triggered in Toxtree for the in vivo mouse micronucleus model can be disregarded. An alert was also activated for nongenotoxic carcinogenicity (SA31a). In Leadscope, identical outputs were obtained for triflumuron and M04 in most of the genotoxicity models investigated. Differences in predictions were obtained in the Chrom Ab CHO v2 and Mouse micronucleus v2 clastogenicity models. Since the studies with triflumuron gave a negative response, the positive predictions for the in vitro Chrom Ab CHL v2 and the in vivo MNT model can be overruled.

The genotoxicity of triflumuron and M04 was assessed using profilers relevant to genotoxicity in the OECD QSAR Toolbox. A summary of the OECD QSAR Toolbox outputs for genotoxicity and organic functional group profilers is presented in Tables 3 and 4, respectively.

Table 3. Genotoxicity profiling of triflumuron and metabolite M04 according to OECD QSAR Toolbox

Profile	General mechanistic profilers		End-point specific profilers
	DNA binding by OECD	Protein binding by OECD	In vivo mutagenicity (micronucleus) alerts by ISS
Alert number	**1**	**2**	**3**
Triflumuron	**x**	**x**	**x**
M04	x	x	x

Bold text: Indicates substances considered to have been adequately tested;

x: Alert is considered to be irrelevant; present in a substance that is considered to have been adequately tested;

Alerts: 1: SN1 >> Nitrenium ion formation >> Aromatic phenylureas

2: Acylation >> Direct acylation involving a leaving group >> Acetates

3: hacceptor-path3-hacceptor

Table 4. Organic functional group profiling of triflumuron and metabolite M04 according to OECD QSAR Toolbox

Substance	Functional groups							
	Alkyl halide	Aryl	Aryl halide	Benzamide	Ether	Imide	Phenol	Urea derivative
Triflumuron	**x**	**x**	**x**	**x**	**x**	**x**		**x**
M04	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**

Bold text: Indicates substances considered to have been adequately tested;

x: Alert is considered to be irrelevant; present in a substance that is considered to have been adequately tested;

Functional group is not present in any substance that is considered to have been adequately tested

Identical alerts were activated for triflumuron and M04 in the profilers relevant to genotoxicity. Because the alert profile of M04 is identical to the parent, all activated alerts were considered to be of no concern. The functional group profile is very similar for both triflumuron and M04, the only difference being that M04 contains a phenol, created by the addition of an OH group, absent in triflumuron. The in silico models (DEREK Nexus, Toxtree, Leadscope, OECD QSAR Toolbox) did not identify any reason why the phenol should be considered of concern regarding genotoxicity.

In summary, M04 is of no greater genotoxicological concern than triflumuron.

No toxicity data are available for metabolites M01 or M04, and M04 (with its phenolic functional group) resulted a in slightly different in silico prediction. The Meeting concluded that dietary exposure to M01 and M04 should be compared to the TTC value for Cramer class III, that is 1.5 µg/kg bw per day.

2. Microbial aspects

There is no information available in the public domain, and no experimental data were submitted that addressed the possible impact of triflumuron residues on the human intestinal microbiome.

Comments

The plant metabolite M01 was negative for genotoxicity in an Ames test and an in vitro micronucleus test in human lymphocytes.

In silico predictions demonstrated that the only genotoxicity alerts given by M04 were also given by triflumuron and hence the Meeting concluded that M04 is unlikely to be genotoxic in vivo, as this had been the conclusion for the parent triflumuron.

No toxicity data were available for metabolite M01 and M04.

The Meeting concluded that dietary exposure to M01 and M04 should be compared to the TTC value for Cramer class III, that is 1.5 µg/kg bw per day.

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Annex: List of participants

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13–22 September 2022

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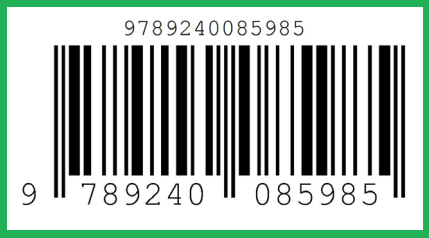
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