Pesticide residues in food — 2021

Joint FAO/WHO Meeting on Pesticide Residues

EVALUATIONS 2021

Part II – Toxicological

Food and Agriculture Organization of the United Nations

World Health Organization

Pesticide residues in food – 2021

Toxicological evaluations

Sponsored jointly by FAO and WHO

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

Virtual meeting, 6–17 September, 4 and 7 October 2021

Food and Agriculture Organization of the United Nations

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2021 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

6–17 September, 4 and 7 October 2021

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Abbreviations and acronyms

AChE	acetylcholinesterase
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
ARfD	acute reference dose
AST	aspartate transaminase
AUC	Area under the concentration-time curve
BROD	benzoxyresorufin-*O*-dealkylase
BrdU	5-bromo-2′-deoxyuridine
BUN	blood urea nitrogen
bw	body weight
ca	circa
CAS	Chemical Abstracts Service (No.)
CCPR	Codex Committee on Pesticide Residues
CHO	Chinese Hamster ovary
C_{max}	maximum concentration
CMC	carboxymethyl cellulose
CPA	cyclophophamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EC3	the amount of test substance needed to induce a 3-fold increase in cell proliferation in a LLNA
EROD	ethoxyresorufin-*O*-deethylase
FAO	Food and Agriculture Organization of the United Nations
FOB	functional observational battery
$GABA_A$	γ-aminobutyric acid, type A
GD	gestation day
GGTP	γ-glutamyl transpeptidase/transferase
GI	gastrointestinal
GIT	gastrointestinal tract
GLP	good laboratory practice
Hb	haemoglobin
HCD	historical control data
HPLC	high-performance liquid chromatography
HPLC-MS/MS	tandem high-performance liquid chromatography/mass spectrometry
Ht	haematocrit
IC_{50}	Half-maximal inhibitory concentration
ICH	International Conference on Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
i.p.	intraperitonial, intraperitonially
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
i.v.	intravenous/intravenously
IWGT	International Working Group on Genotoxicity Testing
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
LC_{50}	median lethal concentration
LC-MS	Liquid chromatography–mass spectrometry
LD	lactation day
LD_{50}	median lethal dose
LLNA	local lymph node assay
LOAEC	lowest-observed-adverse-effect concentration
LOAEL	lowest-observed-adverse-effect level
LOQ	limit of quantitation
LSC	liquid scintillation counting
MCH	mean corpuscular/cell haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular (cell) volume

MetHb	methaemoglobin
MMAD	mass median aerodynamic diameter
MOA	mode of action
MRL	maximum residue limits
MS	mass spectroscopy/spectrometry
MTD	maximum tolerated dose
NCE	normochromic erythrocyte
NMR	nuclear magnetic resonance
NOAEL	no-observed-adverse-effect level
OECD	Organisation for Economic Co-operation and Development
PCE	polychromatic erythrocyte
PND	postnatal day
PP	postpartum
PROD	pentoxyresorufin-*O*-deethylase
PT	prothrombin time
QSAR	quantitative structure–activity relationship
RBC	red blood cell
RTG	relative total growth
SD	standard deviation
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 lucuronosyltransferase
UDS	unscheduled DNA synthesis
V_{max}	maximum rate of reaction
WBC	white blood cell/leucocyte count
WHO	World Health Organization
v/v	volume/volume
w/v	weight/volume
w/w	weight/weight

Introduction

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 virtually, from 6 to 17 September and on the 4th and 7th of October, 2021.

The FAO Panel Members held its pre-meetings in biweekly virtual preparatory sessions from July to September. The WHO Core Assessment Group had organized several preparatory sessions during 2020 and 2021. The Meeting involved 45 participants from five continents spanning most time zones.

The FAO JMPR Secretariat, Ms YongZhen Yang, welcomed all the experts, noting the number of new participants. She expressed her appreciation for the amount of preparatory work already undertaken by the experts in preparing documents and their participation in the virtual pre-meetings with their spare time in the early morning or during the night.

Mr Soren Madsen, WHO JMPR Secretariat, also welcomed the participants noting that the virtual format would make detailed and complex technical discussions challenging and that short concise interventions would be helpful in optimizing the outcome of the meeting. Particularly given the reduced meeting time of a total of 30 hours available for panel discussions during the meeting, necessitated by the multiple time zones in which experts reside.

During the meeting, the FAO Panel of Experts on Pesticide Residues in Food 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. The methodologies are described in detail in the FAO Manual on the submission and evaluation of pesticide residue data for the estimation of maximum residue levels in food and feed (2016) hereafter referred to as the FAO manual. The WHO Core Assessment Group on Pesticide Residues was responsible for reviewing toxicological and related data in order to establish acceptable daily intakes (ADIs) and acute reference doses (ARfDs), where necessary and possible.

The Meeting evaluated 15 pesticides, including five new compounds and two compounds that were re-evaluated for toxicity or residues, or both, within the periodic review programme of the Codex Committee on Pesticide Residues (CCPR). The Meeting established ADIs and ARfDs, estimated maximum residue levels and recommended them for use by CCPR, and estimated supervised trials median residue (STMR) and highest residue (HR) levels as a basis for estimating dietary exposures.

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

In addition, the Meeting considered a number of general issues addressing procedures for the evaluation and risk assessment of pesticide residues in food.

TOXICOLOGICAL MONOGRAPHS AND MONOGRAPH ADDENDA

Fenpyroximate (addendum)

First draft prepared by
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Explanation

Fenpyroximate is the International Organization for Standardization (ISO)-approved common name for *tert*-butyl (*E*)-α-(1,3)-dimethyl-5-phenoxy-1*H*-pyrazol-4-yl methyleneamino-oxy)-*p*-toluate (IUPAC), with Chemical Abstracts Service number 134098-61-6 (the *E* isomer) and 111812-58-9 (unspecified stereochemistry).

Fenpyroximate is a phenoxypyrazole acaricide for application to leaves infested with phytophagous mites. Fenpyroximate acts by inhibiting mitochondrial NADH-coenzyme Q reductase of the electron transport chain in mites.

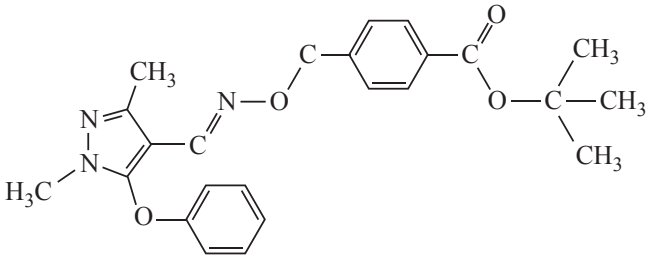
Fenpyroximate was evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1995, when an acceptable daily intake (ADI) of 0–0.01 mg/kg body weight (bw) was established, based upon a no-observed-adverse-effect level (NOAEL) of 1 mg/kg bw per day in a 104-week study in rats and a safety factor of 100. The critical effect in that study was a reduction in body weight gain and plasma protein concentration. Fenpyroximate was re-evaluated by JMPR 2004 in order to determine an acute reference dose (ARfD). An ARfD of 0.01 mg/kg bw was established based on the lowest-observed-adverse-effect level (LOAEL) of 2 mg/kg bw per day for the marginal induction of diarrhoea at the beginning of the 13-week toxicity study in dogs, and using a safety factor of 200 since no NOAEL was identified in this study. The ARfD was re-evaluated by JMPR 2007. The 2007 Meeting established an ARfD of 0.02 mg/kg bw based on the NOAEL of 2 mg/kg bw identified from a study of fenpyroximate where diarrhoea was observed after a single dose in dogs, and using a safety factor of 100. The 2017 Meeting withdrew the existing ARfD and established an new ARfD of 0.01 mg/kg bw on the basis of the LOAEL of 2 mg/kg bw for the induction of diarrhoea seen in a newly submitted single bolus gavage study, and a 13-week study of toxicity in dogs. A safety factor of 200 was used since no NOAEL was identified. The 2017 Meeting also stated that it was unclear whether the diarrhoea was the result of direct irritant or the pharmacological effect of fenpyroximate. In the database that was available, however, histopathological examination of the gastrointestinal tract had not revealed any

evidence of irritation. The JMPR 2017 Meeting re-affirmed the ADI of 0–0.01 mg/kg bw established at JMPR 1995. The 2017 Meeting concluded that the ADI and ARfD can be applied to metabolites M-1, M-3, M-5, M-21, M-22 and Fen-OH.

Fenpyroximate was reviewed by the present Meeting under the periodic review programme of Codex Committee on Pesticide Residues (CCPR) due to a number of proposed additional uses: citrus, banana, celery, cranberry, summer squash, watermelon, bean [succulent shelled], blueberry, plum, apricot, and peach. New studies included a pathological evaluation of tissues from a single bolus dose study in dogs, an incidence report of diarrhoea in beagle dogs at Life Science Research Lab and its analysis, genotoxicity studies with metabolites, sensitivity analysis of selected studies previously reviewed, newly reported pharmacology studies with the parent compound, two published metabolism studies in rats, together with acute and mutagenicity studies on impurities found in fenpyroximate.

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. A search of the open literature did not find any additional relevant publications.

Figure 1. Chemical structure of isoprothiolane



Evaluation for acceptable intake

1. Biochemical aspects

In a published study, absorption, distribution, excretion and metabolism of fenpyroximate was studied in male Sprague Dawley rats at a single oral gavage dose of [*pyrazole*-3-^{14}C]-, [*phenyl*-(*U*) ^{14}C] or [*benzene ring* (*U*)-^{14}C]- labelled fenpyroximate (radiochemical purity > 99.0%) at 1.5 mg/kg bw in olive oil. Blood was collected at specified intervals. The treated rats were housed individually in metabolism cages for collection of carbon dioxide, urine and faeces for 72 hours. For the isolation and identification of faecal metabolites, the total number of 800 rats received fenpyroximate at doses of 15 mg (5 mL/kg bw). The faeces excreted were collected for 48 hours after administration and extracted with ethyl ethanoate, and the extracts concentrated. Metabolites were identified by thin layer chromatography (TLC). The livers from treated rats were removed and homogenates prepared after centrifugation at 9000 × *g*. The pellets were resuspended in the phosphate buffer. The incubation mixture consisted of microsomes (100 mg of liver equivalents), 10 µg of [*pyrazole*-3-^{14}C]fenpyroximate (1.05 kBq) and 2 µmol NADPH in a final volume of 10 mL. After incubation at 37°C, the reaction mixture was extracted twice with 10 mL ethyl ethanoate. The extracts were concentrated for analysis by cochromatography.

After an oral administration of [*pyrazole-3*-^{14}C]-, [*phenyl*-(*U*)-^{14}C] or [*benzene ring* (*U*)-^{14}C]-labelled fenpyroximate, the radiocarbon level in the blood increased to the maximum levels of 0.18, 0.16 and 0.18 µg fenpyroximate equiv./mL at 12 h, 12 h and 9 h, respectively. It then decreased rapidly with a half-life of 11.3 h, 10.6 h and 6.2 h, respectively, to below the detectable limit (0.02 µg fenpyroximate equiv./mL) at 48 hours. The excretion rates of radiocarbon into the urine and faeces within 72 hours following oral administration were, for the three label positions: 26.2% and 65.5% for [*pyrazole-3*-^{14}C], 26.l% and 63.9% for [*phenyl*-(*U*)-^{14}C] and 6.4% and 86.9% for [*benzene ring* (*U*)-^{14}C]. No detectable radioactivity was found in the expired air (under 0.1% of the dose) (Nishizawa et al., 1993).

In a published study, the mode of selective toxicity of fenpyroximate (*tert*-butyl(*E*)-α-(1,3-

dimethyl-5-phenoxypyrazol-4-ylmethyleneamino-oxy)-*p*-toluate), was studied with respect to its detoxification metabolism. Among its metabolites examined, only ester-hydrolyzed metabolites completely lost their inhibitory activity toward NADH–ubiquinone oxidoreductase, which suggested that ester hydrolysis was the key step in detoxification. After a single oral administration of fenpyroximate to rats, two labile intermediates (metabolites A and B) as well as ester-hydrolyzed metabolites were found in the liver and plasma as the major metabolites. These intermediates were also observed in an in vitro metabolism system employing rat liver S9 fraction (9000 × *g* supernatant) in the presence of diisopropyl fluorophosphate, which is a carboxyesterase inhibitor. Metabolites A and B were isolated and identified, respectively, as:

1-hydroxymethyl-1-methylethyl(*E*)-α-(1,3-dimethyl-5-phenoxypyrazol-4-ylmethyleneamino-oxy)-*p*-toluate and
2-hydroxy-2-methylpropyl(*E*)-a-(1,3-dimethyl-5-phenoxypyrazol-4-ylmethyleneamino-oxy)-*p*-toluate.

Under slightly basic conditions or in rat plasma, metabolite A was stoichiometrically and nonenzymatically converted to metabolite B most, likely via intramolecular transesterification. The rate of production in vitro of metabolite A was approximately 10 times greater than that of direct carboxyesterase hydrolysis of fenpyroximate. Metabolite B was hydrolyzed by carboxyesterase approximately 100 times faster than was fenpyroximate. Therefore it is presumed that in the rat fenpyroximate is hydrolyzed principally via hydroxylation of fenpyroximate to metabolite A, followed by transesterification to metabolite B. Hydroxylation of fenpyroximate to metabolite A was observed not only in rat liver, but also in mouse, rabbit, crab-eating monkey, carp, quail liver, and in the mid-gut of *Spodoptera litula* (a noctuid moth). Neither tertiary butyl ester hydrolysis nor formation of metabolite A were detected in *Tetranychus urticae* (Koch) (two-spotted spider mite), which is a fenpyroximate-sensitive organism both in vivo and in vitro. Consequently, the selectivity of fenpyroximate between spider mites and nontarget organisms (especially mammals) would seem to be attributable to species-specific detoxification, ester hydrolysis via microsomal hydroxylation, followed by intramolecular transesterification (Motoba et al. 2000).

2. Toxicological studies

2.1 Short-term studies of toxicity

(a) Oral administration

Rat

In a 90-day toxicity study, fenpyroximate (Batch No. NNI-850; purity 99%) was administered for 13 weeks in the diet to CD rats (10/sex per dose) at dose levels of 0, 20, 100 or 500 ppm (equal to 0, 1.3, 6.57 and 35.22 mg/kg bw per day for males, 0, 1.65, 8.29 and 38.60 mg/kg bw per day for females).

One female at 20 ppm and two males at 500 ppm died during routine blood sampling after week 12. At 500 ppm, 3/10 males and 7/10 females had facial staining, 7/10 males had encrustations of the muzzle, 7/10 females developed large areas of dorsal/ventral hair loss, and 6/10 females had skin encrustations and/or exfoliation. No treatment-related effects were observed in the 20 ppm treatment groups. Both sexes at 100 and 500 ppm had impaired growth performance, reduced food intake and decreased body weights and body weight gains. Body weights for the males at 100 ppm were comparable to the controls until week 10, and then were reduced to 90% of the control weight during weeks 11–13 ($p < 0.05$). body weights for females at 100 ppm were 90–93% of the control weights throughout the study ($p < 0.05$ or $p < 0.001$) except during weeks 0–2 and 10, when they were comparable to the control weights. Body weight gains for both sexes at 100 ppm were 15% lower than the respective control gains. For males and females at 500 ppm, body weights were 49–73% and 62–84%, respectively, of the corresponding control weights during the study ($p < 0.001$), and body weight gains were 32% and 37%, respectively, of the corresponding control gains. Food consumption for both sexes at 100 ppm was 4–5% lower than for the respective controls. Mean food consumption for the males and females at 500 ppm was 59% and 65% respectively of the control value.

No significant abnormalities were found from ophthalmoscopy.

At 100 ppm, there was a decrease in total leukocyte count in males. In males at 500 ppm, haematocrit (Ht), haemoglobin (Hb) and red blood cell (RBC) counts were higher and white blood cell counts were 40% lower than the control values. In females at 500 ppm, Hb, Ht, RBC counts and platelet counts were higher than the control values. Total plasma proteins were 10% lower in males at 500 ppm and 6% and 16% lower in females at 100 and 500 ppm respectively. Females at 500 ppm had alkaline phosphatase (ALP) activity 123% higher and plasma butyrylcholinesterase and plasma acetylcholinesterase activities 72–73% lower than the control values. Increases in sodium level were statistically significant in females at all doses; however, there was no clear dose–response relationship and the magnitude of change was very small (143, 146, 145 and 146 m equiv./L at 0, 20, 100 and 500 ppm, respectively). At 500 ppm males had lower urine volume and pH values.

Differences attributed to the general effect of fenpyroximate on growth, rather than a direct, organ-specific treatment-related effect, were lower relative adrenal gland weights in females at 20 ppm and higher relative heart weights in males and females at 100 ppm. Also recorded were, in males and females at 500 ppm, depressed absolute organ weights for all weighed organs (not statistically significant for testes) and increased relative brain, heart, kidneys (statistically significant for females only), liver, lung and spleen weights, and in males only, adrenal glands, pituitary (not statistically significant) and testis weights ($p < 0.05$ or $p < 0.01$). Treatment-related effects noted in the gross pathology of the 500 ppm groups were facial staining in both sexes In males at 500 ppm there wer encrustations of the muzzle and persistent hyaloid arteries in males, and in females at this dose, dorsal/ventral hair loss, skin encrustations, skin masses, perineal staining and skin exfoliation. Minimal hepatocytic hypertrophy was observed in both sexes at 100 and 500 ppm.

The NOAEL was 20 ppm (equal to 1.3 mg/kg bw per day) based on decreased body weight and body weight gains, reduced feed consumption and minimal hepatocytic hypertrophy in both sexes at the LOAEL of 100 ppm (equal to 6.57 mg/kg bw per day) (Aughton, 1987).

Dog

Study 1

In a single-dose study (not GLP-compliant), fenpyroximate (purity 99.8%) suspended in 0.5% w/v methylcellulose in purified water was administered as a single gavage dose to beagle dogs (four/sex per dose group) at 0, 2 or 5 mg/kg bw. Prior to test article administration, each dog was sham-dosed with the vehicle (0.5% w/v methylcellulose in purified water) for seven days. All animals were observed daily for signs of ill health or overt toxicity and each animal was given a detailed physical examination daily during the dosing period and weekly pre-treatment. All animals were subjected to a battery of examinations to determine neurological function pretreatment and again approximately two hours after dosing on day 1. Body weights were recorded once weekly pretreatment, on day 1 and day 2 before necropsy. Blood samples (for haematology and chemistry) were taken from all animals on day 1 at 0.5, 1, 2, 4, 8 and 24 hours after dosing. Urine samples were collected from all animals by direct catherization of the bladder pretreatment and on day 2 prior to necropsy. Selected organs were weighed before necropsy. Dogs were killed one day after dosing and selected tissues examined histopathologically.

All animals survived to scheduled necropsy. There were no treatment-related effects on body weights and food consumption or on haematological, blood biochemistry or urinary parameters. There were no consistent treatment-related effects on any neurological parameters in the assessment battery at 2 or 5 mg/kg bw on day 1. Organ weight analysis and macroscopic and microscopic analysis showed no treatment-related effects. Soft faeces were observed in 9/24 animals (day −1 and day −2) during the seven-day pretreatment period with vehicle. During the post-dosing period, a single female treated at 5 mg/kg bw vomited two hours after dosing. At 5 mg/kg bw, liquid faeces were seen in 2/4 males and 2/4 females, with onset as early as two hours after dosing and persisting in some cases for up to 24 hours after dosing. At 2 mg/kg bw, liquid faeces were seen in 2/4 male dogs with onset 21 hours after dosing.

No NOAEL could be identified as effects were observed at all doses. The LOAEL was 2 mg/kg bw, the lowest dose tested, based on liquid faeces (diarrhoea) (Harvey, 2006a).

In a newly submitted study, tissues from the previously conducted single bolus gavage study in beagle dogs by Harvey (2006a), the gastrointestinal tract and oesophagus were microscopically examined.

No microscopic findings that suggested local or systemic fenpyroximate-related effects were noted in the oesophagus or gastrointestinal tract following administration of 2 or 5 mg/kg bw single bolus gavage dose in beagle dogs (Walker, 2018).

Study 2

In a dose escalation study, fenpyroximate (purity 99.8%) suspended in 0.5% w/v methylcellulose in purified water was administered orally via bolus gavage to beagle dogs, two of each sex. During the maximum tolerated dose (MTD) phase, the animals were given a single dose of 2 mg/kg bw on day 1. The same animals were then dosed with 5 mg/kg bw on day 8 and with 20 mg/kg bw on day 15. On day 23 the same four dogs were dosed with 5 mg/kg bw (fixed-dose phase) and this dose was repeated for four consecutive days (days 23–27). They were then killed and necropsied. Each dog was dosed with vehicle for seven days prior to the initiation of the treatment to establish the incidence of diarrhoea and the reaction to the vehicle alone. All animals were observed daily for ill health or overt toxicity and given a detailed physical examination daily. All animals were subjected to a battery of examinations to determine neurological function pretreatment (on day 5, during the vehicle dosing) and at approximately two hours after dosing on the day of each incremental change in dose level (nominally days 1, 8 and 15). Body weights were recorded for each animal before treatment, during the vehicle phase and before dosing on the day of each incremental change in dose level. Weighings continued twice weekly during the fixed dose phase and again just before necropsy. Blood samples were taken on day 1 of the fixed-dose phase at 1, 3, 6 and 24 hours after dosing; these were not analysed but stored frozen. Selected organs were weighed. Necropsy were performed on all animals, organs and tissues being prepared for histopathological examination.

There were no treatment-related effects on body weight, food consumption, neurological assessment battery, haematological or clinical chemistry parameters during the MTD and fixed-dose phases. No treatment-related effects were observed on organ weights at scheduled kill. Diarrhoea was caused by a single dose of 20 mg/kg bw in the MTD phase and also after three hours by a single dose of 5 mg/kg bw in 1/2 males and females on the first day, and in all dogs on the five days of the fixed-dose phase of the study. According to the study author it was unclear if this was a residual effect of treatment with 20 mg/kg bw in the MTD phase. However, diarrhoea was not observed from days 16 to 22 after the application of the 20 mg/kg bw dose.

Several dogs had macroscopic findings in the gastrointestinal tract including slightly pale stomach, slightly dark caecum, severe red caecum with slightly red ileum and yellow colon with a red focus. According to the study author it was unclear whether these findings resulted from a residual effect due to prior treatment with 20 mg/kg bw in the escalating dose. There were no concurrent controls. Histopathological examinations were not conducted. No diarrhoea was observed after the single application of 2 mg/kg bw.

The NOAEL was 2 mg/kg bw based on diarrhoea observed at the LOAEL of 5 mg/kg bw (Harvey, 2006b).

Study 3

In a 90-day oral toxicity study, fenpyroximate (purity 98.4–98.9%) was administered via capsule to beagle dogs (four/sex per dose group) at dose levels of 2, 10 or 50 mg/kg bw per day for 13 weeks. Dogs were inspected regularly throughout the working day for evidence of a reaction to treatment or ill-health. Each animal was subjected to a rigorous veterinary examination before dosing commenced and after 4, 8 and 12 weeks of treatment. Once before commencement of treatment, individual electrocardiograph tracings were recorded from each dog. Thereafter further traces were obtained during weeks 6 and 12, at 2 and 24 hours after dosing respectively. The body weight of each animal was determined, before feeding, and at weekly intervals throughout the acclimatization and treatment periods. Food and water consumption were recorded daily in the course of routine observation for other signs. Ophthalmological examination was conducted before treatment and after week 12. Blood was taken before treatment and at weeks 6 and 12 of treatment for haematology and clinical chemistry analysis. Urinalysis was performed before treatment and after 11 weeks of treatment. Selected organs were weighed at necropsy. Macroscopic examinations were performed on all dogs. Selected organs and tissues were examined histopathologically.

During weeks 4 or 5, two females at 50 mg/kg bw per day were euthanized in extremis after

a period of treatment-related inappetence and body weight loss. Diarrhoea occurred most frequently in females at 10 mg/kg bw per day and in both sexes at 50 mg/kg bw per day (Table 1). One female at 2 mg/kg bw per day was emaciated during the latter part of the study. At 50 mg/kg bw per day, 3/4 males were emaciated during the latter half of the study and 4/4 females were emaciated beginning at week 2. Emesis was observed in 2/4 males and 4/4 females at 10 mg/kg bw per day and in 4/4 males and 4/4 females at 50 mg/kg bw per day. Emesis was generally observed between 30 minutes and five hours after dosing, with the greatest incidence occurring during week 1. Torpor was observed in 1/4 females at 2 mg/kg bw per day, 2/4 females at 10 mg/kg bw per day, and 2/4 males and 4/4 females at 50 mg/kg bw per day. Slight bradycardia was noted in several dogs from all treated groups two hours and 24 hours after dosing (Tables 2a and 2b). The level of bradycardia was essentially the same at both intervals after dosing and was primarily observed at 10 and 50 mg/kg bw per day. There were no treatment-related effects on the wave intervals or amplitudes.

Table 1. Group incidence of diarrhoea in 13-week oral toxicity study in dogs treated with fenpyroximate

	Group incidence of diarrhoea per sex and dose (%)							
	Male				**Female**			
Dose (mg/kg bw/day)	**0**	**2**	**10**	**50**	**0**	**2**	**10**	**50**
Week number								
–2	0	0	0	0	0	0	5	0
–1	0	0	5	0	10	5	0	5
1	10	25	40	80	10	45	45	45
2	5	25	45	75	0	55	50	35
3	5	30	20	65	5	35	40	45
4	5	25	15	70	0	15	65	40
5	15	30	15	80	5	40	30	40
6	5	10	20	70	5	35	40	50
7	10	20	15	70	10	40	70	65
8	5	20	30	65	0	20	50	30
9	0	10	10	70	0	20	30	35
10	10	30	10	70	0	35	65	70
11	20	20	25	70	0	30	55	70
12	5	30	25	55	0	30	60	55
13	15	20	5	75	0	0	50	45

Source: Broadmeadow, 1988, 1989a

Table 2a. Electrocardiography in male dogs in 13-week toxicity study

Dose (mg/kg bw per day)	Heart rate[a] (min^{-1})	Intervals (ms)						Amplitude (mV)					
		P	PR	QRS	ST	QT	QTc	P	Q	R	S	T	R:T
Before treatment													
0	150	30	90	30	130	160	250	0.27	0.40	2.34	0.29	0.18	14.4
	[27]	[2]	[9]	[2]	[14]	[12]	[14]	[0.12]	[0.28]	[0.47]	[0.31]	[0.06]	[4.6]
2	128	31	89	30	146	175	255	0.24	0.38	2.27	0.45	0.27	10.8
	[10]	[5]	[11]	[2]	[13]	[15]	[18]	[0.04]	[0.26]	[0.44]	[0.28]	[0.12]	[6.7]
10	144	28	90	31	141	172	266	0.24	0.34	1.96	0.36	0.17	13.0
	[10]	[1]	[15]	[4]	[9]	[10]	[8]	[0.02]	[0.09]	[0.44]	[0.08]	[0.06]	[4.2]
50	143	33	87	33	132	165	254	0.20	0.48	2.82	0.26	0.16	20.6
	[8]	[3]	[14]	[3]	[21]	[20]	[32]	[0.05]	[0.11]	[0.45]	[0.22]	[0.07]	[9.2]
After treatment (during week 6 of treatment)													
0	155	39	87	35	135	170	272	0.34	0.42	2.72	0.25	0.25	11.4
	[18]	[3]	[8]	[4]	[10]	[9]	[14]	[0.05]	[0.29]	[0.54]	[0.41]	[0.07]	[3.4]
2	124	33	85	32	147	179	256	**0.26***	0.58	2.88	0.52	0.29	12.6
	[9]	[3]	[9]	[2]	[5]	[6]	[14]	[0.2]	[0.41]	[0.70]	[0.25]	[0.12]	[8.3]
10	**118***	37	96	34	146	180	251	0.29	0.38	2.51	0.45	0.29	13.2
	[13]	[7]	[16]	[4]	[8]	[11]	[6]	[0.05]	[0.09]	[0.55]	[0.16]	[0.17]	[10.7]
50	**122***	37	86	33	144	177	249	**0.26***	0.39	2.94	0.35	0.33	12.3
	[34]	[4]	[13]	[2]	[10]	[9]	[22]	[0.04]	[0.10]	[0.54]	[0.16]	[0.19]	[7.4]

[a] Heart rate is in beats per minute; Source: Broadmeadow, 1988, 1989a

* Significantly different from controls, $p < 0.05$; [ ] Standard deviations are shown in square brackets

Table 2b. Electrocardiography in female dogs in 13-week toxicity study

Dose (mg/kg bw per day)	Heart rate[a] (min^{-1})	Intervals (ms)						Amplitude (mV)					
		P	PR	QRS	ST	QT	QTc	P	Q	R	S	T	R:T
Before treatment													
0	143	34	88	30	137	166	249	0.24	0.22	2.26	0.57	0.41	6.2
	[48]	[10]	[4]	[3]	[18]	[15]	[28]	[0.06]	[0.21]	[0.30]	[0.39]	[0.13]	[2.6]
2	148	31	83	29	135	164	257	0.22	0.36	2.46	0.69	0.31	8.7
	[7]	[3]	[8]	[1]	[4]	[4]	[4]	[0.05]	[0.17]	[0.45]	[0.15]	[0.12]	[2.5]
10	157	28	88	28	129	157	249	0.22	0.34	2.45	0.42	**0.21***	12.2
	[41]	[5]	[9]	[2]	[18]	[18]	[10]	[0.07]	[0.30]	[0.66]	[0.22]	[0.06]	[4.2]
50	165	31	87	30	126	156	255	0.30	0.40	2.87	0.23	**0.19****	15.3
	[34]	[5]	[8]	[3]	[21]	[22]	[14]	[0.05]	[0.15]	[0.34]	[0.11]	[0.02]	[1.8]
After treatment (during week 6 of treatment)													
0	127	40	91	34	144	178	254	0.26	0.34	2.44	0.53	0.32	10.1
	[40]	[4]	[3]	[2]	[12]	[11]	[29]	[0.05]	[0.32]	[0.81]	[0.29]	[0.15]	[8.0]
2	136	38	87	35	138	173	253	0.25	0.38	2.36	0.46	0.33	7.8
	[50]	[6]	[15]	[4]	[18]	[18]	[30]	[0.04]	[0.05]	[0.57]	[0.13]	[0.15]	[2.7]
10	133	35	89	31	141	172	247	0.27	0.22	2.97	0.40	0.20	22.7
	[52]	[4]	[11]	[2]	[24]	[23]	[17]	[0.08]	[0.11]	[0.69]	[0.18]	[0.16]	[13.3]
50	110	32	**120***	34	149	183	245	0.20	0.37	2.80	**0.14***	0.15	22.0
	[25]	[3]	[8]	[3]	[27]	[30]	[12]	[0.06]	[0.07]	[0.58]	[0.09]	[0.07]	[14.3]

[a] Heart rate is in beats per minute; Source: Broadmeadow, 1988, 1989a

[ ] Standard deviations are shown in square brackets

Significantly different from controls; * $p < 0.05$, ** $p < 0.01$

Body weights and body weight gains for low- and mid-dose males were comparable to controls. Body weights of high-dose males were lower than controls, but not with statistical significance. Body weights of all treated females were lower than controls, reaching statistical significance at 50 mg/kg bw per day ($p < 0.05$). Total body weight gain for the females at 2 mg/kg bw per day was 6% lower than the control weight gain; this was attributed to weight loss in one female that had short periods of inappetence. Food consumption was decreased by 5–7% in low- and mid-dose females and by 34% in high-dose females. Feed consumption in males was unaffected by treatment. Water consumption was unaffected by treatment. No treatment-related ophthalmological abnormalities were observed. High-dose females had 34.2–34.7% lower total white blood cell counts and 19–34% longer partial thromboplastin time with kaolin (PTTK) at weeks 6 and 12 ($p < 0.05$, 0.01 or 0.001) and 20% higher platelet counts at week 12 ($p < 0.05$) than controls. No treatment-related effects on clinical chemistry parameters were observed in low-dose males. In mid- and high-dose males, however, glucose levels at weeks 6 and/or week 12 were 7–11% lower than in controls ($p < 0.05$). No treatment-related effects on clinical chemistry parameters were observed in low- or mid-dose females, but in high-dose females blood urea concentrations at weeks 6 and 12 were 32–46% higher ($p < 0.05$) than in controls. The two decedents (both females at 50 mg/kg bw per day) showed high blood urea concentrations and low plasma butyrylcholinesterase activities, and one had low concentrations of blood glucose. No intergroup differences were seen in urine analysis results. In high-dose males absolute and relative adrenal gland weights were 25–36.8% higher and relative liver weights were 13% higher than controls. In high-dose females relative adrenal gland weights were 23% higher and relative liver weights were 32.7% higher than controls. High-dose females had depleted hepatocytic glycogen and fine vacuolation of the cell cytoplasm in the renal medullary rays. One or both of the high-dose females euthanized in extremis exhibited first and second degree heart block, increased urea concentration, low glucose concentration, disturbances in plasma electrolyte levels, depleted hepatocytic glycogen and fine vacuolation of the cell cytoplasm in the renal medullary rays.

In this study the LOAEL was 2 mg/kg bw per day, the lowest dose tested, based on slight bradycardia and an increased incidence of diarrhoea in both sexes (weekly observations), and in females only, reduced food consumption, body weight and body weight gain and increased emaciation and torpor No NOAEL could be identified (Broadmeadow, 1988, 1989a).

Study 4

In a one-year toxicity study, fenpyroximate (purity 98.0%) was administered to beagle dogs (four per sex per dose) via capsule at dose levels of 0, 0.5, 1.5, 5.0 or 15.0 mg/kg bw per day for 52 weeks. Dogs were inspected regularly throughout the working day for evidence of reaction to treatment or ill health. Each animal was subjected to a rigorous veterinary examination before dosing commenced and then after 12, 24, 38 and 49 weeks of treatment. Once, before start of treatment, individual electrocardiograph tracings were recorded for each dog. Thereafter further traces were obtained during weeks 11, 24 and 50, at 2 hours and 24 hours after dosing respectively. The body weight of each animal was determined, before feeding, at weekly intervals throughout the acclimatization and treatment periods. Food and water consumption were observed daily in the course of routine observation for other signs. Ophthalmological examination was performed before the start of treatment and after 49 weeks. Blood samples were taken before commencing treatment and after 12, 24 and 50 weeks of the treatment for the evaluation of haematology and clinical chemistry parameters. Analysis of urine samples was performed before the start of the study and during weeks 12, 24 and 50 of treatment. Selected organs were weighed and selected tissue samples preserved in fixative.

There were no deaths during the study. Diarrhoea was observed in animals from all groups, including controls, but was more frequent in males at 5.0 mg/kg bw per day and in males and females at 15 mg/kg bw per day (Table 3). Salivation was noted in a few dogs from all treated groups as was emesis in isolated animals at 1.5 mg/kg bw per day or above. Vomiting (from time of dosing up to five hours after) was observed in one female at 1.5 mg/kg bw per day (day 1), one female at 5.0 mg/kg bw per day (days 1 and 3), and one male and two females at 15.0 mg/kg bw per day (all on day 1 and one female also on days 2 and 3). Three of the four male dogs at 15.0 mg/kg bw per day gained weight more slowly during the first three months of the study than dogs in the control group. The difference in average weight gain (33% lower in the treated animals) was statistically significant ($p < 0.05$). The body weight gains of male dogs at 0.5, 1.5 and 5.0 mg/kg bw per day and female dogs in all treatment groups did not differ

significantly from the respective controls. Periods of inappetence, occasionally marked, were recorded for three males and one female at 15.0 mg/kg bw per day. Neither the food consumption of animals at 0.5, 1.5 and 5.0 mg/kg bw per day nor the water consumption by all treated animals were affected by treatment. Veterinary examination confirmed increased salivation and thin appearance in some treated dogs. There were no treatment-related ocular lesions. Electrocardiographic examinations in weeks 11, 24 and 50, indicated slight bradycardia with a prolonged ST interval two hours and 24 hours after dosing in male dogs receiving 15.0 mg/kg bw per day. There was no clear evidence of a similar effect in females. Compared with controls, all four male dogs treated at 15 mg/kg bw per day had significantly slower ($p < 0.05$ or 0.01) heart rates two hours and 24 hours after dosing. The heart rates of females at the highest dose were also slower than the controls, but the difference was not statistically significant.

Table 3. Group incidence of diarrhoea in one-year study in dogs treated with fenpyroximate

	Group incidence of diarrhoea per sex and dose (%)									
	Male					Female				
Dose (mg/kg bw/day)	**0**	**0.5**	**1.5**	**5**	**15.0**	**0**	**0.5**	**1.5**	**5**	**15.0**
Weeks										
5–8	10	5	20	30	65	15	20	0	15	55
9–12	15	20	25	30	45	15	20	5	20	60
13–16	10	15	20	45	60	10	20	10	15	60
17–20	0	5	20	40	50	5	15	0	5	50
21–24	5	10	5	15	35	0	5	0	5	40
25–28	0	10	5	25	40	0	15	0	0	60
29–32	5	10	20	30	55	15	20	0	10	55
33–36	5	10	5	20	45	5	5	0	0	50
37–40	0	0	5	15	40	0	5	0	5	50
41–44	0	0	10	20	30	5	5	0	10	45
45–48	0	0	0	15	30	0	5	0	5	30
49–52	5	0	5	15	20	5	0	0	0	35

Source: Broadmeadow, 1989b

Most haematological differences between control and treated dogs occurred randomly and could be attributed to normal variation. Both sexes in all treated groups had blood cholesterol concentrations 26–45% lower than those in the control groups after 50 weeks of dosing; no accompanying changes in liver function or pathology were noted. Urinary analysis found no treatment-related changes. Males in all treated groups had prostate glands an average of 60% heavier than those in the control group, but with no dose–response relationship. There were no macroscopic or histopathological findings that could be attributed to treatment.

The NOAEL was 0.5 mg/kg bw per day based on bradycardia in males at the LOAEL of 1.5 mg/kg bw per day (Broadmeadow, 1989b).

The incidence of diarrhoea in beagle dogs administered fenpyroximate by capsule at Life Science Research during 1988 and 1989 were provided. The report presents the incidences for all groups on each of these studies in the week preceding the commencement of treatment (week –1) and for untreated (control) animals in the first week of treatment (week 1). With the exception of one study (where the vehicle was a 0.1% w/v aqueous Nikkol HC0-60, a PEG60 hydrogenated castor oil solubiliser, and the administration method was gavage), the controls in the listed studies were given empty gelatine capsules. The summary is provided in the following Table 4.

Table 4. Summary of overall incidence of diarrhoea reported in Week –1 and Week 1 in studies in beagle dogs conducted at Life Science Research in 1988/1989

		Week –1	Week 1
Males	Affected/total males [as a %]	21/156 [13%]	15/41 [37%]
Females	Affected/total males [as a %]	26/156 [17%]	13/41 [32%]

Source: Broadmeadow, 2018a,b

This information demonstrates that a considerable background incidence of diarrhoea can be expected in toxicity studies conducted in beagle dogs (Broadmeadow, 2018a, b).

Table 5. Incidence of diarrhoeaa, severity and time course observed in the 13-week toxicity study by oral (capsule) in beagle dogs with fenpyroximate (dose shown in mg/kg bw per day)

Group [sex]	Dose	Animal number	Time after first dose administration (h)	Comment
1 [M]	0	2534	4.75	Moderate
2 [M]	2	2522	4.75	Moderate
		2542	>21	Moderate (reported in the morning after the first dose)
3 [M]	10	2526	>21	Moderate (reported in the morning after the first dose)
		2528	2.25	Moderate
4 [M]	50	2524	0.25	Moderate, becoming marked later, but was reported before dose administration and is therefore less clearly attributable to treatment
		2530	0.25	Marked
		2548	4.25	Moderate
1 [F]	0	2503	>21	Moderate (reported in the morning after the first dose) but was also reported before the first dose
2 [F]	2	2485	>21	Moderate (reported in the morning after the first dose)
		2489	>21	Marked (reported in the morning after the first dose) but moderate diarrhoea was also reported before the first dose
		2495	>21	Moderate (reported in the morning after the first dose)
3 [F]	10	2497	>21	Moderate (reported in the morning after the first dose)
		2499	4.75	Marked, persisting to the following morning (that is >21 hours after dosing)
4 [F]	50	2487	>21	Slight (reported in the morning after the first dose)
		2491	4.75	Moderate, but was reported before dose administration (moderate on Day –1 and slight before dosing) and is therefore not attributable to treatment
		2507	4	Moderate

M: Male; F: Female;

Source: Broadmeadow, 2018a,b

Table 6. Incidence of diarrhoeaa, severity and time course observed in the 52-week toxicity study by oral (capsule) in beagle dogs with fenpyroximate (dose shown in mg/kg bw per day)

Group [sex]	Dose	Animal number	Time after first dose administration (h)	Comment
1M	0	2796	>20 hours	Moderate (reported in the morning after the first dose)
2M	0.5	-	-	No animal displayed diarrhoea within the 24-hour period after the first dose.
3M	1.5	2784	3.25	Moderate
4M	5	2770	>20	Moderate (reported in the morning after the first dose)
		2778	3.5	Moderate
		2804	3.5	Moderate
		2808	>20	Slight (reported in the morning after the first dose)
5M	15	2768	3.25	Moderate
		2776	3.25	Moderate
		2780	3.5	Slight (this animal had moderate diarrhoea on Day –2)
		2802	3.5	Slight, becoming moderate at >20 hours post-dose (this animal also had moderate diarrhoea on Day –1)
IF	0	-	-	No animal displayed diarrhoea within the 24-hour period after the first dose
2F	0.5	2747	>20	Slight (reported in the morning after the first dose)
		2769	>20	Moderate (reported in the morning after the first dose)
3F	1.5	-	-	No animal displayed diarrhoea within the 24-hour period after the first dose.
4F	5	2743	3.75	Moderate
5F	15	2733	3.5	Marked
		2755	3.5	Marked
		2765	3.75	Marked

M: Male; F: Female; Source: Broadmeadow, 2018a,b

In a 13-week study, diarrhoea was observed in one male and one female animals in the control group and observed within 4.75 hours (male), and more than 21 hours (female) after administration of the dose. Marked diarrhoea was observed in the one male and moderate diarrhoea in two males at 50 mg/kg bw per day (Table 5).

In a 52-week study, diarrhoea was observed in one male in the control group and observed more than 20 hours after administration of the dose (Table 6; Broadmeadow, 2018a,b).

2.2 Genotoxicity

The results of studies on the genotoxicity of fenpyroximate as summarized by JMPR in 2017, are shown in Table 7. The JMPR Meeting in 2017 (FAO, 2017) concluded that no evidence was found of genotoxicity due to fenpyroximate based on an adequate range of assays, both in vitro and in vivo.

Table 7. Overview of genotoxicity tests with fenpyroximate from JMPR 2017

End-point	Test object	Concentration range	Purity	Result	Reference
		In vitro			
Bacterial gene mutation assay	*Salmonella typhimurium* TA 98, TA 100, TA 1535, TA 1537 and TA 1538 *Escherichia coli* WP2*uvrA*	± *metabolic activation (S9):* 50, 158, 500, 1580 and 5000 µg/plate in DMSO	97.3%	Negative	May, 1988

End-point	Test object	Concentration range	Purity	Result	Reference
Mammalian cytogenetic test	Chinese hamster V79 lung cells	± *metabolic activation (S9):* 3, 10, 30, 100 and 330 µg/mL in acetone	97.3%	Negative	Hodson-Walker, 1988a
Mammalian cell gene mutation	Human lymphocytes	± *metabolic activation (S9):* 1.25, 5, and 20 µg/mL in acetone	97.3%	Negative	Hodson-Walker, 1988b
Mammalian cytogenetic assay	Mouse lymphoma L5178Y tk+/−	± *metabolic activation (S9):* 0.059–30 µg/mL[a] in 10% v/v sodium CMC	99.2%	Negative	Kajiwara, 2016a
DNA repair test	*Bacillus subtilis* (H17 (rec+), M45 (rec−))	± *metabolic activation (S9):* 10, 20, 50, 100, 200 and 500 µg/disk in DMSO	97.3%	Negative	Watanabe, 1988
Unscheduled DNA synthesis	Rat hepatocytes	0.025, 0.051, 0.102, 0.255, 0.509 and 1.02 µg/mL in DMSO	97.3%	Negative	Cifone, 1989
		In vivo			
Micronucleus test in mice	CD-1 mice	80, 400 or 2000 mg/kg bw in 0.5% methylcellulose	97.3%	Negative	Hodson-Walker, 1988c

CMC: Carboxymethyl cellulose; bw: Body weight; DMSO: Dimethyl sulfoxide; Source: FAO, 2017

S9: 9000 × *g* supernatant fraction from induced rats (metabolic activation);

tk: Thymidine kinase locus; v/v: volume for volume; w/v: weight for volume

[a] At 3 hours, −S9: 0, 0.059, 0.12, 0.23, 0.47, 0.94, 1.88, 3.75, 7.5, 15 and 30 µg/mL;
At 3 hours, +S9: 0, 0.12, 0.23, 0.47, 0.94, 1.88, 3.75, 7.5, 15 and 30 µg/mL;
At 24 hours –S9: 0, 0.029, 0.059, 0.12, 0.23, 0.47, 0.94, 1.88, 3.75, 7.5 and 15 µg/mL

2.3 Special studies

(a) General pharmacology

General pharmacological parameters were assessed with fenpyroximate in mice, rats, rabbits and Guinea pigs using oral administration and in vitro experiments.

Effects on central nervous system

When mice were orally administered the test substance at 100 mg/kg bw per day or 1000 mg/kg bw per day (in 1% Tween 80 aqueous solution), the following observations were made: lower reactivity, alertness, pain response, righting reflex, grip strength, limb and abdominal tone, ipsilateral flexor reflex; increased passivity, abnormal body posture, palpebral drooping; decreased spontaneous motor activity. In the 1000 mg/kg bw per day dose group, some animals deaths also occurred. No effects were noted at 10 mg/kg bw per day.

When rabbits were orally administered the test substance at 100 or 300 mg/kg bw per day (1% Tween 80 aqueous solution), lower body temperature and deaths were noted. No effects were seen at 30 mg/kg bw per day.

When rabbits were intravenously administered the test substance at 0.3 mg/kg bw per day, electroencephalograms showed a temporal awake pattern. At 0.5 mg/kg bw per day, the electroencephalograms showed high amplitude and slow waves after the temporal awake pattern and flattening occurred gradually. The animals died subsequently. No effects were noted at 0.1 mg/kg bw per day and under.

Effects on respiration and circulatory systems

When rats or rabbits were intravenously administered the test substance at 0.1, 0.3 or 1.0 mg/kg bw per day (in rats) and 0.03, 0.1, 0.3 or 0.5 mg/kg bw per day (sequentially in rabbits), lower blood pressure

and heart rate, and electrocardiogram abnormalities were observed at 1 mg/kg bw in rats and 0.5 mg/kg bw in rabbits. Such respiratory excitation was observed for a short while, transitioned to inhibition and subsequently returned to normal. At 0.3 mg/kg bw per day, lower blood pressure and heart rate, changes in electrocardiogram, and respiratory excitation were observed. At 0.1 mg/kg bw per day, lower diastolic blood pressure was noted in rabbits only. No effects were noted at 0.03 mg/kg bw per day.

Effects on the autonomic nervous system

In isolated ileum from Guinea pigs and isolated uterus from rats, inhibition of contraction, which was considered a direct effect on the muscles, was noted at 1×10^{-8} g/mL and 1×10^{-7} g/mL, respectively.

Effects on digestive organs

When mice were orally administered the test substance at 1000 mg/kg bw per day, lower transport activity in the small intestine and deaths were noted. No effects were noted at 100 mg/kg bw per day and below.

Effects on skeletal muscle

When rats were intravenously administered the test substance at 1.0 mg/kg bw per day, reduced muscle contraction was observed in sciatic nerve–gastrocnemius muscle specimens. No effects were noted at 0.3 mg/kg bw per day and below.

Effects on blood

No blood clotting and haemolysis effects were noted even at 1×10^{-7} g/mL.

Effects on isolated mitochondria in rat liver

Inhibition of electron transport Complex I was observed.

Based on the above, the effects of the test substance inhibited the electron transport system of mitochondria lowered body weight, temperature, blood pressure and heart rate, caused changes in electrocardiograms and respiratory excitation, and caused non-specific effects on biological functions at the lethal dose and dose levels close to the lethal dose (Uchida, 1990).

(b) Studies on metabolites

Genotoxicity

The results of genotoxicity studies with fenpyroximate metabolites are summarized in Table 8 (FAO, 2017).

Table 8. Overview of genotoxicity tests with fenpyroximate metabolites

End-point	Test object	Concentration range	Purity	Result	Reference
		Metabolite M-1: in vitro			
Bacterial gene mutation assay	*Salmonella typhimurium* TA 98, TA 100, TA 1535 and TA 1537 *Escherichia coli* WP2*uvrA*	± *metabolic activation (S9):* 0, 313, 625, 1250, 2500 and 5000 µg/plate in DMSO	99.7%	Negative	Watanabe, 1990
Mammalian cytogenetic test	Chinese hamster V79 lung cells	6 h –S9: 0–2.5 µg/mL 6 h +S9: 0–40 µg/mL 24 h –S9: 0–0.7 µg/mL Vehicle: DMSO Precipitation at 62.5 µg/mL	99.5%	Negative	Kajiwara, 2016b

End-point	Test object	Concentration range	Purity	Result	Reference
Mammalian cytogenetic assay	Mouse lymphoma L5178Y $tk^{+/-}$	3 h –S9: 0–30 µg/mL 3 h +S9: 0–70 µg/mL 24 h –S9: 0–1.5 µg/mL Vehicle: DMSO	99.5%	Negative	Kajiwara, 2016c
		Metabolite M-1: in vivo			
Mouse micronucleus	CD-1 male mice	0.5, 1.0 and 1.5 mg/kg bw i.v. in saline containing 2.5% v/v dimethylacetamide and Cremophor EL®	99.8%	Negative	Tsukushi, 2016
		Metabolite M-3: in vitro			
Bacterial gene mutation	*Salmonella typhimurium* TA 98, TA 100, TA 1535 and TA 1537 *E. coli* WP2*uvrA*	± *metabolic activation (S9):* 0, 156, 313, 625, 1250, 2500 and 5000 µg/plate in DMSO	97.9%	Negative	Enomoto, 2016
Mammalian cytogenetic assay	Chinese hamster V79 lung cells	6 h –S9: 0–275 µg/mL 6 h +S9: 0–150 µg/mL 24 h –S9: 0–150 µg/mL Vehicle: DMSO Precipitation at 500 µg/mL	97.9%	Negative	Kajiwara, 2016d
Mammalian cytogenetic assay	Mouse lymphoma L5178Y $tk^{+/-}$	3 h –S9: 0–1000 µg/mL 3 h +S9: 0–1000 µg/mL 24 h –S9: 0–250 µg/mL Vehicle: DMSO	97.9%	Negative	Munechika, 2016
		Metabolite M-12: in vitro			
Bacterial gene mutation	*Salmonella typhimurium* TA 98, TA 100, TA 1535 and TA 1537 *E. coli* WP2*uvrA*	± *metabolic activation (S9):* 0, 313, 625, 1250, 2500 and 5000 µg/plate in DMSO	98.5%	Negative	Watanabe, 1989a

i.v.: Intravenous; bw: Body weight; DMSO: Dimethyl sulfoxide; v/v: Volume for volume Source; FAO, 2017

S9: 9000 × *g* supernatant fraction from induced rats (metabolic activation); *tk*: Thymidine kinase locus;

M-1: *tert*-butyl (*Z*)-4-[(1,3-dimethyl-5-phenoxypyrazole-4-yl) methylene-ammooxymethyl] benzoate;

M-3: *E*-4-[(1,3-dimethyl-5-phenoxypyrazol-4-yl)-methyleneaminooxymethyl] benzoic acid;

M-12: *tert*-butyl (*E*)-4-[(3-methyl-5-phenoxypyrazole-4-yl) methylene-ammooxymethyl]benzoate;

The Registrant provided sensitivity data regarding the S9 fraction prepared by the testing facility for metabolites M-1 and M-12 using *Salmonella typhimurium* strains TA 98, and TA 100, employing 7,12-dimethylbenz[α]anthracene (DMBA) as well as 2-aminoanthracene (2-AA) as positive controls.

The results indicate that the number of revertant colonies induced by DMBA and 2-AA in both assays was much greater than the historical control data for the solvent, indicating that that the S9 fractions used for M-1 and M-12 were sensitive to the known mutagens DMBA and 2-AA (Matsumoto, 1989).

A statistical analysis was performed to evaluate the data from the previously conducted in vitro chromosomal aberration study by Kajiwara (2016d) with M-3. A statistical analysis (Fisher's exact probability test) was performed to detect differences in the incidence of aberrant cells between the negative control and each of the test substance treatment groups, and positive control, for each condition, with and without S9 mix and for the 24-hour assay. Statistically significant differences were evaluated at a two-tailed significance level of 5%.

No significant difference was detected in the incidence of aberrant cells between the negative control value and any test substance treatment group. A significant difference was detected in the incidence of structurally aberrant cells between the negative control value and the positive controls (Kajiwara, 2018).

The genotoxicity studies summarized in Table 9 were conducted with metabolite M-1, (fenpyroximate *Z*-isomer).

Table 9. Genotoxicity study with metabolite M-1

End-point	Test object	Concentration range	Purity	Result	Reference
		Metabolite M-1: in vivo			
Mouse micronucleus test: (range-finding study)	ICR mice (male and female)	0, 0.75, 1.5 and 3.0 mg/kg bw/day (i.p.) daily for 2 consecutive days in (w/v) 1% CMC and 0.1% Tween 80	99.8%	Negative[a]	Tsukushi, 2019a
Mouse micronucleus test: (main study)	ICR mice (male)	0, 75, 150 and 300 mg/kg bw/day (gavage) daily for 2 consecutive days in (v/v) 2.5% dimethylacetamide and 2.5% Cremophor EL®	97.51%	Negative[b]	Tsukushi, 2019b
Comet assay (range-finding study)	Crl:CD (SD) rats (male)	0, 1.04, 1.25, 1.5 and 1.80 mg/kg bw/day (i.v.) daily for 2 consecutive days in 10% dimethylacetamide and 10% Cremophor EL®	99.8%	Negative[c]	Tsukushi, 2019c
Comet assay: (main study)	Crl:CD (SD) rats (male)	0, 0.45, 0.90 and 1.80 mg/kg bw/day (i.v.) daily for 2 consecutive days in 10% dimethylacetamide and 10% Cremophor EL®	99.8%	Negative[d]	Tsukushi, 2017a, b

CMC: Carboxymethyl cellulose; i.p.: Intraperitoneally; i.v.: Intravenously; bw: body weight;

DMSO: Dimethyl sulfoxide; v/v: Volume for volume w/v: Weight for volume;

S9: 9000 × *g* supernatant fraction from induced rats (metabolic activation)

[a] One male was found dead at 10 minutes after the first dosing, and two males were killed in extremis at 20 minutes after the first dosing with 3.0 mg/kg bw per day of the test substance. These animals showed abdominal position or morbidity in clinical observation. Clinical signs of toxicity at 1.5 mg/kg bw per day.

[b] At 300 mg/kg bw per day, no death, but the hypopnea was observed after second dosing. At 150 mg/kg bw per day, one animal was dead after first dosing. In the 75 mg/kg bw per day group, there were no changes related to the test substance treatment.

[c] Severe clinical signs such as abdominal position, dyspnoea, irregular respiration, and decreased locomotor activity were observed within 20 minutes after each dosing in the animals treated at 1.50 and 1.80 mg/kg bw of the test substance.

[d] Clinical signs of toxicity such as abdominal position and abnormal respiration were observed and ceased within 30 minutes at 1.80 mg/kg bw per day. Decreased motor activity was seen after administration at 0.90 mg/kg bw per day, and no signs were apparent at 0.45 mg/kg bw per day.

In silico prognoses for fenpyroximate (parent) and a series of impurities (NN-4, NN-9, NN-10, NN-12, NN-13 and NN-14) was carried out using the toxicity prediction models Derek Nexus and Toxtree. Additionally, the Organisation for Economic Co-operation and Development (OECD) QSAR Toolbox v.4.1 was used to profile the chemicals through its general mechanistic and end-point specific profilers.

The outcome of the in silico predictions for parent and impurities did not activate toxicologically meaningful alerts in any of the predictive tools (Suraez-Rodrigez & Fowler, 2018).

(c) Studies on impurities of fenpyroximate

1,3-dimethyl-5-phenoxy-4-pyrazolecarbaldehyde oxime (NH-4)

In an acute oral toxicity study, Sprague Dawley rats (five/sex per dose) were administered a single gavage dose of NH-4 (purity 98.7%) at 0, 444, 667, 1000, 1500 2250 and 3375 mg/kg bw in an aqueous solution of 0.5% CMC and 0.1% Tween 80. Clinical signs of overt toxicity were observed at 0.25, 0.5,

1, 3 and 6 hours after dosing, then at least once daily thereafter for 14 days. Body weights were recorded before treatment and on days 3, 7 and 14 after treatment. Animals were autopsied when found dead.

The mortality in males was as follows: all five rats died at 1500 mg/kg bw, four rats died at 3375 mg/kg bw, three rats each died at 2250 and 1000 mg/kg bw, and two rats died at 667 mg/kg bw. The mortality in females was as follows: all five rats died at 2250 mg/kg bw, four rats died at 1500 mg/kg bw, three rats died at 1000 mg/kg bw, and one rat each died at 667 and 444 mg/kg bw. General observations showed that reduced spontaneous movement, ataxic gait, and lying on side occurred. Furthermore, tonic-clonic convulsions were observed in the rats which died within 15 minutes of administration. Signs disappeared by the second day after administration. The body weight gain for the male rats that received 2250 mg/kg bw or more and of the female rats that received 1500 mg/kg bw was suppressed for three days after administration, but the increased normally thereafter. At autopsy after the end of the 14-day observation period, a splenic abscess was observed in one male rat that received 444 mg/kg bw, and grey white spots on the liver were observed in one female rat that received 296 mg/kg bw. Pulmonary congestion, brownish-yellow spots on the lung, and bubbles in the trachea were observed in the rats that died during the observation period.

Under the study conditions, the acute oral LD_{50} for NH-4 in male rats was 1016 mg/kg bw (95% confidence interval: 412–1833 mg/kg bw) For females the LD_{50} for NH-4 was 881 mg/kg bw (95% confidence interval: 615–1277 mg/kg bw) (Mitsubishi-Kasei Institute, 1990a).

A reverse mutation test using *Salmonella typhimurium* strains TA 1535, TA 1537, TA 1538 and TA 100 and *Escherichia coli* WP2 *uvrA* was conducted for NH-4 (purity 98.7%) in the presence and absence of metabolic activation taken from Sprague Dawley rat S-9 liver homogenate . The test concentrations ranged from 0 to 5000 µg/plate in DMSO.

No increase in the number of revertant colonies was observed for the test substance in the presence or absence of metabolic activations.

Under the study conditions, NH-4 was considered to have no reverse mutagenic potential (Ohtsuka, 1989).

tert-Butyl 4-chloromethylbenzoate (NN-9)

In an acute oral toxicity study, Sprague Dawley rats (five/sex per dose) were administered single gavage doses of NN-9 (purity 97.9%) at 0, 4000, 4400, 4800, 5300, 5900 or 6400 mg/kg bw. in aqueous solution of 0.5% CMC and 0.1% Tween 80. Clinical signs of overt toxicity were observed at 0.5, 1, 3 and 6 hours after dosing and then at least once daily thereafter for 14 days. Body weights were recorded before treatment and on days 3, 7 and 14 after treatment. Animals were autopsied when found dead.

The mortality in males was as follows: two rats died at 4400 mg/kg bw, one rat each died at 4800 and 5300 mg/kg bw, and four rats died at 6400 mg/kg bw. The mortality in females was as follows: one rat died at each of 4800 and 5300 mg/kg bw, and all five rats died at 6400 mg/kg bw. General observations showed that reduced spontaneous movement, emaciation, anaemia, incontinence of urine, and crouching occurred. Reduced spontaneous movement was observed from the day of administration, and emaciation was observed from three days after administration. These signs, however, disappeared by the tenth day after administration. Decrease in body weight and suppression of body weight gain in both sexes that received 4400 mg/kg bw or more were observed from three days after administration until seven days after administration, but body weight increased normally thereafter. At autopsy after the end of the 14-day observation period, slight atrophy of the thymus was observed in one male rat that received 6400 mg/kg bw, and discolouration of the liver was observed in one female rat that received 4400 mg/kg bw. Focal or diffuse haemorrhage of the lungs, thymus and kidney, bloody fluid in the thorax, atrophy of the thymus and spleen, congestion of the liver, focal haemorrhage in the gastric wall and in the intestinal mucosa were observed in the rats found dead during the observation period.

Under the study conditions, the acute oral LD_{50} for NN-9 in male rats was 5609 mg/kg bw (95% confidence intervals 4767–6599 mg/kg bw). For females the LD_{50} for NN-9 was 5972 mg/kg bw (95% confidence intervals 5464–7518 mg/kg bw) (Mitsubishi-Kasei Institute, 1990b).

The results of genotoxicity studies for NN-9 are summarized in the Table 10 below.

Table 10. Results of the genotoxicity study with fenpyroximate impurity NN-9

Test system	Test object	Concentration range	Purity	Result	Reference
		Metabolite NN-9: in vitro			
Bacterial reverse mutation assay	*Salmonella typhimurium* strains TA 100, TA1535, TA1537 and TA1538 *E. coli* WP2*uvrAp*	*With and without metabolic activation:* 0, 1, 4, 20, 100, 500 and 5000 µg/plate, in DMSO	99.6	Weak positive	Ohtsuka, 1989
Chromosomal aberration test	Chinese hamster lung fibroblasts	*Without metabolic activation:* 0, 1.33, 4.0 and 12 µg/mL *With metabolic activation:* 0, 10, 30 and 100 µg/mL ± S9 both in DMSO	99.6	Negative	Kajiwara, 1989a
		Metabolite NN-9: in vivo			
Mouse bone marrow micronucleus test	ICR-CD1 mice (male)	0, 750, 1500 and 3000 mg/kg bw/day (oral gavage) in aqueous solution of 0.5% CMC and 0.01% Tween 80	99.6	Negative	Kajiwara, 1989b

DMSO: Dimethyl sulfoxide; CMC; Carboxylmethyl cellulose

Metabolite NN-10, 1,3-dimethyl-5-phenoxy-4-pyrazolecarbonitrile

In an acute oral toxicity study, Sprague Dawley rats (five/sex per dose) were administered a single gavage dose of NN-10 (purity 99.9%) at 0, 1300, 1800, 2600, 3600, 4300 or 5000 mg/kg bw for males and 0, 1800, 2600, 3600, 5000 or 7000 mg/kg bw for females The metabolite was administered in aqueous solution of 0.5% CMC and 0.1% Tween 80. Observations for overt clinical signs of toxicity were made at 0.5, 1, 3 and 6 hours after dosing, then at least once daily thereafter for 14 days. Body weights were recorded before treatment and then on days 3, 7 and 14. Animals were autopsied when found dead.

The mortality in males was as follows: one rat died at 3600 mg/kg bw, and all five rats died at both 4300 and 5000 mg/kg bw. The mortality in females was as follows: one rat died at 3600 mg/kg bw, and three rats died at each of 5000 and 7000 mg/kg bw. All of the dead rats were found between the day of administration and day 3 after administration. General observations showed that reduced spontaneous movement, ataxic gait, crouching, lying on side, gasping, salivation, and clonic convulsion occurred. These signs disappeared by the day 3 after administration. The body weight gain in female rats that received 3600 or 5000 mg/kg bw was suppressed for three days after administration, but body weight increased normally thereafter. Pulmonary congestion, scattered focal haemorrhage of the thymus, discolouration of the liver, scattered petechial haemorrhage in the gastric wall, distension of the stomach and intestine with gas, retention of the test substance in the stomach, atrophy of the spleen, dark red urine in the bladder, and focal haemorrhage in the wall of the urinary bladder were observed in rats that died during the observation period. Macroscopic findings were comparable between controls and rats killed at the end of observation period.

Under the study conditions, the acute oral LD_{50} for NN-10 in male rats was 3741 mg/kg bw (95% confidence interval 3558–3934 mg/kg bw) and for females 4539 mg/kg bw (95% confidence interval 4089–5039 mg/kg bw) (Mitsubishi-Kasei Institute, 1990c)

A reverse mutation test using *Salmonella typhimurium* strains TA 1535, TA 1537, TA 100, TA 98 and *Escherichia coli* WP2 *uvrA* was conducted for NN-10 (purity 99.9%) in the presence and absence of metabolic activation from Sprague Dawley rat S9 liver homogenate. The test concentrations ranged from 0 to 5000 µg/plate in DMSO.

No increase in the number of revertant colonies was observed for the test substance, neither in the presence nor absence of metabolic activation.

Under the study conditions NN-10 was considered to have no reverse mutagenic potential (Watanabe, 1989b).

Metabolite NN-14,
tert-*Butyl* E-*4-[(5-chloro-1,3-dimethylpyrazol-4-yl)methyleneaminooxymethyl]benzoate*

In an acute oral toxicity study, Sprague Dawley rats (5/sex per dose) were administered single gavage dose of NN-14 (purity 99.9%) at 0 or 7000 mg/kg bw in aqueous solution with 0.5% CMC and 0.1% Tween 80. Observations for overt clinical signs of toxicity were made at 0.5, 1, 3 and 6 hours after dosing and then at least once daily thereafter for 14 days. Body weights were recorded before treatment and on days 3, 7 and 14. Animals were autopsied when found dead.

No treatment-related clinical signs were observed. No mortality occurred during the study. Body weights increased normally except for one female rat that showed depressed body weight for the first three days, but which increased normally thereafter. No changes were observed at necropsy.

Under the study conditions the acute oral LD_{50} for NN-14 in rats was > 5000 mg/kg bw (Mitsubishi-Kasei Institute, 1990d).

A reverse mutation test using *Salmonella typhimurium* strains TA 1535, TA 1537, TA 100, TA 98 and *Escherichia coli* WP2 *uvrA* was conducted for NN-14 (purity 99.7%) in the presence and absence of metabolic activation from Sprague Dawley rat S-9 liver homogenate . The test concentrations ranged from 0 to 5000 µg/plate in DMSO.

No increase in the number of revertant colonies was observed for the test substance, neither in the presence nor absence of metabolic activation.

Under the study conditions, NN-14 was considered to have no reverse mutagenic potential (Watanabe, 1989c).

3. Observations in humans

No new data available.

Comments

Biochemical aspects

In a published study, a metabolite which had not been previously identified was found when liver microsomes were incubated with fenpyroximate; this was referred to as R-UL-1 (1-hydroxymethyl-1-methylethyl (*E*)-α-(1,3-dimethyl-5-phenoxypyrazol-4-yl)-methyleneaminooxy)-*p*-toluate). The metabolite R-UL-1 (detected during an early phase in vitro study) is an unstable intermediate, especially in relation to butyl ester oxidation. The metabolite R-UL-1 converts to:
2-hydroxy-2-methylpropyl (*E*)-α-([1,3-dimethyl-5-phenoxypyrazol-4-yl]methyleneaminooxy)-*p*-toluate under neutral and basic conditions.

In a published study, it was suggested that the selectivity of fenpyroximate for spider mites as against non-target organisms, especially mammals, could be attributable to a species-specific detoxification, described as ester hydrolysis via microsomal hydroxylation, followed by intramolecular transesterification (Motobo et al., 2000).

Toxicological data

In repeat-dose toxicity studies, bradycardia, diarrhoea and decreased body weight in dogs, and decreased body weight and liver effects in rats, were the key findings.

In a 13-week toxicity study in rats using dietary fenpyroximate concentrations of 0, 20, 100 or 500 ppm (equal to 0, 1.3, 6.57 and 35.2 mg/kg bw per day for males, 0, 1.65, 8.29 and 38.6 mg/kg bw per day for females) the NOAEL was 20 ppm (equal to 1.3 mg/kg bw per day) based on decreased body weight and body weight gains, and reduced feed consumption in both sexes at 100 ppm (equal to 6.57 mg/kg bw per day) (Aughton, 1987).

In a single-dose oral toxicity study in dogs, fenpyroximate was administered as a bolus gavage dose of 0, 2 or 5 mg/kg bw. As diarrhoea was seen at all dose levels, the NOAEL could not be identified (Harvey, 2006a).

In a dose escalation oral toxicity study in the same dogs, fenpyroximate was administered via bolus gavage dose of 2, 5, 20 and 25 mg/kg bw. The NOAEL was 2 mg/kg bw per day based on diarrhoea seen at 5 mg/kg bw per day (Harvey, 2006b).

In a 90-day oral toxicity study in dogs using oral (capsule) fenpyroximate doses of 0, 2, 10 or 50 mg/kg bw per day, the LOAEL was 2 mg/kg bw per day, the lowest dose tested, based on slight bradycardia and an increased incidence of diarrhoea in both sexes (weekly observations), and in females only, reduced feed consumption, body weight and body weight gain and increased emaciation and torpor (Broadmeadow, 1989a).

In a one-year oral toxicity study in dogs using oral (capsule) fenpyroximate doses of 0, 0.5, 1.5, 5.0 or 15.0 mg/kg bw per day, the NOAEL was 0.5 mg/kg bw per day based on bradycardia observed in male dogs at the LOAEL of 1.5 mg/kg bw per day (Broadmeadow, 1989b).

Re-examination of gastrointestinal tract tissues from a single-bolus study by Harvey in 2006 showed no microscopic findings that suggested local or systemic fenpyroximate-related effects had been noted in the oesophagus or gastrointestinal tract following the administration of a 2 or 5 mg/kg bw single-bolus gavage dose to beagle dogs (Walker, 2018).

Fenpyroximate was tested for genotoxicity in an adequate range of assays, both in vitro and in vivo. No evidence of genotoxicity was found.

The 2017 Meeting concluded that fenpyroximate was unlikely to be genotoxic, and this was confirmed by the 2021 Meeting.

A special study conducted in mice, rats, rabbits and guinea pigs aimed at evaluating pharmacological parameters with fenpyroximate indicated that the test substance inhibited the electron transport system of mitochondria, lowered body weight, temperature, blood pressure and heart rate, caused changes in electrocardiograms and respiratory excitation, and caused non-specific effects on biological functions at the lethal dose and dose levels close to the lethal dose (Uchida, 1990).

Toxicological data on metabolites and/or degradates

Metabolite M-1

Fenpyroximate metabolite M-1 (the *Z*-isomer) was negative for mutagenicity according to in vivo mouse micronucleus assay (Tsukushi, 2019a, b) and comet assay (Tsukushi, 2019a, b).

Metabolite NH-4

The fenpyroximate impurity, 1,3-dimethyl-5-phenoxy-4-pyrazolecarbaldehyde oxime (NH-4), was shown to have an LD_{50} in rats of 881 mg/kg (Misubushi-Kasei Institute, 1990a), and was negative in the reverse mutation assay (Ohtsuka, 1989).

Metabolite NN-9

The fenpyroximate impurity, *tert*-butyl 4-chloromethylbenzoate (NN-9), was shown to have an acute oral LD_{50} in rats of > 5000 mg/kg bw (Misubushi-Kasei Institute, 1990b). It showed weak positive mutagenicity in reverse mutation assays (Ohtsuka, 1989), was negative in the chromosomal aberration test (Kajiwara, 1989a), and negative in the in vivo mouse micronucleus assay (Kajiwara, 1989b).

Metabolite NN-10

The fenpyroximate impurity, 1,3-dimethyl-5-phenoxy-4-pyrazolecarbonitrile (NN-10), was shown to have an acute oral LD_{50} in rats of 3741 mg/kg bw (Misubushi-Kasei Institute, 1990c). It was negative in bacterial reverse mutation assays (Watanabe, 1989a).

Metabolite NN-14

The fenpyroximate impurity, *tert*-butyl (*E*)-4-[(5-chloro-1,3-dimethylpyrazol-4-yl)methylene amino oxymethyl]benzoate (NN-14), was shown to have an acute oral LD_{50} in rats of > 5000 mg/kg bw (Misubushi-Kasei Institute, 1990d) and to be negative in the bacterial reverse mutation assay (Watanabe, 1989b).

Metabolite R-UL-1

Metabolite R-UL-1 was not identified in rat metabolism studies. It is plausible that Fen-OH and/or R-UL-1 occur as unstable intermediate metabolites in the rat when the parent is oxidized at its *t*-butyl moiety to give M-22, or hydrolysed at its *t*-butyl moiety to give M-3 (Nishizawa et al., 1993). No toxicological data were available on Fen-OH or R-UL-1, but an additional hydroxy group usually renders a molecule more likely to undergo further phase II metabolism and be more rapidly excreted.

The Meeting concluded that the toxicity of plant and livestock metabolites M-1, M-3, M-5, M-21, M-22, Fen-OH and R-UL-1 would be covered by the acceptable daily intake (ADI) established for fenpyroximate.

Microbiological data

No data was available.

Human data

No data was available.

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

Toxicological evaluation (addendum)

The Meeting withdrew the previous ARfD and established a new ARfD of 0.005 mg/kg bw on the basis of a NOAEL of 0.5 mg/kg bw per day, with a LOAEL of 1.5 mg/kg bw per day for bradycardia seen in males in a one-year study of toxicity in dogs. A safety factor of 100 was used.

Although it was not clear from the study design whether or not the bradycardia already occurs after a single dose, the effect was used as the basis for an ARfD in a cautious and conservative approach.

The new ADI and ARfD can be applied to M-1, M-3, M-5, M-21, M-22, Fen-OH and R-UL-1.

Addendum to levels relevant to risk assessment of fenpyroximate

Species	Study	Effect	NOAEL	LOAEL
Dog	One-year study of toxicity[a]	Toxicity	0.5 mg/kg bw per day	1.5 mg/kg bw per day[c]

[a] Capsule administration

Acceptable daily intake (ADI) applies to fenpyroximate and to M-1, M-3, M-5, M-21, M-22, Fen-OH and R-UL-1, expressed as fenpyroximate

0–0.005 mg/kg bw

Acute reference dose (ARfD) applies to fenpyroximate and to M-1, M-3, M-5, M-21, M-22, Fen-OH and R-UL-1, expressed as fenpyroximate

0.005 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.

Addendum to critical end-points for setting guidance values for exposure to fenpyroximate

Short-term studies of toxicity	
Target/critical effect	Bradycardia (dog)
Lowest relevant oral NOAEL	0.5 mg/kg bw per day (dog)
Genotoxicity	No evidence of genotoxicity
Studies on toxicologically relevant metabolites	
M-1	Ames test, mammalian cytogenetic assay in Chinese hamster lung cells, mouse lymphoma assay, micronucleus test and comet assay: all negative

Summary

	Value	Study	Safety factor
ADI	0–0.005 mg/kg bw[a]	One-year study of toxicity in dogs	100
ARfD	0.005 mg/kg bw[a]	One-year study of toxicity in dogs	100

[a] Applies to fenpyroximate and to M-1, M-3, M-5, M-21, M-22, Fen-OH and R-UL-1, expressed as fenpyroximate

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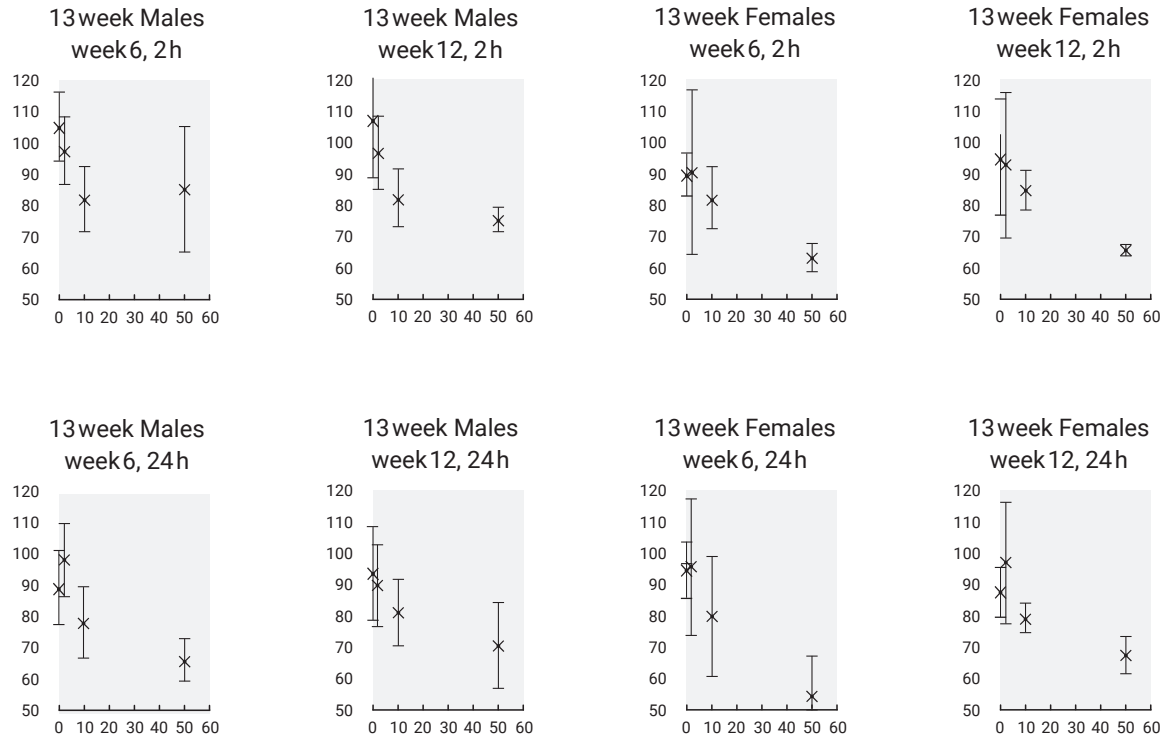
Appendix 1. Detailed data from toxicity studies on dogs by Broadmeadow, 1989

Thirteen-week dog study

Heart rate of males and females ($n = 4$) was measured 2 h and 24 h after dosing, before dosing period and at weeks 6 and 12.

For each animal the heart rate was calculated as a percentage of its prestudy rate. The plots depict the average group heart rate expressed as a percentage of the heart rate before the dosing period.

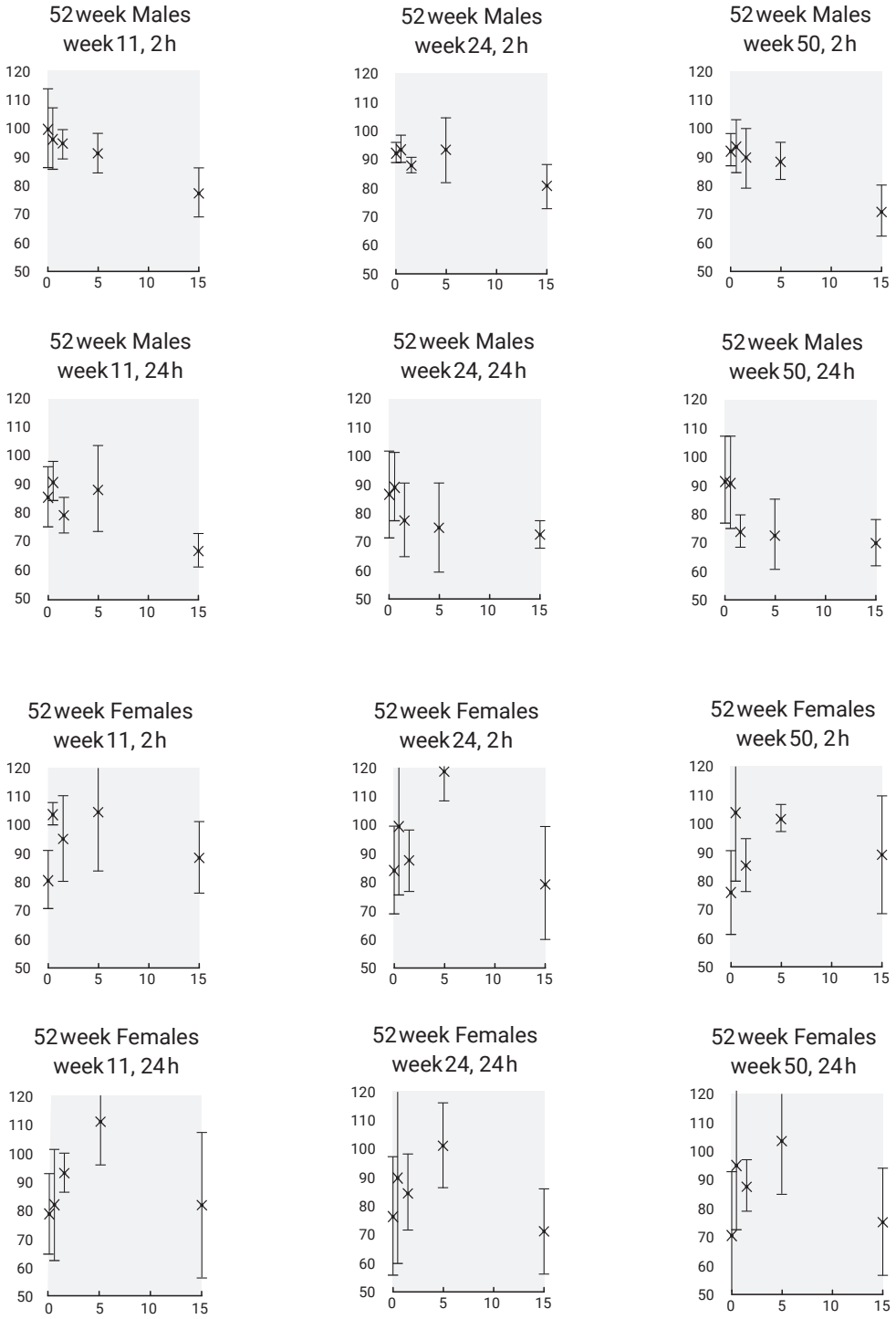


(*Redrawn from Broadmeadow, 1989*)

Fifty-two week dog study

Heart rate of males and females ($n=4$) was measured 2 h and 24 h after dosing before dosing period and at weeks 6 and 12.

For each animal, the heart rate was calculated as a percentage of its prestudy rate. The plots depict the average group heart rate expressed as a percentage of the heart rate before the dosing period.



(*Redrawn from Broadmeadow, 1989*)

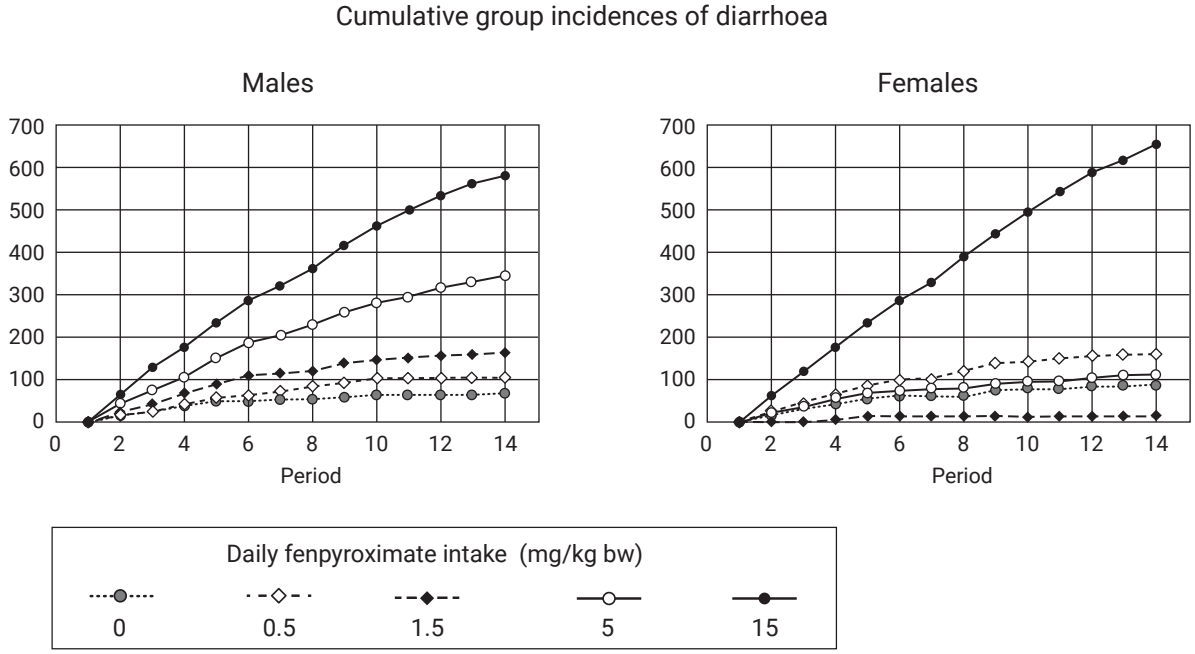
In the 13 weeks study, males have lower heart rates at all dose levels compared to their prestudy heart rate, females only at 10 and 50 mg/kg bw.

Conclusion

Regarding heart rate effects, the both studies are quite consistent. Males are more sensitive than females and the effect level is somewhere > 0.5 and < 2 mg. No earlier time point of measurement than 6 weeks is available; therefore, it could be argued that the effect could be an immediate pharmacological effect also related to the diarrhoea.

Fifty-two week dog study diarrhoea

The two plots below depict the dose group cumulative diarrhoea events (y-axis) per period (x-axis)



(*Redrawn from Broadmeadow, 1989*)

Conclusion

In males, 1.5 mg is a quite clear response level. In females, only the 15 mg dose is a clear response level.

Fipronil

First draft prepared by
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Explanation

Fipronil (M&B 46030) is the International Organization for Standardization (ISO)–approved name for 5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(*RS*)-(trifluoromethyl)sulfinyl]-1H-pyrazole-3-carbonitrile (IUPAC), for which the Chemical Abstracts Service number is 120068-37-3.

Fipronil was previously evaluated by the FAO/WHO Joint Meeting on Pesticide Residues (JMPR) in 1997 and 2000. It was reviewed by the present Meeting as part of the periodic review programme of the Codex Committee on Pesticide Residues (CCPR).

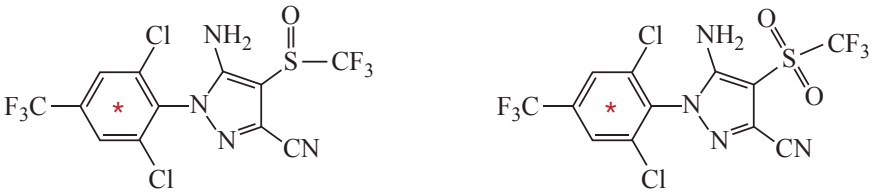
All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with current test guidelines, unless otherwise noted. Additional information from a literature search was identified that complemented the toxicological information submitted for the current assessment.

Evaluation for acceptable daily intake

1. Biochemical aspects

The structures of fipronil and its major metabolite fipronil-sulfone are shown in Fig. 1

Figure 1. Fipronil and fipronil sulfone (M&B 46136)



* phenyl ring uniformly labelled with ^{14}C

Fipronil is a broad-spectrum insecticide and acaricide of the fiprole (phenylpyrazole) group. It acts in the nervous system as a γ-aminobutyric acid, type A glutamate and glycine receptor-gated ($GABA_A$-gated) chloride channel blocker. Its actions at receptor-gated chloride channels are responsible for its neurotoxicity. Fipronil displays selective toxicity towards invertebrates due to its higher affinity for $GABA_A$-gated chloride channels in these animals, its affinity for the glutamate-gated chloride channels and metabolic and kinetic differences between mammals and invertebrates.

Fipronil was first evaluated by JMPR in 1997, when an acceptable daily intake (ADI) of 0–0.000 2 mg/kg body weight (bw) and an acute reference dose (ARfD) of 0.003 mg/kg bw (for fipronil and fipronil-desulfinyl, expressed as fipronil) was established based on the neurotoxicity of these chemicals. The 1997 Meeting concluded that toxicity of the other mammalian metabolites of fipronil was comparable with, or less than, that of fipronil. Because the photodegradation product fipronil-desulfinyl was of toxicological concern and not a mammalian metabolite of fipronil it was reviewed separately. A temporary ADI of 0–0.000 03 mg/kg bw was established for fipronil-desulfinyl. At JMPR 2000, after considering additional data, a group ADI was established of 0–0.000 2 mg/kg bw for fipronil and fipronil-desulfinyl, alone or in combination. The group ARfD, established by the 1997 JMPR, of 0.003 mg/kg bw for fipronil and fipronil-desulfinyl, alone or in combination, was reaffirmed.

Fipronil was reviewed by the Meeting in 2021 as part of the periodic review programme of the CCPR. For the 2021 periodic review, data on dermal absorption, disposition and kinetics, genotoxicity, repeat dose toxicology, effects on the thyroid, reproductive toxicity, metabolites, photometabolites, high-throughput screening and peer-reviewed published literature were supplied. An updated literature search was also performed. All studies were evaluated and, where appropriate, summarized below.

The submitted data was of sufficient breadth and quality for evaluation with all critical studies being compliant (except as noted) with good laboratory practices (GLP). The purity of fipronil in all the test articles used was > 95%. All studies were validated by the appropriate use of positive and negative controls.

1.1 Absorption, distribution and excretion

(a) Oral route

Rat

Young adult male and female Sprague Dawley (SD) rats were given a single oral gavage dose of 4 or 131.4 mg/kg bw of [^{14}C]fipronil, or repeated daily oral gavage dosing of fipronil (14 daily doses of 4 mg/kg bw of fipronil followed by a single oral 4 mg/kg bw dose of [^{14}C]fipronil). Results showed dose- and dose regimen-dependent, but sex-independent, systemic absorption. The highest estimated absorption, at 53% of administered dose (AD), occurred following single oral dosing at 4 mg/kg, followed by repeat oral dosing at 4 mg/kg (approximately 39% AD) and then single oral dosing at 150 mg/kg bw (ca 34% AD). Fipronil underwent rapid and extensive first pass metabolism with mostly fipronil-sulfone (M&B 46136) and fipronil conjugates (mostly glucuronides) reaching the systemic circulation. The conjugates were rapidly excreted in urine whereas fipronil-sulfone distributed to the tissues, especially lipophilic ones. Fipronil-sulfone was the major chemical form in the tissues, especially in the fat, liver, kidneys, muscle and uterus; equivalent to 90–100% of radioactive dose). About 46% of the radioactive dose (mostly as fipronil-sulfone) persisted in the tissues for 168 hours post dose. In blood maximum concentration (C_{max}) of radioactivity and and area under the concentration–time curve for 0–168 hours ($AUC_{0-168\,h}$) were both approximately dose-proportional. The radioactivity mean terminal elimination half-life ($t_{½}$) was about 175 h, likely due to the slow release of fipronil-sulfone from lipophilic tissues. The major route of elimination was in faeces which contained fipronil and up to 10 other metabolites. Given the faecal metabolite profile, substantial biliary elimination was considered likely (Powles, 1992; Powles, 1994).

In two [^{14}C]fipronil tissue kinetics studies where young adult SD rats were administered single oral gavage doses of 4 or 50 mg/kg bw the rate of gastrointestinal (GI) absorption was dose-dependent (blood radioactivity T_{max} at the low dose of 5–6 h, but 34–38 h with the high dose), but sex-independent. Except for the GI tract, maximal tissue radioactivity coincided with the blood radioactivity T_{max}. High levels of GI radioactivity persisted for up to 168 h post dose. Irrespective of the dose, the highest tissue radioactivity levels were in the abdominal fat, adrenal glands, pancreas, thyroid, skin and fur, ovaries, uterus and the liver. The $t_{½}$ for terminal elimination of radioactivity was about 135–171 h in high-dose males and females, respectively and about 183–245 h in low-dose males and females, respectively (Totis & Fisher, 1994), about 217 h following dosing at 50 mg/kg bw, and 268 h following dosing at 4 mg/kg bw (Fisher, 1999a). Tissue persistence in these studies amounted to about 9% of radioactivity retained at 168 h in the high-dose group with about 55% of radioactivity retained in the low-dose group; in both cases values were dose-dependent.

In a [^{14}C]fipronil daily repeat dose oral gavage bioaccumulation study, where young adult male SD rats were dosed either at 4 mg/kg bw per day for 35 days, or dosed at 0.025, 0.075 or 4 mg/kg bw per day for 21 days, radioactivity was widely distributed in the tissues, concentrated in fat and was persistent (present in the tissues for at least 99 days post dose). The mean $t_{½}$ for tissue radioactivity was 545 ± 56 h with a value about 1.4-fold higher in bone and marrow, residual carcass, skin and fur. Fipronil-sulfone (M&B 46136) was the major tissue metabolite, representing > 90% of the radioactivity in the liver, kidneys, brain, fat, plasma, skin and fur (Fisher, 1999b).

In a bile duct excretion study conducted in adult SD rats administered single oral gavage doses of 4 or 40 mg/kg bw of [^{14}C]fipronil, the substance was well absorbed. Estimated oral absorption was about

90%, and this was dose-independent. The extent of biliary excretion was both dose- and sex-dependent (11% and 24 % in high-dose males and females respectively; 7% in both sexes at the low dose). Biliary excretion consisted of conjugated (majority) and non-conjugated metabolites. The mammalian metabolite and food residue fipronil-carboxy (RPA 200761) was present in bile at 0.1–0.3% of AD. Fipronil-sulfone (M&B 46136) was present in bile at 0.05–0.2 % of AD (Totis, 1995).

In a [^{14}C]fipronil biliary reabsorption study in which adult SD rats were administered a single oral gavage dose of 4 mg/kg bw, the disposition of radioactivity was consistent with previous studies. Fipronil-derived radioactivity underwent considerable enterohepatic recirculation. Notably, once fipronil-derived radioactivity was incorporated into bile it was more likely to be recirculated and eventually excreted than stored within the body fat. About 18% of the administered radioactivity (likely consisting of non-absorbed material plus biliary excreta) was excreted in the faeces (Kemp 1999a).

Rabbit

Following single oral gavage dosing of adult female New Zealand White rabbits with [^{14}C]fipronil at a dose of 5 mg/kg bw, the radioactivity was well absorbed (estimated absorption about 80%) and widely distributed; the highest were in bone marrow, GI contents and Harderian glands, with moderate levels in the abdominal fat, stomach and thyroid. Radioactivity was persistent, with up to about 2.6% of the administered radioactivity remaining in the tissues at 60 days post dose and detectable levels of tissue radioactivity at 75 days; $t_{½}$ for blood radioactivity was up to about 49.5 days. The major route of excretion was in the faeces. Excretion via urine and faeces was protracted, likely reflecting the slow release of radioactivity from fatty tissues (Viswaligam & Stewart, 1993).

Human

Based on in vitro human blood binding studies, the radioactivity derived from [^{14}C]fipronil and its metabolites [^{14}C]fipronil-desulfinyl (M&B 46513), [^{14}C]fipronil-sulfide (M&B 45950) and [^{14}C]fipronil-sulfone (M&B 46136) was largely distributed to the plasma. Distribution was not affected by concentration or blood triglyceride content (Giudicelli 1998).

Comparative studies in rabbits, rats and mice

Comparative disposition was evaluated in adult female New Zealand White rabbits, SD rats and CD-1 mice which were administered [^{14}C]fipronil as single oral gavage doses of 5 mg/kg bw. Radioactivity was widely distributed and concentrated in fat in all species. Outside of fat the highest levels of radioactivity were found in thyroid, liver, and kidneys in all species and in muscle in rabbits. The elimination $t_{½}$ for blood radioactivity was about three days in rodents and 14 days in rabbits. Metabolism of fipronil followed the same pathways in all species but was more extensive in rats. Faecal excretion predominated in rodents (about 24–37% of AD) but was lower in rabbits (about 12% of AD). Urinary excretion was low (about 20% of AD in rats, about 5.5% of AD in rabbits and mice). The major faecal metabolites in rabbits were fipronil sulfide (M&B 45950) and fipronil-sulfone (M&B 46136), whereas in rats the major metabolite was just fipronil-sulfone (Lowden, 1999).

Autoradiographic tissue distribution was evaluated in adult Dutch strain rabbits, in SD rats andin Swiss Webster mice given a single oral gavage dose of 5 mg/kg bw of [^{14}C]fipronil. The disposition of radioactivity in all three species was consistent with other studies and the highest levels of radioactivity were associated with the fat (Whitby 1991).

A comparative toxicokinetic study of fipronil in CD-1 mice, in SD rats and in New Zealand White rabbits orally gavage dosed with 0.4 mg/kg bw per day (for 15 days) or 40 mg/kg bw per day (for 23 days) was supplied but was not considered reliable due to the presence of high levels of metabolites in the negative control samples, anomalies regarding tissue distribution of of fipronil-sulfide (M&B 45950) compared with other more reliable toxicokinetics studies, and the presence of multiple study amendments. (Broadmeadow 1991a, b, c, d; Cummins 1991, 1993; Maycey, 1993).

(b) Dermal route

Based on mass balance studies conducted in male SD rats topically treated (non-occlusive) with [^{14}C]fipronil as single doses of 0–8 mg/animal, systemic absorption at 24 h post dose was minimal at less than 3% of AD (Cheng 1996).

1.2 Biotransformation

(a) In vitro studies

Rat, rabbit and human liver microsomal preparations rapidly biotransformed fipronil to fipronil-sulfone (M&B 46136) but were incapable of further metabolism. The rate of fipronil-sulfone formation by rat and human microsomes was similar but was about 1.6-fold higher with rabbit microsomes than with the other species (Valles, 1997).

Based on in vitro studies CYP3A family enzymes (CYP3A4 in humans, CYP3A2 in rats) are the major catalysts for the metabolism of fipronil to fipronil-sulfone. CYP3A1 also contributed activity in the rat. Minor catalysts were CYP2C19 (humans), CYP3A1, 2A2, 2C6 and 2D1 (rats). Human CYPs 2D6 and 3A5 were relatively inactive. Metabolism of fipronil to fipronil-sulfone was faster in rat than in human preparations. Fipronil-sulfone (M&B 46136) was a potent inhibitor of members of the CYP2C subfamily (Le Lain, 2001a, b).

Cultured fresh rat and rabbit hepatocytes and cultured cryopreserved human hepatocytes converted [^{14}C]fipronil primarily to fipronil-sulfone, but were incapable of further biotransformation. No species differences were noted in the metabolites formed. Rat hepatocytes metabolized fipronil to fipronil-sulfone at a faster rate, confirming the results of the hepatic microsomal and recombinant CYP enzyme studies (Valles, 1998).

Enantioselective metabolism of fipronil to fipronil-sulfone by human liver microsomes in vitro has been reported. Maximum rate of reaction (V_{max}) for *S*-fipronil was about 1.6-fold greater than for *R*-fipronil or racemic fipronil. However, the calculated human hepatic clearance for *S*-fipronil (0.83 mL/minute per mg) is similar to that for *R*-fipronil (0.98 mL/minute per mg) and racemic fipronil (1.02 mL/minute per mg). The calculated human hepatic extraction rate for *S*-fipronil (4.1%) is slightly lower than that for *R*-fipronil (4.9%) or racemic fipronil (5.1%). Overall enantioselective metabolism is unlikely to be of toxicologic importance in humans since enantiomeric differences in human hepatic clearance and extraction was less than two-fold (Carrao et al., 2019).

(b) In vivo studies

Three major patterns were apparent:

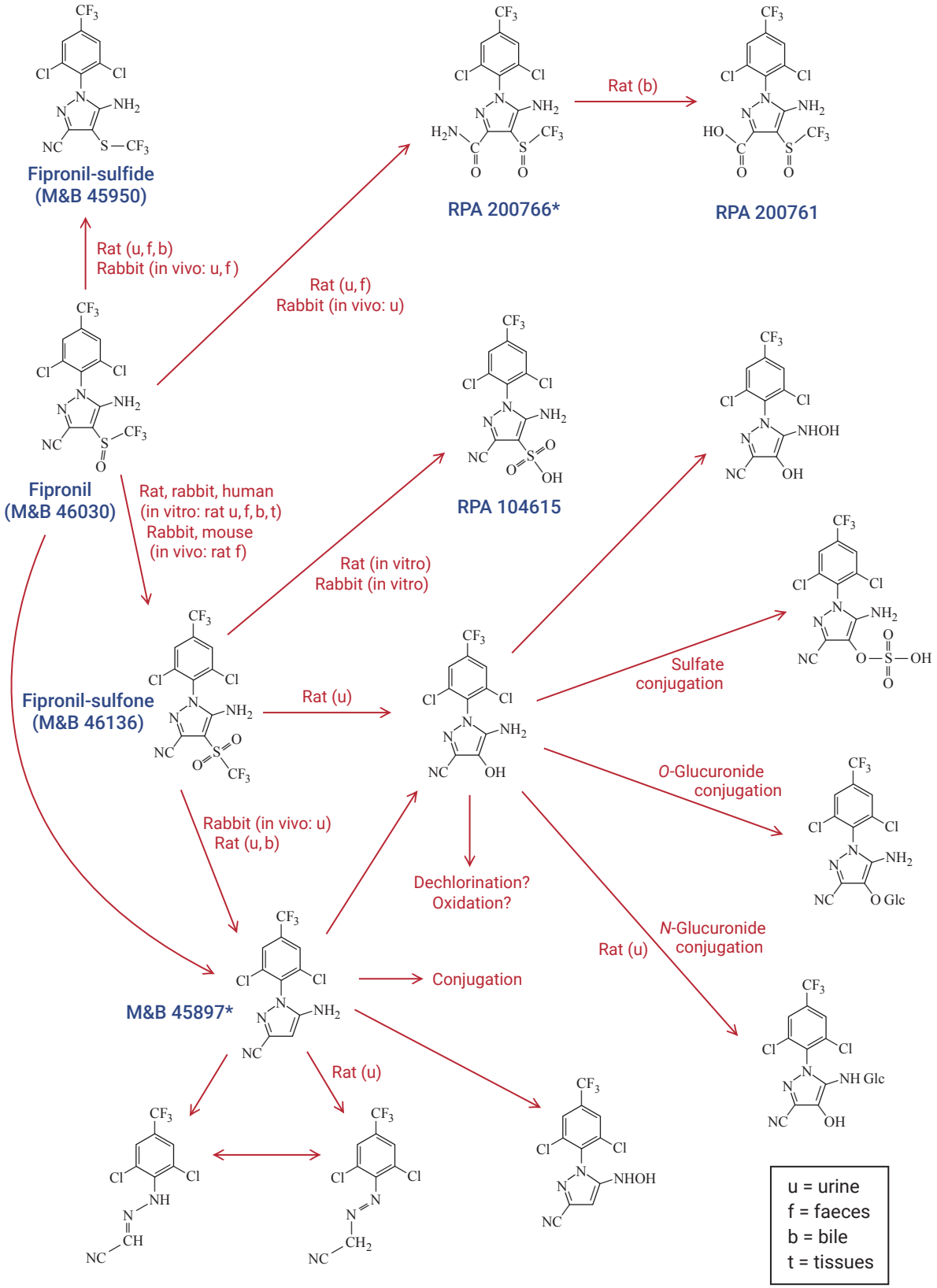
(i) Consistent with the in vitro studies the predominant metabolic pattern was the rapid first pass hepatic metabolism of fipronil to fipronil-sulfone (M&B 46136), the dominant in vivo circulating and tissue metabolite, with systemic exposure representing > 90% of the oral dose with the parent compound.

(ii) Conversion of fipronil to fipronil-sulfone (M&B 46136) was more dominant in rats than in humans.

(iii) Metabolism of fipronil by the rat was more extensive when compared with other species (including humans). A large number of minor metabolites were also generated in vivo. Many of these metabolites, and the metabolic pathways that generate them, are not fully characterized.

A partial description of the metabolism of fipronil in the rat is shown in Fig. 2.

Figure 2. Partial summary of proposed metabolic pathways for fipronil in the rat



* M&B 45897: 5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-1H-pyrazole-3-carbonitrile

* RPA 200766: 5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfinyl]-1H-pyrazole-3-carboxamide

1.3 Effects on enzymes and other biochemical parameters

In vitro studies in human hepatocytes demonstrated that fipronil at concentrations ranging from 0.1–25 μM induced CYP1A1, CYP2B6, CYP3A4 and CYP3A5 (Das et al., 2006). Based on the evaluation of the ToxCast high-throughput in vitro screening data (summarized below), fipronil was a possible agonist of the constitutive androstane receptor. In addition, fipronil increased the catalytic activity of CYP2B6, CYP2C9, CYP2C19 and CYP3A4 in a human hepatocyte cell line (consistent with CAR activation).

Consistent with the in vitro studies, micro-array screening of the liver of SD rats orally dosed with fipronil (3 mg/kg bw per day for 14 days) demonstrated the upregulation of gene expression for CYP2B1, CYP2B2 and CYP3A1, the carboxylesterases Ces2 and Ces6, the phase II enzymes UDP-glucuronosyltransferase 1A1 (UGT1A1), SULT1B1, and glutathione *S*-transferase α2 (GSTA2), and the membrane transporters ATP-binding cassette subfamily C Member 2 (Abcc2), Abcc3, Abcg5, Abcg8, solute carrier organic anion transporter 1a1 (Slco1a1) and Slco1a4. These findings in rats are consistent with a phenobarbital-like activation of both CAR and the pregnane X receptor (PXR). Conclusions were confirmed in CAR- and PXR-deficient mice orally dosed with fipronil at 5 mg/kg bw per day for 14 days (Roques et al., 2013).

Repeated oral gavage dosing of CD rats with 5 mg/kg bw per day of fipronil resulted in an approximately two-fold increase ($p < 0.050$) in hepatic microsomal ethoxyresorufin-*O*-deethylase levels, *p*-nitrophenol oxidation rate, ethylmorphine-*N*-demethylase levels, and total P450 levels. These findings are consistent with fipronil-mediated induction of hepatic microsomal CYP1A1, CYP2E1 and CYP3A4 (Shavila et al., 1990).

2. Toxicological studies

2.1 Acute toxicity

All studies listed below complied with relevant test guidelines and were GLP compliant except as noted.

(a) Lethal doses

Table 1. Acute oral and dermal toxicity

Route	Species	Sex		Result	References
Oral	Rat	Males	LD_{50}	92 mg/kg bw	Gardner, 1988a
		Females	LD_{50}	103 mg/kg bw	
	Mouse[a]	Males	LD_{50}	49 mg/kg bw	Oba, 1994
		Females	LD_{50}	57 mg/kg bw	
	Mouse[a]	Males	LD_{50}	98 mg/kg bw	Dange, 1995a
		Females	LD_{50}	91 mg/kg bw	
Dermal (occlusive)	Rat	Males and females	LD_{50}	>2000 mg/kg bw	Gardner, 1988b
	Rabbit	Males	LD_{50}	445 mg/kg bw	Myers, 1992
		Females	LD_{50}	354 mg/kg bw	
Inhalation dust aerosol, nose only; four-hour exposure	Rat	Males and females	LC_{50}	0.68 mg/L	Cracknell, 1991
	Rat	Males	LC_{50}	0.36 mg/L	Nachreiner, 1995
		Females	LC_{50}	0.42 mg/L	

[a] Study was not compliant with GLP

LD_{50}: Median lethal dose; LC_{50}: Median lethal concentration;

(b) Dermal irritation

Occlusive dermal exposure of New Zealand White rabbits to 0.5 g of fipronil moistened with water for four hours was non-irritant and non-toxic (Liggett, 1988a). However, when corn oil was used as the vehicle, mild to moderate oedema and erythema (both fully reversible) occurred (Myers & Christopher, 1993a).

Fipronil under aqueous conditions was not irritating to the skin but under lipophilic conditions it was slightly irritating.

(c) Ocular irritation

Administration of 82 mg (approximately 0.1 mL) of fipronil into the conjunctival sacs of three male New Zealand White rabbits induced mild conjunctival redness and chemosis in all animals (fully reversible within three days post exposure). No corneal damage or iritis occurred (Liggett, 1988b).

In a second study, administration of 90 mg of fipronil into the conjunctival sacs of three male and three female New Zealand White rabbits induced transient, moderate corneal opacity in 2/6 of the animals and iritis in 5/6 animals. Mild to moderate conjunctival redness occurred in all six rabbits with conjunctival chemosis occurring in 5/6 animals, both fully reversible within 14 days post exposure (Myers & Christopher, 1993b). Overall fipronil is slightly irritating to the eye in rabbits.

(d) Dermal sensitization

Fipronil did not induce delayed-type hypersensitivity reactions; induction consisted of 30% w/v fipronil in paraffin oil (minimally irritant), challenge was with 5% and 30% fipronil Nor was fipronil toxic in a Guinea pig three-fold induction Buehler test (Smith, 1990). In further studies fipronil was non-toxic and not a sensitizer in a Guinea pig Magnusson & Kligman maximization test. Induction here consisted of 5% w/v fipronil in propylene glycol, the challenge being 3%, 10% and 25% w/v fipronil. (Rees, 1993, 2007).

2.2 Short-term studies of toxicity

(a) Oral administration

Oral short-term toxicity studies with fipronil were conducted in the rat and the dog and consisted of four-week studies, ninety-day studies and, in the dog, two one-year studies.

Rat

In a four-week oral toxicity study, six groups of five male and five female SD rats were fed diets incorporating fipronil at 0, 25, 50, 100, 200 or 400 ppm (equal to 0, 3.4, 6.9, 13, 24 and 45 mg/kg bw per day for males, 0, 3.5, 6.7, 13, 25 and 55 mg/kg bw per day for females). One out of five females in the 400 ppm dose group was found dead on day 4 of the study. The cause of this death was not definitively established. Transiently decreased feed efficiency (resolved after 1–3 weeks) and effects on body weight parameters occurred following dosing at 100 ppm and above. Marginal, non-adverse effects occurred in clinical chemistry parameters, namely increased platelet counts at 200 ppm and above, increased serum total protein and globulin at all exposure levels, reduced serum albumin in females dosed at 400 ppm and small increases in serum calcium in males dosed at 400 ppm, and increased serum cholesterol at 400 ppm. Significant ($p < 0.05$) increases in liver weight (increased relative weight in all fipronil-exposed females, increased non-adjusted weight in males at 200 ppm and above) was accompanied by hepatocellular hypertrophy in males dosed at 100 ppm and above and in females exposed at 200 ppm and above. All levels of fipronil exposure resulted in minimal to moderate thyroid follicular hypertrophy with an incidence 80% or more in males at all exposure levels and 60% or more in females at all exposure levels. The lowest-observable-adverse-effect level (LOAEL), when the effects on the thyroid were taken into account, was 25 ppm (equal to 3.4 and 3.5 mg/kg bw per day in males and females, respectively; Peters et al., 1990). For this study JMPR 1997 reports that no NOAEL was identified due to the effects on the thyroid. However, taking into account the findings of the human relevance mode of action/adverse outcome pathway analysis (see Appendix 1) a NOAEL of 50 ppm was established at JMPR 2021 (equal to 6.7 mg/kg bw per day) due to reduced body weight gain during the first week of treatment at 100 ppm and greater.

In a 13-week oral toxicity study five groups of 10 male and 10 female SD rats were fed diets incorporating fipronil at 0, 1, 5, 30 or 300 ppm (equal to 0, 0.07, 0.33, 1.9 and 20 mg/kg bw per day for males, 0, 0.07, 0.37, 2.3 and 24 mg/kg bw per day for females). No unplanned deaths, behavioural effects, changes in appearance, neurological abnormalities or ophthalmoscopic abnormalities occurred.

Transient, reversible effects on body weight gain, (correlating with reduced food intake) occurred following dosing at 30 ppm and greater. Small ($p < 0.05$), non-adverse reductions in haematological parameters, reflecting reduced circulating erythron and circulating Hb (Hb) masses, occurred after dosing at 300 ppm. Increased ($p < 0.05$) relative liver weight (no anatomic pathology correlates) occurred after dosing at 30 ppm and greater. Increased body fat deposition occurred in males dosed at 300 ppm and thyroid follicular hypertrophy and thyroid hyperplasia occurred after dosing at 300 ppm. Taking into account the effects on the thyroids, the NOAEL was 5 ppm (equal to 0.33 mg/kg bw per day). This was the same as the value from JMPR 1997 (Holmes, 1991a). Taking into account, however, the findings of the human relevance mode of action evaluation for the thyroid effects (see Appendix 1) the NOAEL was 30 ppm (equal to 1.9 mg/kg bw per day).

Dog

Study1
In a repeat oral exposure toxicity study, four groups each consisting of two male and two female beagle dogs were oral capsule dosed with fipronil at 1 or 20 mg/kg bw per day for four weeks, or 0 or 10 mg/kg bw per day for six weeks. There were no unplanned deaths, ophthalmoscopic abnormalities, effects on organ weights or anatomic pathology findings. Food consumption and body weight parameters were reduced following dosing at 20 mg/kg bw per day (particularly during treatment weeks 2 and 3). Dosing at 10 mg/kg bw per day and above resulted in clinical signs (abnormal postures, head nodding, facial twitching, continuous swallowing, forelimb jerking, exaggerated flexor reflexes, aggressive behaviour, convulsions) consistent with neurotoxicity. The erythrocyte parameters Ht (Ht), Hb concentration and erythrocyte count (EC), were increased following dosing at 10 mg/kg bw per day and above. The NOAEL was 1 mg/kg bw per day (Holmes, 1991b).

Study2
In a 13-week repeat oral exposure toxicity study, four groups each consisting of four male and four female beagle dogs were dosed via an oral capsule with fipronil at 0, 0.5, 2 or 10 mg/kg bw per day. Unplanned deaths included one high-dose male and three high-dose females, due to inappetence, body weight loss, emaciation and/or neurotoxicity). Dogs dosed at 10 mg/kg bw per day developed clinical signs consistent with neurotoxicity (subdued behaviour, underactivity, disorientation, apparent disturbances in visual perception/awareness, excessive salivation, hind limb extension, ataxia, abnormal limb motions, irregular heartbeat, tremor and muscular twitches, facial twitches, exaggerated blink and gag reflexes, head nodding, decreased tactile placing reflexes and convulsions) and emaciation. Clinical signs of neurotoxicity were apparent within the first week of treatment but there was insufficient data to determine whether or not neurotoxicity occurred following the first dose. Reduced blood cholesterol occurred in males dosed at 10 mg/kg bw per day. Dosing of female dogs at 2 mg/kg bw per day resulted in reduced body weight gain (about −17% compared with controls). The NOAEL for this study was 0.5 mg/kg bw per day (Holmes, 1991c).

Study3
In a one-year repeat oral exposure toxicity study four groups each consisting of six male and six female beagle dogs were oral capsule dosed with fipronil at 0, 0.2, 2.0 or 5.0 mg/kg bw per day. Two male dogs in the high-dose group and one male dosed at 2.0 mg/kg bw per day were euthanized during the study due to neurotoxicity (manifesting as nervous behaviour, aggression, tremor, limb spasticity, hyperesthesia, facial twitching, abnormal gait and convulsions), inappetence and weight loss. Clinical signs of neurotoxicity were apparent within the second week of treatment, but there was insufficient data to determine whether or not neurotoxicity occurred following the first dose. Similar clinical signs of neurotoxicity occurred in surviving dogs dosed at 2.0 mg/kg bw per day and above. The NOAEL was 0.2 mg/kg bw per day (Holmes, 1992).

Study4
In a one-year dietary exposure toxicity study five groups each consisting of five male and five female beagle dogs were fed fipronil in food at concentrations that achieved doses equal to 0, 0.075, 0.3, 1 or 3 mg/kg bw per day; the high dose was reduced to 2 mg/kg bw per day after 38 days. One high-dose female

was euthanized due to clinical signs of neurotoxicity (tremor, underactivity, convulsions, prostration, reduced menace and startle reactions and abnormal gait), inappetence and clinical pathologic evidence of dehydration (increased Ht, Hb concentration and EC, combined with increased total plasma protein concentration). Clinical signs of neurotoxicity occurred in surviving animals dosed at 1 mg/kg bw per day and above. Clinical signs of neurotoxicity were apparent within the first week of treatment but there was insufficient data to determine whether or not neurotoxicity occurred following the first dose. Fipronil and fipronil sulfone were detected in the plasma of all fipronil-reated groups. The plasma concentration of fipronil sulfone was higher than that of fipronil. There was no evidence of a significant sex difference or of accumulation of either fipronil or fipronil sulfone. The NOAEL was 0.3 mg/kg bw per day (Holmes, 1993).

Based on the combined findings of the 13-week and one-year studies the overall NOAEL for repeat-dose toxicity in the dog was 0.3 mg/kg bw per day based on the occurrence of clinical signs of neurotoxicity occurring at doses of 1 mg/kg bw per day or greater.

(b) Dermal application

Rat

Semi-occlusive dermal exposure of SD rats to fipronil at 0, 100, 500, 1000 mg/kg bw per day (10 rats per sex per dose) for six hours per day for 28 days did not induce adverse toxicological effects. Increased ($p < 0.05$) liver absolute and/or relative weights (adaptive and not accompanied by any adverse anatomic pathology changes) occurred at doses of 500 mg/kg bw per day and greater. No fipronil-associated dermal irritation occurred.

The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Henwood, 1997).

Rabbit

In a 21-day repeat dermal exposure toxicity study, five groups each consisting of six male and six female New Zealand White rabbits were topically (occlusive) exposed for 6 hours per day, five days per week (total of 15 doses) to 0, 0.5, 1, 5 or 10 mg/kg bw per day of fipronil. No unscheduled deaths occurred. Two rabbits in the high-dose group exhibited a period of extreme hyperactivity (considered adverse) following the 14th (one male) or 15th (one female) dose. Observed in the high-dose groups were reduced overall body weight gain ($p < 0.05$; −47.4 g compared with +225.2 g for controls), and reduced food consumption between days 15 and 21 (44% in males, $p < 0.05$; 22% in females). No skin irritation was observed.

The NOAEL was 5 mg/kg bw per day (Wagner & Hermansky, 1993).

(c) Exposure by inhalation

Rat

In a 10-day dose-ranging inhalation toxicity study a nose-only, powder aerosol was used to deliver fipronil of mass median aerodynamic diameter 2.6–3.0 µm, geometric standard deviation 1.7 µm. Five groups, each consisting of five male and five female SD rats, were exposed to fipronil at 0, 0.005, 0.010, 0.050, or 0.101 mg/L for four hours per day. There were two unplanned deaths in the high-dose group, one male, one female. Evidence of toxicity observed at exposures of 0.050 mg/L and above included convulsions, dilated pupils, decreased activity, excessive salivation, dyspnea, reduced body weight ($p < 0.05$; −12% compared with controls), reduced body weight gain ($p < 0.05$; −40% compared with controls) and reduced food consumption over the first eight days of treatment ($p < 0.05$; −12% compared with controls). Increased liver weight ($p < 0.05$; +10% compared to controls) occurred in all fipronil-exposed males, and after dosing at 0.050 mg/L and greater, in females too (+19% compared to controls). No microscopic anatomic pathology examinations were performed.

Based on the findings in other rat studies the effects on relative liver weight were likely to have been adaptive. The NOAEC was 0.010 mg/L (Viau, 1997).

A 28-day, four hours per day, nose-only aerosol inhalation study was performed in rats but this was not available for evaluation.

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

In the 78-week oncogenicity phase of a repeat oral exposure combined chronic toxicity and oncogenicity study, five groups of CD-1 mice, each consisting of 52 males and 52 females, were fed diets containing fipronil at 0, 0.1, 0.5, 10 or 30 ppm (equal to 0, 0.011, 0.055, 1.181 and 3.430 mg/kg bw per day in males, 0, 0.012, 0.063, 1.230 and 3.616 mg/kg bw per day in females). An additional cohort of 72 male and 72 female mice were fed diets containing 60 ppm (dose in mg/kg bw per day not determined). In the chronic toxicity arm of the study, five groups each consisting of 20 male and 20 female CD-1 mice were fed fipronil in the diet at concentrations of 0, 0.1, 0.5, 10, 30 for 53 weeks. Mice in the 60 ppm cohort either died (premortem convulsions observed in one male) during the first nine weeks of exposure or were euthanized after 10 weeks of treatment due to poor body condition, reduced weight gain and reduced food consumption. The lesions that had contributed to death in this cohort were not identified at necropsy, but all of the animals had high relative liver weights. Several of the males had convulsions near the beginning of the study. No statistical ($p > 0.05$) difference in survival in males assigned to the carcinogenicity study arm was noted. However, in females assigned to the carcinogenicity study arm there was a fipronil-associated, dose-related statistical trend ($p < 0.05$) towards increased survival (see Table 2).

Feeding fipronil at 10 ppm or greater resulted in reduced body weight gain ($p < 0.05$; up to −12%) over the first 13 weeks when data from the chronic toxicity and carcinogenesis study arms were combined. However, over the 0–52 week period reduced body weight gain ($p < 0.05$) occurred only in the 30 ppm dose groups when data from both the chronic toxicity and carcinogenesis study arms were combined. By the end of the carcinogenesis study (78 weeks) reduced body weight gain only occurred in the high-exposure females ($p < 0.05$).

At the end of the chronic toxicity phase (week 54) increased relative liver weights ($p < 0.05$) occurred in males exposed at 0.5 ppm or greater, and in females exposed at 30 ppm. By the end of the carcinogenesis study (78 weeks) increased relative liver weight ($p < 0.05$) occurred in males dosed at 10 ppm and greater and in females dosed at 30 ppm. When data from both the chronic toxicity and carcinogenesis study arms were combined an increased incidence, compared to controls, of liver enlargement and of changes to the surface of the liver was seen in males given 30 ppm. By the end of the chronic toxicity study (week 54) an increased incidence compared with controls ($p < 0.05$) of hepatocellular microvesicular periacinar vacuolation occurred in males dosed at 10 ppm and above. By the end of the carcinogenesis study (78 weeks) an increased incidence of hepatocellular microvesicular periacinar vacuolation compared with the controls ($p < 0.05$) occurred in males dosed at 10 ppm and above, and in females dosed at 0.5 ppm and above. An increased incidence of hepatocellular hyperplasia and hepatic degenerative changes ($p < 0.05$) occurred in male decedents dosed at 30 ppm in the carcinogenesis study arm. In the carcinogenesis study arm, there were no treatment-related neoplastic changes in females, but males at 30 ppm had an increased incidence ($p < 0.05$) of malignant hepatocellular carcinomas in comparison with concurrent controls. An additional hepatocellular carcinoma was observed in one male at 30 ppm in the toxicity study. The incidence of hepatocellular adenomas alone or combined with carcinomas was not significantly increased. Since the increase in the incidence of carcinomas in males at 30 ppm was within the range seen in historical controls from the testing laboratory, and the incidence among male concurrent controls was lower than the mean incidence in male historical controls, the neoplastic findings in males were considered not to be related to treatment.

The NOAEL for the study was 0.5 ppm (equal to 0.055 mg/kg bw per day) (Broadmeadow, 1993a, 1994a, b). This NOAEL is numerically the same as that identified by JMPR in 1997.

Table 2. Findings from the long-term mouse study of toxicity and carcinogenicity

	Dose level (ppm)									
	0	0.1	0.5	10	30	0	0.1	0.5	10	30
	Males					Females				
Incidence of unscheduled premature mortality in the 78 week carcinogenesis cohort (mortality/number of animals)										
0–78	28/52	21/52	26/52	26/52	26/52	20/52	20/52	26/52	15/52	14/52
Mean body weight change (g [% of control])										
Weeks 0–13 (chronic and carcinogenesis cohorts)	15.9 [100]	14.7 [92]	15.1 [95]	14.0* [88]	11.7** [74]	10.9 [100]	10.5 [96]	10.7 [98]	9.1* [83]	9.1* [83]
Weeks 13–26 (chronic and carcinogenesis cohorts)	4.9 [100]	5.0 [102]	5.3 [108]	5.9 [120]	4.4 [90]	6.7 [100]	6.4 [96]	7.4 [110]	5.8 [87]	5.9 [88]
Weeks 0–26 (chronic and carcinogenesis cohorts)	21.1 [100]	19.8 [94]	20.5 [97]	20.0 [95]	16.1** [76]	17.6 [100]	16.9 [96]	18.1 [103]	14.9 [85]	14.7* [84]
Weeks 0–52 (chronic and carcinogenesis cohorts)	23.4 [100]	23.0 [98]	23.0 [98]	22.0 [94]	19.7** [84]	22.2 [100]	21.0 [95]	23.7 [107]	21.1 [95]	19.1 [86]
Weeks 0–78 (carcinogenesis cohorts)	23.1 [100]	21.9 [95]	23.7 [103]	21.3 [92]	19.9 [86]	25.4 [100]	21.7 [85]	25.8 [102]	22.1 [87]	20.6* [81]
Mean liver weights (absolute weight										
Week 54,										
chronic cohorts: absolute weight	2.61 [100]	2.64 [101]	2.70 [103]	3.02 [116]	3.37** [129]	1.67 [100]	1.77 [106]	1.86 [111]	1.84* [110]	2.00** [120]
chronic cohorts: relative weight	5.079 [100]	5.663 [111]	5.719* [113]	6.132* [121]	7.675** [151]	4.363 [100]	4.417 [101]	4.497 [103]	4.666 [107]	5.392** [124]
Week 79,										
carcinogenesis cohorts: absolute weight	2.77 [100]	2.78 [100]	2.92 [105]	3.30 [119]	3.81** [138]	1.99 [100]	1.93 [97]	2.06 [104]	2.02 [102]	2.13 [102]
carcinogenesis cohorts: relative weight	5.634 [100]	5.744 [102]	5.977 [106]	7.008* [124]	8.261** [147]	4.5356 [100]	4.575 [101]	4.451 [98]	4.799 [106]	5.294** [117]
Incidence of hepatic macroscopic anatomic pathology findings for combined chronic toxicity and carcinogenesis cohorts (incidence/number of animals)										
Areas of surface change	0/52	1/52	3/52	3/52	8/52**	0/52	0/52	1/52	0/52	1/52
Enlarged	2/52	2/52	1/52	4/52	7/52	1/52	0/52	1/52	0/52	1/52
Incidence of hepatic microscopic anatomic pathology findings										
Microvesicular periacinar vacuolation in the chronic toxicity cohorts after 53 weeks	0/14	2/15	2/19	7/16**	12/18***	1/18	1/19	4/15	1/17	4/13
Microvesicular periacinar vacuolation in the carcinogenesis cohorts after 78 weeks	5/24	7/31	7/26	13/26*	13/26*	0/32	0/32	4/26*	3/37	7/38*
Hepatocyte hyperplasia in the carcinogenesis cohort decedents[a]	0/28	2/21	1/26	0/26	4/26*	0/20	0/20	0/26	0/15	0/14
Hepatic degenerative changes in the carcinogenesis cohort decedents	3/28	5/21	3/26	5/26	11/26*	6/20	4/20	5/26	4/15	2/14

	Dose level (ppm)									
	0	0.1	0.5	10	30	0	0.1	0.5	10	30
	Males					Females				
Incidence of neoplastic changes in the carcinogenesis cohorts at 78 weeks										
Hepatocellular carcinoma	1/28	0/21	0/26	0/26	2/26[b]	0/20	0/20	0/26	0/15	0/14
Hepatocelluar ademoma	3/28	1/21	2/26	0/26	1/26[b]	0/20	0/20	0/26	0/15	0/14
% total hepatic neoplasias	14.3%	4.8%	7.7%	0/26	11.5	0%	0%	0%	0%	0%
Historical control range for hepatic tumours	2.9%–25.0%	-	-	-	-	-	-	-	-	-

Significantly different from control: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

[a] Degenerative changes included necrosis of occasional cells and apoptosis, increased ploidy, hypertrophy and degeneration of periacinar hepatocytes, chronic inflammation and bile stasis

[b] One male at 30 ppm had both an adenoma and a carcinoma

Rat

In a combined chronic toxicity and oncogenicity study, groups of Sprague Dawley CD rats were given dietary concentrations of 0, 0.5, 1.5, 30 or 300 ppm of fipronil (equal to 0, 0.019, 0.059, 1.27 and 12.68 mg/kg bw per week in males, 0, 0.025, 0.078, 1.61 and 16.75 mg/kg bw per day in females, measured over study weeks 0–90). Dosing lasted 89 or 91 weeks in the oncogenicity study arm, with 50 rats per sex per dose. In the chronic toxicity study arm dosing lasted 52 weeks, with 15 rats per sex per dose, which included the satellite groups used for evaluation of the effects of a 13-week 'off-dose′ recovery period following 52 weeks of treatment. Satellite animals were used to measure thyroid hormone levels during treatment and in the 'off-dose′ recovery period. The duration of the study was intended to be 104 weeks, however, males from the oncogenicity phase were killed after 89 weeks and females after 91 weeks when survival reached 25% (in high-dose males and 30 ppm females). Survival was consistent with that seen in other contemporary SD rat studies and was not affected by fipronil treatment in the absence of a dose–response relationship.

Body weight gain the high-dose group was reduced after 52 weeks of treatment ($p < 0.05$; −85% in males, −82% in females compared to controls). The body weight gain in females (but not males) fed fipronil at 30 ppm for more than 52 weeks was also reduced ($p < 0.05$; −23% compared to controls). The effects on weight gain following dosing at 30 ppm or more was not fully reversed by the end of the recovery period. Clinical signs consistent with neurotoxicity (convulsive episodes, irritability, overactivity, excessive vocalization, excessive salivation, aggression and teeth grinding) occurred following dosing at 1.5 ppm or more (Table 3), but did not occur during the reversibility phase. Haematology findings consistent with small (non-adverse) reductions in circulating erythron mass occurred following dosing at 300 ppm, and less frequently following dosing at 1.5 or 30 ppm. These effects did not occur during the reversibility phase. Prothrombin times were generally slightly shorter following dosing at 300 ppm, and intermittently shorter in females dosed at 30 ppm. Increased liver weights ($p \leq 0.05$; no microscopic anatomic correlates) occurred following dosing at 30 ppm or greater, and were accompanied by changes in plasma protein, cholesterol, and shorter prothrombin times.

Serum thyroxine (T4) levels were reduced in all fipronil-treated groups, but were more markedly reduced following dosing at 300 ppm (T4 was undetectable during the first week of treatment). Serum T4 remained low in males dosed at 30 ppm or greater for two weeks post dose, but the change was reversed by week 11 of the recovery phase. The effects of fipronil on serum triiodothyronine (T3) were less consistent although an increase in serum T3 occurred during the recovery phase in females dosed at 30 ppm or more. Increased serum thyroid-stimulating hormone (TSH) occurred in males dosed at 30 ppm and above and in females dosed at 300 ppm. Elevated serum TSH was still apparent at the end of the recovery phase in males dosed at 30 ppm. The effects on serum thyroid-associated hormones were accompanied by increased thyroid weights ($p < 0.05$) following dosing at 30 ppm and 300 ppm, and in the high-dose group did not fully resolve by the end of the recovery period.

No treatment-related neoplastic lesions were found in rats from the chronic toxicity phase. A significantly increased incidence of thyroid follicular neoplasia ($p < 0.05$) occurred in rats fed fipronil at 300 ppm.

The NOAEL for fipronil-induced thyroid follicular neoplasia in rats was 30 ppm (equal to 1.27 mg/kg bw per day). While fipronil is a thyroid carcinogen in rats, based on the mode of action/adverse outcomes analysis shown in Appendix 1, this effect would have only limited if any relevance to humans exposed to fipronil in the diet.

Increased kidney and adrenal weights ($p < 0.05$) occurred following dosing at 30 ppm or more, and increased relative spleen weights ($p < 0.05$) occurred at the end of the oncogenicity study phase following the dosing of males at 300 ppm. The effects on kidney weight following dosing at 300 ppm did not resolve by the end of the recovery phase. The effect on kidney weights was accompanied by an increased incidence of large and pale kidneys. An increased chronic progressive nephropathy ($p < 0.05$) occurred in males dosed at 0.5 ppm and above, and in females dosed at 30 ppm and above. Chronic progressive nephropathy is a spontaneous renal disease of rats. It is a progressive disease with known physiological factors that modify disease progression, such as high dietary protein. The weight of evidence supports an absence of a renal counterpart in humans (Hard, Johnson & Cohen, 2009; Hard et al., 2013).

The NOAEL for the study was 0.5 ppm (equal to 0.019 mg/kg bw per day) due to the presence of neurotoxicity at 1.5 ppm (Aughton, 1993).

Table 3. Findings in the rat combined chronic toxicity and oncogenicity study

	Dose level (ppm)									
	Males[a]					**Females[a]**				
	0	**0.5**	**1.5**	**30**	**300**	**0**	**0.5**	**1.5**	**30**	**300**
Incidence of clinical findings (rats affected/total animals)										
Thin build	14/50	8/50	12/50	14/50	22/50	9/50	11/50	13/50	18/50	9/50
Obese	9/50	8/50	6/50	4/50	0/50	4/50	5/50	4/50	2/50	0/50
Convulsion	0/50	0/50	3/50	1/50	5/50	0/50	0/50	0/50	2/50	11/50
Aggressive	0/50	3/50	1/50	2/50	4/50	0/50	0/50	4/50	2/50	9/50
Irritable	3/50	4/50	3/50	4/50	6/50	2/50	2/50	5/50	6/50	18/50
Overactive	0/50	0/50	0/50	0/50	0/50	0/50	0/50	1/50	0/50	3/50
Excessive vocalisation	4/50	9/50	3/50	6/50	10/50	4/50	2/50	11/50	7/50	19/50
Excessive salivation	0/50	0/50	1/50	0/50	1/50	0/50	0/50	0/50	2/50	8/50
Group mean body weight change (g)										
Treatment week										
0–1	62	62	61	58**	26**	28	29	27	25*	13**
1–52	650	666	646	612	578**	310	321	312	306	265**
0–52	712	728	707	670	603**	338	350	339	330	278**
0–88/90	699	771	781	652	576**	451	420	438	346*	339**
(% of control)	(100)	(110	(112	(93)	(82)	(100)	(93)	(97)	(77)	(75)
Recovery phase (52–64)	−12	−10	37	−4	3	36	50	30	42	21
Group mean haematology values										
Packed cell volume (%)										
Treatment week										
24	46	45	45	45	43**	44	45	45	44	41**
50	47	47	47	46	43***	46	45	44*	43***	41***
76	47	44	42*	41*	42*	43	42	44	42	39*
88/90	46	46	46	42	41*	42	44	44	42	39
Recovery phase	48	49	46	46	48	45	47*	46	45	44

	Dose level (ppm)									
	Males[a]					Females[a]				
	0	0.5	1.5	30	300	0	0.5	1.5	30	300
Haemoglobin (g%)										
Treatment week										
24	16.1	16.0	16.1	15.7	15.0***	15.5	15.9	15.8	15.3	14.7**
50	16.1	16.1	16.1	15.7	14.9***	15.7	15.7	15.2	15.1*	14.2***
76	16.2	15.1	14.6	14.0*	14.4*	15.0	14.8	15.5	14.4	13.6*
88/90	15.6	15.3	15.1	13.9*	13.7*	14.2	15.1	15.1	14.5	13.1
Recovery phase	15.7	15.9	15.3	15.0	15.7	14.8	15.5*	15.0	15.0	14.5
Red blood cell count (10^6/mm^3)										
Treatment week										
24	9.37	9.23	9.01*	8.95*	8.90*	8.11	8.41	8.37	8.14	8.11
50	9.26	9.10	8.97	8.82	8.76*	8.15	8.16	8.01	7.90	7.87
76	9.01	8.60	8.14	7.96*	8.68	8.02	7.82	8.32	7.82	8.58
88/90	8.80	8.61	8.75	8.00	8.14	7.73	8.05	8.17	7.95	7.47
Recovery phase	9.05	9.25	8.69	8.75	8.71	7.93	8.20	7.82	8.05	7.86
Mean corpuscular haemoglobin (pg)										
Treatment week										
24	17	17	18	18	17	19	19	19	19	18***
50	18	18	18	18	17	20	19	19	19	18***
76	18	18	18	18	17***	19	19	19	18	18
88/90	18	18	17	17	17	18	19	19	18	18
Recovery phase	17	17	18	17	18	19	19	19*	19	19
Mean corpuscular cell volume (fL)										
Treatment week										
24	49	49	51	50	48	54	53	54	54	51***
50	51	52	53	52	50	57	56	55*	55*	52***
76	52	51	52	51	49**	54	54	53	53	53
88/90	53	54	53	52	50	54	55	54	53	54
Recovery phase	53	53	54	53	55	57	58	58	56	56
Prothrombin time (s)										
Treatment week										
24	14.1	14.6*	14.0	14.1	13.6*	13.4	13.8*	13.5	13.0*	12.7**
50	15.2	16.0	15.0	15.5	14.7	14.3	14.9	14.1	13.9	12.9**
76	13.5	14.1	13.7	13.3	13.0	13.4	12.3***	12.7*	12.2***	12.0***
88/90	13.1	14.1*	14.0*	13.1	13.0	12.1	12.2	11.9	11.9	11.8
Recovery phase	14.0	14.9*	14.1	14.3	14.1	13.2	13.7	13.2	12.7*	12.5*
Platelets (1000/µL)										
Treatment week										
76	1042	1048	1121	1296*	1246*	852	1058*	958	1021	1193***
88/90	918	917	1050	1185*	1338***	921	1133*	956	1017	1061

	Dose level (ppm)									
	Males[a]					Females[a]				
	0	0.5	1.5	30	300	0	0.5	1.5	30	300
Group mean clinical chemistry values										
Cholesterol (mmol/L)										
Treatment week										
24	56	67	65	69	82*	76	66	63	79	135***
50	88	82	103	102	117	113	101	114	137	229***
76	104	125	140	135	149*	95	169*	128	169*	228***
88/90	134	127	135	174	170	143	170	178	231*	230*
Recovery phase	117	110	122	126	133	106	100	107	128	210***
Calcium (mmol/L)										
Treatment week										
24	2.5	2.5	2.5	2.6**	2.6**	2.5	2.6	2.5	2.6**	2.7***
50	2.7	2.6	2.7	2.7	2.8*	2.7	2.7	2.7	2.7	2.8***
76	2.6	2.7	2.7	2.8*	2.7	2.7	2.7	2.7	2.7	2.9**
88/90	2.7	2.6	2.6	2.7	2.8	2.8	2.8	2.8	2.7	2.9*
Recovery phase	2.6	2.6	2.6	2.7	2.6	2.6	2.6	2.6	2.7	2.7**
Total protein (g/dL)										
Treatment week										
24	2.5	2.5	2.5	2.6**	2.6**	2.5	2.6	2.5	2.6**	2.7***
50	2.7	2.6	2.7	2.7	2.8*	2.7	2.7	2.7	2.7	2.8***
76	2.6	2.7	2.7	2.8*	2.7	2.7	2.7	2.7	2.7	2.9**
88/90	2.7	2.6	2.6	2.7	2.8	2.8	2.8	2.8	2.7	2.9*
Recovery phase	2.6	2.6	2.6	2.7	2.6	2.6	2.6	2.6	2.7	2.7**
Albumin (g/dL)										
Treatment week										
24	2.9	2.9	2.9	2.9	2.5***	3.9	3.6	3.7	3.8	3.6
50	2.8	3.0	2.7	2.8	2.7	3.7	3.9	3.8	3.8	3.5
76	2.9	2.5*	2.5*	2.5**	2.4**	3.5	3.2	3.3	3.1	3.0*
88/90	3.1	2.9	2.7**	2.4***	2.3***	3.8	3.5	3.4	3.0**	3.0**
Recovery phase	2.9	2.8	2.7	2.5**	2.6	3.4	3.7	3.7	3.8	3.2
Albumin : globulin ratio										
Treatment week										
24	0.8	0.7	0.7	0.7*	0.5***	1.1	1.1	1.0	1.0*	0.9***
50	0.7	0.8	0.6	0.7	0.6**	1.0	1.1	1.0	0.9	0.8***
76	0.7	0.6*	0.6*	0.6**	0.5***	0.9	0.8	0.9	0.8	0.6***
88	0.7	0.7	0.6*	0.5***	0.5***	0.9	0.8	0.7*	0.6**	0.6***
Recovery phase	0.7	0.7	0.6	0.6*	0.6	0.8	1.0	0.9	0.9	0.7
Alpha-1 globulin (g/dL)										
Treatment week										
24	1.4	1.6	1.5	1.8**	1.9***	1.2	1.2	1.3	1.4	1.7***
50	1.6	1.5	1.7	1.8	2.0**	1.4	1.3	1.5	1.9**	2.1***
76	1.4	1.7*	1.7*	1.9**	1.9**	1.3	1.7	1.5	1.7	2.0**
88	1.7	1.6	1.8	2.1*	2.3**	1.4	1.8	1.9*	2.3**	2.1**
Recovery phase	1.6	1.6	1.8	1.9*	1.8	1.4	1.2	1.4	1.6	2.3***

	Dose level (ppm)									
	Males[a]					Females[a]				
	0	0.5	1.5	30	300	0	0.5	1.5	30	300
Alpha-2 globulin (g/dL)										
Treatment week										
24	0.5	0.5	0.5	0.5	0.6***	0.4	0.4	0.5	0.5*	0.5***
50	0.4	0.4	0.5	0.5	0.5	0.5	0.5	0.5	0.6	0.8***
76	0.4	0.4	0.4	0.5**	0.6***	0.5	0.6	0.6	0.6**	0.7***
88	0.5	0.5	0.5	0.6	0.6*	0.7	0.7	0.7	0.8*	0.9***
Recovery phase	0.5	0.4	0.4	0.5	0.6	0.6	0.5	0.5*	0.6	0.8*
Beta globulin (g/dL)										
Treatment week										
24	1.8	1.7	1.7	1.7	2.2***	1.5	1.4	1.5	1.6	1.7*
50	1.8	1.7	1.8	1.7	2.0*	1.5	1.5	1.6	1.6	1.6
76	1.8	1.9	1.9	1.9	1.8	1.6	1.8	1.7	1.8	2.0*
88	2.0	2.0	2.1	2.1	2.1	1.9	1.9	1.8	1.9	2.2***
Recovery phase	2.0	2.0	1.9	2.0	1.9	2.1	1.8	1.9	1.7*	1.7*
Group mean serum thyroid hormone concentrations										
Triiodothyronine, T3 (ng/mL)										
Treatment week										
1	0.61	0.59	0.61	0.58	0.53*	0.78	0.75	0.77	0.75	0.80
4	0.75	0.83	0.85	0.84	0.72	0.77	0.83	0.75	0.70	0.81
12	0.92	0.91	0.95	1.10*	1.01	1.17	1.17	1.11	1.03	1.17
24	0.69	0.73	0.74	0.70	0.64	0.93	0.92	0.84	0.82	0.84
50	0.70	0.67	0.84*	0.82*	0.69	0.87	0.88	0.83	0.84	0.87
2R[b]	0.64	0.58	0.64	0.69	0.62	0.98	0.87	0.95	0.89	1.10
4R	0.70	0.59	0.68	0.74	0.77	0.88	0.91	0.88	0.95	1.13***
7R	0.80	0.77	0.78	0.81	0.89	0.91	1.03	0.87	1.10*	1.19**
11R	0.56	0.52	0.59	0.62	0.64	0.75	0.81	0.83	0.95**	1.11***
Thyroxine, T4 (µg/dL)										
Treatment week										
1	2.93	3.02	2.23*	1.16***	0.00***	2.32	1.86	2.58	1.26**	0.00***
4	3.14	2.70*	2.56**	1.84***	0.39***	3.03	2.48*	2.36*	1.46***	0.79***
12	5.18	4.74	3.96**	3.50***	1.22***	3.62	2.85**	2.87*	2.05***	1.10***
24	4.58	3.81*	3.35***	2.43***	0.76***	2.85	3.09	3.49**	2.98	1.46***
50	5.95	5.51	4.83**	3.90***	2.07***	3.31	3.46	3.00	2.06***	1.38***
2R	3.97	3.71	3.45	3.05**	2.56***	1.67	1.70	1.27	1.39	1.59
4R	4.10	3.45*	2.79***	2.64***	3.21**	2.54	2.53	2.05	2.04	2.21
7R	3.80	3.31	2.94*	2.66**	3.12	2.16	2.30	1.71*	2.04	1.85
11R	3.70	3.58	3.25	3.29	3.52	2.95	3.60*	3.27	3.65*	3.09
Thyroid-stimulating hormone, TSH (ng/mL)										
Treatment week										
1	4.7	7.1	6.2	11.8***	20.3***	3.5	3.5	3.2	3.6	7.6***
4	5.2	8.0	6.5	11.2**	22.9***	3.8	3.9	3.3	3.9	7.5***
12	5.7	7.2	5.8	6.1	18.4***	3.4	3.4	2.9	3.5	8.7***
24	7.2	10.0	6.9	8.6	21.0***	3.2	3.7	3.2	3.9	6.6***
50	13.0	17.1	12.4	26.6*	57.3***	6.2	8.0	5.5	6.1	13.5***

	Dose level (ppm)									
	Males[a]					Females[a]				
	0	0.5	1.5	30	300	0	0.5	1.5	30	300
2R	7.2	7.1	5.7	7.4	12.7**	3.5	3.6	3.5	3.5	3.8
4R	5.6	6.4	6.0	7.3	10.8**	3.7	3.5	3.6	3.3	3.7
7R	5.9	6.3	4.4	5.2	9.1*	3.0	3.2	3.1	2.7	3.1
11R	3.8	4.6	4.6	5.1	8.4**	2.7	3.1	2.9	2.7	3.1
Group mean organ weights										
Liver; absolute weight (g)										
Treatment week										
52	29.5	30.0	29.1	34.3	40.5**	16.4	17.3	17.1	19.1	23.4**
89/91	28.3	32.4	32.1	33.9	39.4**	23.0	22.0	21.5	25.0	27.9*
13R	30.3	30.3	32.9	30.9	30.6	18.5	19.4	17.8	21.0	19.7
Liver; relative weight (% body weight)										
Treatment week										
52	3.53	3.44	3.40	4.32**	5.26**	3.56	3.62	3.70	4.31**	5.49**
89/91	3.30	3.24	3.48	4.40**	5.41**	3.88	3.98	3.82	5.12**	6.14**
13R	3.50	3.70	3.44	3.69	4.55*	3.42	3.60	3.54	3.81*	4.68**
Thyroid; absolute weight (g)										
Treatment week										
52	0.039	0.035	0.042	0.047	0.056**	0.027	0.031	0.030	0.032	0.045**
89/91	0.042	0.051*	0.053*	0.063**	0.094**	0.036	0.038	0.036	0.044	0.072*
13R	0.038	0.039	0.043	0.045	0.045	0.031	0.031	0.030	0.034	0.035
Thyroid; relative weight (% body weight)										
Treatment week										
52	0.0047	0.0040	0.0050	0.0058*	0.0073**	0.0059	0.0064	0.0065	0.0073*	0.0107**
89/91	0.0049	0.0052	0.0056	0.0082**	0.0129**	0.0060	0.0070	0.0065	0.0090**	0.0156*
13R	0.0045	0.0047	0.0045	0.0054	0.0067*	0.0059	0.0058	0.0061	0.0063	0.0080**
Kidney; absolute weight (g)										
Treatment week										
52	5.81	5.40	6.26	6.18	5.87	3.78	3.59	3.50	3.76	3.89
89/91	6.32	7.29	7.24	8.56**	9.86*	4.23	4.51	4.15	5.75	4.89
13R	6.03	6.01	6.80	8.22	6.20	3.92	3.72	3.71	4.16	4.32
Kidney; relative weight (% body weight)										
Treatment week										
52	0.702	0.627	0.732	0.799	0.764	0.831	0.764	0.763	0.850	0.916
89/91	0.737	0.741	0.791	1.144**	1.354**	0.716	0.829*	0.751	1.207**	1.119**
13R	0.719	0.745	0.716	0.994	0.940	0.742	0.701	0.744	0.774	1.038*
Adrenal; absolute weight (g)										
Treatment week										
52	0.063	0.060	0.068	0.068	0.067	0.090	0.089	0.087	0.078	0.096
89/91	0.074	0.088	0.086	0.092	0.109**	0.108	0.138	0.123	0.125	0.128
13R	0.065	0.063	0.063	0.068	0.065	0.092	0.191	0.097	0.092	0.097
Adrenal; relative weight (% body weight)										
Treatment week										
52	0.0076	0.0069	0.0081	0.0092	0.0088	0.0198	0.0192	0.0190	0.0178	0.0229
89/91	0.0086	0.0088	0.0094	0.0124*	0.0155*	0.0188	0.0256	0.0222	0.0262*	0.0299*
13R	0.0077	0.0078	0.0066	0.0082	0.0098	0.0177	0.0316	0.0199	0.0173	0.0233*

	Dose level (ppm)									
	Males[a]					Females[a]				
	0	0.5	1.5	30	300	0	0.5	1.5	30	300
Spleen; absolute weight (g)										
Treatment week										
52	1.229	1.161	1.276	1.121	1.248	0.710	0.714	0.669	0.646	0.647
89/91	1.224	1.939*	1.540*	1.790	1.558*	0.858	0.987	0.822	0.918	0.876
13R	1.235	1.244	1.374	1.515	1.176	0.908	0.812	0.831	0.769	0.714
Spleen; relative weight (% body weight)										
52	0.1471	0.1333	0.1498	0.1413	0.1630	0.1561	0.1502	0.1455	0.1471	0.1529
89/91	0.1416	0.1963	0.1633	0.2378	0.2122**	0.1453	0.1802*	0.1468	0.1900**	0.1939**
13R	0.1407	0.1505	0.1440	0.1812*	0.1757	0.1677	0.1523	0.1673	0.1411*	0.1692
Non-neoplastic findings										
Incidence of chronic progressive nephropathy (incidence/total animals)										
Week 52	5/12	3/14	5/14	7/15	9/12	4/14	5/14	3/14	6/14	7/13
Week 89/91	8/20	11/14*	17/22*	17/20**	11/12**	6/23	12/21	5/21	9/13*	12/22
Week 13R	7/13	7/15	7/13	8/13	6/10	5/13	4/13	5/11	11/15	9/10*
Week 52	5/12	3/14	5/14	7/15	9/12	4/14	5/14	3/14	6/14	7/13
Week 89/91	8/20	11/14*	17/22*	17/20**	11/12**	6/23	12/21	5/21	9/13*	12/22
Neoplastic findings										
Malignant follicular cell carcinoma, incidence (incidence/total animals, [%])										
Week 89/91	0/49 [0]	0/48 [0]	0/50 [0]	0/50 [0]	5/50* [10]	0/50 [0]	1/50 [2.0]	0/50 [0]	1/50 [2.0]	2/50 [10]
Recovery phase	0/15 [0]	0/15 [0]	0/15 [0]	1/14 [7.1]	1/12 [8.3]	0/14 [0]	0/13 [0]	0/15 [0]	0/15 [0]	0/14 [0]
Benign follicular cell adenoma, incidence (incidence/total animals, [%])										
Week 89/91	0/49 [0]	1/48 [2.1]	5/50* [10]	3/50 [6]	12/50*** [24]	0/50 [0]	0/50 [0]	0/50 [0]	0/50 [0]	8/50** [16]
Reversibility period	0/15 [0]	0/15 [0]	0/15 [0]	0/14 [0]	1/12 [8.3]	0/14 [0]	0/13 [0]	1/15 [6.7]	0/15 [0]	2/14 [14.3]
Total thyroid tumours, incidence (incidence/total animals, [%])										
Week 89/91	0/49 [0]	1/48 [2.1]	5/50* [10]	3/50 [6]	17/50*** [34]	0/50 [0]	1/50 [2]	0/50 [0]	1/50 [2]	10/50*** [20]
Recovery phase	0/15 [0]	0/15 [0]	0/15 [0]	1/14 [7.1]	2/12 [16.7]	0/14 [0]	0/13 [0]	1/15 [6.7]	0/15 [0]	2/14 [14.3]
Rat thyroid tumour historical control range (as %)										
Malignant follicular cell carcinoma										
	0 – 6					0 – 10				
Benign follicular cell adenoma										
	2 – 10					0 – 4				

[a] Male values: weeks 0–88; Female values: weeks 0–90; R: denotes week of recovery phase

[b] week of recovery/reversibility phase (specified week "off-dose") * $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.001$

2.4 Genotoxicity

(a) In vitro studies

All assays were validated by the appropriate use of positive and negative controls as appropriate.

Table 4. Summary of in vitro genotoxicity studies

Study type	Test system	Dose levels and comments	Purity (%)	Result	Reference
Bacterial reverse mutation test	*S. typhimurium* strains TA 1535, TA 1537, TA 98 and TA 100	Test 1: 0, 0.8, 4, 20, 100 and 500 µg/plate (± S9) Test 2: 0, 25, 50, 100, 200 and 400 µg/plate (± S9) Toxicity observed at ≥ 400 µg/plate	95–97	Negative	Clare, 1988a
	E.coli strain WP2 uvrA	0, 20, 100, 500, 2500, and 5000 µg/plate Toxicity observed at ≥ 2500 µg/plate	98.9	Negative	Engelhardt & Leibold, 2005a
	S. typhimurium strains TA 1535, TA 1537, TA 98 and TA 100 *E.coli* strain WP2 uvrA	All strains: 20–5000 µg/plate (± S9) TA 1537: – S9: 10–2500 µg/plate + S9: 4–1000 µg/plate	79.1[a]	Negative	Schulz & Hellwig, 2006
Rec assay	*B. subtilis*	500–20000 µg/disk	95	Negative[b]	Kurita, 1994
Chromosome aberration	Human lymphocytes	0, 75, 150 and 300 µg/ml (± S9) Limit of solubility = 300 µg/ml	95–97	Negative[c]	Marshall, 1988a
	Chinese hamster Lung cells	Without S9 mix: 0, 30, 45 and 60 µg/ml; (6 h exposure); 0, 7.5, 15, 22.5 and 30 µg/ml (24 h and/or 48 h exposures) With S9 mix: 0, 15, 30 and 60 µg/ml; (6 h exposure) In all cases, positive only at cytotoxic concentrations	98.3	Positive[d]	Wright, 1995
Gene mutation in mammalian cells	Chinese hamster lung V79 cells	0, 0.8, 4, 20, 100 and 500 µg/ml (± S9) Toxic concentration ≥ 100 µg/ml	97.2	Negative	Lloyd, 1993
	GADD45a-GFP GreenScreen HC assay in human lymphoblastoid TK6 cell line	0, 50, 100, 200 µM Positive (AC_{50} = 44.76 µM) but only at cytotoxic concentrations; lower limit of cytotoxicity 8.74 µM	> 90	Positive[e]	Knight et al., 2009
CellCiphr Cytotox Profiling Panel	Human-derived HepG2 cells	10 concentrations (two-fold dilutions) from 200 to 0.39 µM	> 90	Negative[e]	Knight et al., 2009

(continued on next page)

Study type	Test system	Dose levels and comments	Purity (%)	Result	Reference
Invitrogen CellSensor p53RE-bla HCT-116 assay	HCT-116 cells with a stably-integrated β-lactamase reporter gene under control of p53 response elements	15 concentrations ranging from 1.2 nM to 92 µM	>90	Negative[e]	Knight et al., 2009

AC_{50}: Concentration that gives 50% maximal activity

[a] Formulation containing approximately 80% fipronil (supplemental information)

[b] Assay currently considered obsolete and no longer included in the test batteries for genotoxicity evaluations

[c] The study essentially complies with the current OECD TG 473 except that 200 instead of 300 metaphases per test point were scored

[d] Positive at six-hour exposure without S9 mix at toxic concentration (60 µg/mL).
The ToxCast TOX21_DT40_100 assay findings indicated upregulation of Ku70–Rad54 gene expression with AC_{50} of 2.34 µM. This is about the same as the AC_{50} for mitochondrial membrane depolarization (2.02 µM), a key marker of depleted ATP production, cellular stress and potential cell death. Given that both Rad54 and Ku proteins are involved in the repair of DNA double-strand breaks (their upregulation is a response to genomic damage) this supports the conclusion that chromosomal aberrations due to DNA double-strand breaks associated with fipronil are likely associated with cytotoxicity rather than direct effects on DNA.

[e] Assay not validated and currently not included in the test batteries for genotoxicity evaluations

(b) In vivo studies

Table 5. Summary of in vivo genotoxicity studies

Study type	Test system	Dose levels Comments	Purity (%)	Result	Reference
Mouse micronucleus	CD-1 mice erythrocyte bone marrow cells	0, 1, 5 and 25 mg/kg bw, oral gavage No bone marrow toxicity; maximum dose was 25% of the acute oral LD_{50}	97.2	Negative	Edwards, 1993
	CD-1 mice erythrocyte bone marrow cells	0, 12.5, 25 and 50 mg/kg bw, oral gavage No bone marrow toxicity; maximum dose was 50% of the acute oral LD_{50}	96.2	Negative	Edwards, 1995
	NMRI mice erythrocyte bone marrow cells	0, 12.5, 25 and 50 mg/kg bw per day, oral gavage	78.8[a]	Negative	Schulz, 2011
Unscheduled DNA synthesis (UDS assay)	Rat primary hepatocytes	0, 12.5, 25 and 50 mg/kg, oral gavage; maximum dose was 50% of the acute oral LD_{50}	91.7	Negative[b]	Engelhardt & Leibold, 2004a

[a] Formulation containing approximately 80% fipronil (supplemental information)

[b] Based on the DNA lesion and tissue specificity, it is widely accepted that the in vivo UDS assay is not sufficiently sensitive (EFSA, 2017)

Many of the submitted in vitro studies are historical, and while they were performed in accordance with guidelines which were relevant at the time they were conducted, these assays are not consistent with current international guidelines and standards. Studies using the BAS 350 00 I WG formulation, containing approximately 80% fipronil, have been summarized as supplemental information. All assays were validated by the appropriate use of positive and negative controls. The positive finding

in the GADD45a-GFP GreenScreen HC assay in human lymphoblastoid TK6 cells was only seen accompanied by substantial concurrent cytotoxicity. In the in vitro chromosomal aberration test in Chinese hamster lung cells, positive results were only obtained in the presence of substantial cytotoxicity following six hours of exposure. A second in vitro chromosomal aberration assay in human lymphocytes, which evaluated fipronil concentrations up to the limit of solubility, was negative. Fipronil did not induce clastogenic effects in two mouse in vivo micronucleus assays which used systemically toxic oral gavage doses up to 50% of the acute oral LD_{50}. Based on the dispositional information for fipronil summarized in section 1.1, bone marrow exposure would have occurred in these studies. Furthermore, a formulation containing approximately 80% fipronil did not induced clastogenic effects in a third mouse in vivo micronucleus assay. Nor did Fipronil induce an increase in unscheduled DNA synthesis (UDS) in an in vivo rat hepatocyte UDS assay.

Overall, based on the weight of evidence, it is concluded that fipronil at levels likely to occur via dietary exposure, does not raise concerns with respect to genotoxicity.

2.5 Mechanistic studies: thyroid function studies in rats

Three studies of thyroid function in the rat were reported. An [^{125}I]thyroxine plasma clearance study is summarized in Table 6a, a perchlorate discharge study in Table 6b and a biliary [^{125}I]thyroxine excretion study in Table 6c. The human relevance of these findings is discussed in Appendix 1.

Table 6a. Summary of the effects of fipronil on thyroid function in in rats; [^{125}I]thyroxine plasma clearance in male SD rats (Peters, 1991a)

Group *n*	Fipronil dose regimen	Positive control dose regimen	[^{125}I] T4 plasma kinetics			
			Group	**Terminal $t_{½}$ (h)**	**Clearance (mL/minute)**	**Volume of distribution (mL)**
6	1 or 14 oral doses of 0 or 10 mg/kg bw per day	1 or 14 oral doses of phenobarbital at 80 mg/kg bw per day	**Single dose (mean ± SD)**			
			Negative control	17.2 ± 2.5	0.0548 ± 0.0052	80.54 ± 6.55
			Fipronil	15.6 ± 3.0	0.0606 ± 0.0073	80.43 ± 4.10
			Phenobarbitone	14.1 ± 0.5**	0.0722 ± 0.0053**	87.83 ± 5.91*
			Multiple dose (mean ± SD)			
			Negative control	22.5 ± 2.4	0.0568 ± 0.0050	110.05 ± 2.41
			Fipronil	11.8 ± 1.5**	0.1484 ± 0.0174**	150.31 ± 14.41**
			Phenobarbitone	15.5 ± 2.6**	0.1045 ± 0.0168**	137.83 ± 12.79**

SD: Standard deviation' T4: Thyroxine

Statistical evaluation: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; Student′s *t*-test

For control groups, levels of significance are for comparisons of data between day 14 and day 1 control group animals.

Summary

Administering fipronil in14 daily oral doses of 10 mg/kg bw per day to male rats stimulated whole blood clearance of T4-derived radioactivity when compared to the controls (a 48% decrease in terminal half-life, 161% increase in clearance and 37% increase in the volume of distribution). Under these circumstances the effects of fipronil were similar to those of phenobarbital. Little change in the pharmacokinetic parameters of T4-derived radioactivity occurred following a single oral dose of 10 mg/kg bw of fipronil.

Table 6b. Summary of the effects of fipronil on thyroid function in in rats; perchlorate discharge test in male SD rats (Peters 1991b)

Group *n*	Fipronil dose regimen	Positive control dose regimen	^{125}I-derived radioactivity in thyroids (total percentage of dose/pair of thyroids)			
			Dose group	**Saline [% control]**	**10 mg/kg bw PC [% control]**	**25 mg/kg bw PC [% control]**
27	14 oral doses of 0 or 10 mg/kg bw per day	14 oral doses of 10 mg/kg bw per day of propylthiouracil (PTU) 14 IP doses of 50 mg/kg bw per day of noxythiolin (NT)	Negative control	3.078 [100]	3.191 [100]	2.795 [100]
			Fipronil	5.541** [180]	5.245** [164]	7.086** [254]
			PTU	1.388** [45]	0.316** [10]	0.237** [8]
			NT	6.355** [206]	7.267** [228]	7.777** [278]
			Thyroid weight			
			Group	**Overall group mean (g [%])**		
			Negative control	0.017 [100]		
			Fipronil	0.021** [124]		
			PTU	0.054** [318]		
			NT	0.023** [135]		

PB: Phenobarbital; PTU: propylthiouracil; IP: intraperitoneal; PC: perchlorate;
NT: noxythiolin, a thiourea inhibitor of thyroid iodide organification;

Statistical evaluation: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; Student's *t*-test
For control groups, levels of significance are for comparisons of data between day 14 and day 1 control group animals.

Summary

Fourteen consecutive daily doses of 10 mg/kg bw per day of fipronil to male rats stimulated thyroid follicular activity as evidenced by slightly increased thyroid weight and increased thyroid accumulation of ^{125}I. However, there was no evidence of an inhibition of the organification of iodide since no efflux of thyroidal ^{125}I was seen following potassium perchlorate administration.

Table 6c. Summary of the effects of fipronil on thyroid function in in rats; [^{125}I] thyroxine biliary excretion study in bile duct-cannulated male SD rats

Dose group *n*	Number of daily doses	Dose level (mg/kg bw per day)			
		Control	Fipronil[a]		Phenobarbital[b]
		0	1	10	80
Weight of bile fluid excreted during 0–5 h post dose (g [% control])					
3	1	2.91 [100]	3.29 [113]	3.31 [114]	3.72 [128]
3	14	8.1990 [100]	4.23 [99]	6.02** [141]	6.06** [142]
Amount of [^{125}I] T4 excreted in bile during 0–5 h post dose (total ng equivalents [% control])					
3	1	14.1306 [100]	16.8799 [120]	18.4011 [130]	21.2047 [150]
3	14	8.1990 [100]	23.1201 [182]	28.0671 [242]	33.1797 [305]
Biliary clearance of radioactivity after intravenous infusion of [^{125}I] T4 (mL/hour [% control])					
3	1	0.799 [100]	0.991 [124]	1.027 [129]	1.619 [203]
3	14	2.421 [100]	3.669 [152]	9.830 [406***]	4.356 [180*]
Amount of conjugated [^{125}I] T4 in pooled bile samples (ng [% control])					
3	1	20.1588 [100]	29.8059 [148]	31.5063 [156]	35.0131 [174]
3	14	11.5983 [100]	36.0189 [311]	46.7362 [403]	551.7349 [446]

[a] Fipronil dose regimen: 1 or 14 oral doses of 1 or 10 mg/kg bw per day; Source: Taylor, 1993

[b] Positive control dose regimen: 1 or 14 oral phenobarbital doses of 80 mg/kg bw per day; T4: Thyroxine;

Statistical evaluation: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; Student's *t*-test

For control groups, levels of significance are for comparisons of data between day 14 and day 1 control group animals.

Summary

Fourteen consecutive daily doses of 10 mg/kg bw/d of Fipronil to male rats resulted in increased biliary clearance of T4 and in T4-conjugated biliary products

The combined results of the mechanistic studies summarized in Table 6 demonstrate that the effects of fipronil on thyroid hormones results from a disturbance of thyroid–pituitary hormonal feedback secondary to increased biliary clearance of T4 and not from the direct effects on the thyroid. Rats are known to be much more sensitive to these effects than humans due to what is described as the lower thyroid efficiency in rats, associated with differences in the metabolic catabolism in rats compared to humans, the shorter terminal half-life of circulating thyroid hormones in rats and the lower levels of circulating/extracellular thyroid hormone molecular chaperone proteins in rats which render circulating thyroid hormones more available for catabolism. These effects in rats render them more sensitive than humans to non-genotoxic thyroid neoplasia, secondary to disturbances of the hypothalamic–pituitary–thyroid axis.

Contrasting with the in vivo data summarized in Table 7 are the findings of the ToxCast high throughput screening NHEERL_MED_hDIO3_dn assay (see below). This enzyme reporter assay evaluates the regulation of the DIO3 gene which codes for human Type III iodothyronine deiodinase. The AC_{50} for this assay was 1.16 µM (scaled top approximately 5; compare this with the AC_{50} for mitochondrial membrane depolarization of 2.02 µM) with an analysis direction of negative. The relationship of this finding to human health remains uncertain.

2.6 Reproductive and developmental toxicity

(a) Multigeneration studies

Rat

In a rat multigenerational reproductive toxicity study, SD rats were fed fipronil at dietary concentrations of 0, 3, 30 or 300 ppm (equal to 0, 0.25, 2.54 and 26.03 mg/kg bw per day in males [combined generations], 0, 0.27, 2.74 and 28.40 mg/kg bw per day in females [combined generations]) continuously for two generations. Two litters were derived from the first (F0) generation and a single litter from the second F1 generation. At the 300 ppm high-dose level, mortality, convulsions, reduced body weight gain and food consumption were seen in F0 and F1 adults. Mating performance of the F1 generation was slightly reduced at this dose level (83% compared with 100% in controls) with a consequent reduction in the fertility index. Increased liver and thyroid weights (absolute and body weight relative) were seen at both 30 ppm and 300 ppm in adults of both generations. The increased liver weight at 300 ppm was accompanied by increased incidences of hepatic centriacinar fatty vacuolation. Increased thyroid weights at 30 ppm and 300 ppm were associated with follicular epithelial hypertrophy in the F0 and F1 adults (except for females fed 30 ppm). This was also seen in F1 males given 3 ppm fipronil. Absolute pituitary weights were low in F1 females fed 30 ppm and 300 ppm. Toxicity was observed in F1A and F2 generation offspring at the 300 ppm high dose as evidenced by reduced body weight gain during lactation and an associated slight delay in tooth eruption in F1A litters and pinna unfolding in F2 litters. Prenatal and postnatal viability and body weight gain were also reduced in both the F1A and F2 offspring. Viability indices at birth and day 4 postpartum (PP; prior to culling) were 83% and 89%, respectively, for the F1A offspring, and 78% and 73% respectively for the F2 offspring, compared with control values of 97–100%. In addition, convulsions were observed in thirteen F1A offspring and five F2 pups between days 14 and 20 PP, when they were starting to consume the diet. No other abnormalities were evident. At 30 ppm of fipronil there were increases in liver and thyroid weights (absolute and relative) in both sexes of both adult generations. Follicular epithelial hypertrophy in the thyroid, the only histopathological finding, was seen in F0 males and both sexes of the F1 adults.

Taking into account the findings of the human relevance mode of action framework evaluation on thyroid effects in rats (see Appendix 1) the NOAEL for systemic toxicity, offspring toxicity and reproductive toxicity was 30 ppm (equal to 2.54 mg/kg bw per day) (King, 1992).

(b) Developmental toxicity

Rat

In a rat prenatal toxicity study, dose levels of 0, 1, 4 or 20 mg/kg bw per day of fipronil were administered by oral gavage to groups of 25 mated SD rats from days 6–15 postcoitum (PC). A low level of maternal toxicity, manifesting as a decrease in body weight gain following dosing at 20 mg/kg bw per day, was observed (about 10% over days 6–20 PC; about 61% over days 6–10 PC) . Water consumption was slightly increased from days 8–20 PC Marginally lower weight gain was also observed at 4 mg/kg bw per day, but this was considered to be of doubtful biological significance. There was no effect upon litter parameters or on fetal weight nor treatment-related effects on fetal morphology.

The NOAEL for maternal toxicity was 4 mg/kg bw per day and the NOAEL for developmental toxicity was 20 mg/kg bw per day, the highest dose tested (Brooker & David, 1991).

Rabbit

In a rabbit prenatal toxicity study, dose levels of 0, 0.1, 0.2, 0.5 or 1.0 mg/kg bw per day were administered by oral gavage from days 6–19 *p.c.*to groups of 22 mated New Zealand White rabbits. Dose levels $\geq$ 0.2 mg/kg bw per day were maternally toxic, as demonstrated by consistent, significant ($p < 0.05$) reductions in body weight gain (up to about 50%) over days 6–18 *p.c.* (and over days *p.c.* 6–28 for doses $\geq$ 0.5). Marginally low body weight gain at 0.1 mg/kg bw per day over days 6–10 *p.c.* was considered to be of doubtful biological significance bearing in mind the inherent variability in rabbits. There was no effect on litter parameters, fetal and placental weights or fetal morphology. The NOAEL for maternal toxicity was 0.2 mg/kg bw per day. The NOAEL for developmental toxicity was 1.0 mg/kg bw per day, the highest dose tested (King 1990). This NOAEL is the same as that identified by JMPR in 1997.

(c) Effect of hypothalamic–pituitary–thyroid axis disturbance on rat fetal development

Groups of 45 presumed pregnant female SD rats received a basal diet containing 3 ppm of 6-propylthiouracil (6-PTU; positive controls) or fipronil at 0.1, 0.3, 1.0 or 3.0 mg/kg bw per day from gestation day 6 (GD 6) until weaning of the offspring on postnatal day 21 (PND 21). On GDs 20 and 21 dams per group and their fetuses were terminated to gather blood samples for the determination of thyroid hormones. The remaining dams were allowed to litter and rear their pups until PND 4 or PND 21. On PND 4 or PND 21 blood samples were collected from pups and on PND 21 additionally from dams, for the determination of thyroid hormones. Thyroid glands were examined histopathologically in all dams and selected offspring. The validity of the study was confirmed by the responses of the treated dams to the 6-PTU thyrotoxic positive control.

There were no adverse effects on maternal survival, clinical condition, food consumption or body weight development during gestation and lactation. Nor were there any adverse effects of fipronil or 6-PTU administration on the survival, general physical condition or body weight development of the F1 offspring. Prenatal and postnatal development was normal in all dose groups. Serum T4 level was significantly decreased and TSH level significantly increased (both at $p < 0.05$)in the high dose dams on GD 20. However, no toxicologically relevant changes in thyroid hormones were noted up to the end of the lactation period at lactation day 21 (LD 21). Treatment with fipronil had no effect on thyroid weights in the dams, although increased liver weight (adaptive) was apparent in the high-dose dams on GD 20 and in the dams dosed at 1 mg/kg bw per day or more on LD 21. Increased thyroid follicular height and larger colloid area was also observed on LD 21 in dams dosed at 1 mg/kg bw per day and above.

In the high-dose GD 20 fetuses T4 levels were decreased but TSH levels were unaffected by fipronil exposure. Fetal thyroid and liver weights were not affected, and no relevant histopathological changes were observed in the thyroids of those fetuses. In the high-dose PND 4 offspring T4 and T3 levels were decreased, but TSH levels were unaffected by fipronil treatment. Liver weight was increased in male pups in the treatment cohorts receiving 1.0 mg/kg bw per day or more, however, no relevant organ weight or histopathological changes were observed in thyroids of suckling pups from fipronil-treated dams. In examining PND 21 weanlings, which in addition to potential transmammary exposure had dietary exposure to fipronil for about one week, increased follicular height and larger colloid area was apparent in the cohorts receiving 1.0 mg/kg bw per day or more despite the absence of effects on thyroid hormones and thyroid weights. Increased liver weight occurred in the treatment cohorts receiving 0.3 mg/kg bw per day and above.

The NOAEL for maternal and developmental thyrotoxicity was 0.3 mg/kg bw per day (Coder, 2019). Based on the mode of action/adverse outcomes pathaway evaluation (see Appendix 1) these findings have only limited if any relevance to humans exposed to fipronil via the diet.

2.7 Special studies

(a) Neurotoxicity

Rat

Study 1

In an acute neurotoxicity study groups of 15 male and 15 female SD rats were given a single oral gavage dose of 0, 0.5, 5.0 or 50.0 mg/kg bw of fipronil. Dosing at 50 mg/kg bw resulted in convulsion-associated deaths starting two days post dose. Convulsions also occurred in five males and one female high-dose animals which survived. Reduced body weight ($p < 0.05$) occurred in the high-dose group along with other clinical signs of neurotoxicty. These were mostly limited to a 7–8 h period post dose and consisted of the following: ptosis, abnormal gait, fine tremors, myoclonis, head bobbing and decreased hind leg splay, arousal and rearing activity in the open field test and decreased approach response, tail pinch and air righting reflexes. Reduced muscle tone, pupil size, body temperature and motor activity were also noted. Some evidence of neurotoxicity (decreased hind leg splay, decreased rearing in females) occurred in the 5.0 mg/kg bw dose cohort. Signs of neurotoxicity were not present at 14 days post dose. The NOAEL for the study was 0.5 mg/kg bw (Gill, Wagner & Driscoll, 1993).

Study 2
In a second acute neurotoxicity study, groups of groups of 10 male and 10 female SD rats were administered a single oral gavage dose of 0, 2.5, 7.5 or 25 mg/kg bw of fipronil. Effects in the high-dose group included reduced body weight gain ($p < 0.05$), impaired food efficiency and clinical signs consistent with neurotoxicity. The symptoms were mostly limited to within seven hours post dose and consisted of: decreased hind limb splay, increased foregrip strength in males, unusual behaviour, reduced motor activity and decreased body temperature). Reduced body weight gain ($p < 0.05$) in females and decreased hind leg splay in males (7 h post dose) occurred following dosing at 7.5 mg/kg bw. The NOAEL for the study was 2.5 mg/kg bw (Hughes, 1997). Taking into account the different dose ranges evaluated by Gill et al. (1993) and Hughes (1997) the overall combined NOAEL for the two studies was 2.5 mg/kg bw.

Study 3
In a 90-day repeat oral dose neurotoxicty study, groups of 15 male and 15 female SD rats were administered fipronil in the diet at concentrations of 0, 0.5, 5.0 or 150 ppm (equal to 0, 0.03, 0.3 and 8.89 mg/kg bw per day in males, 0, 0.04, 0.35 and 10.78 mg/kg bw per day in females). At 150 ppm, body weight gain was reduced ($p < 0.05$) in the first two weeks of the study by as much as 60% compared with the control for both sexes in week 1 (correlated with reduced food consumption) and by about 17% in males in week 2. However, this effect was completely reversed by the end of the study. Given that dietary palatability was affected in the 28-day repeat dose dietary study in rats at dietary concentrations greater than 100 ppm it is likely that the effects on body weight and food consumption in this study relate to palatability. There was no evidence of any neurological effect.

In 1997 JMPR noted:

> *"Functional changes seen in animals at the high dose in week 4, 9, and/or 13 were increased urination and an increased incidence of exaggerated response to tail pinching in males, an increased incidence of exaggerated startle responses during manipulation in animals of each sex, and increased forelimb grip strength in females at week 13. No treatment-related findings were seen at necropsy or during histopathological examination of nervous tissues. The changes in animals at the high dose may not have been related to treatment; however, these findings, although relatively minor when taken separately, represent a minimal effect of treatment when taken in toto. In addition, the exaggerated responses to touch and sound, particularly in males, and the increased urination of males at the high dose were considered to be related to treatment in the preceding study. Therefore, the NOAEL for both neurotoxicity and systemic toxicity was 5 ppm, equal to 0.3 mg/kg bw per day."*

These findings were re-evaluated at JMPR 2021 and the following conclusions reached:

A statistically significant increase in the number of male animals with urine present in the observation arena was observed at the week 4 evaluation regarding the 150.0 ppm dose group. This increased incidence was not considered to be biologically significant due to the lack of accompanying autonomic changes and the corresponding increase in the incidence of the presence of urine in the observation area in all other groups since the pretreatment evaluation. During the week 4 evaluation, 4/10 males in the 150.0 ppm group displayed an exaggerated startle and tail pinch response. No other parameters seemed to be affected and the exaggerated responses were only transient. Overall these changes were not considered to be treatment-related. A statistically significant increase in forelimb grip strength for females in the 150.0 ppm group was observed at the week 13 evaluation. This increase was believed to be the result of a decrease in control values and was not considered biologically significant since the values for the 150.0 ppm group were consistent with the values obtained at the two previous evaluations. Based on the reconsideration of these data the NOAEL for neurological effects was 150 ppm (equal to 8.9 mg/kg bw per day), the highest dose tested (Driscoll & Hurley, 1993).

Study 4
In a postnatal developmental neurotoxicity study, fipronil was administered continuously via the diet to groups of 30 parental female SD rats at dietary concentrations of 0, 0.5, 10 or 200 ppm (equal to 0, 0.05, 0.91 and 15.17 mg/kg bw per day) from day 6 PC to day 10 of lactation. At 200 ppm, there were two maternal deaths during lactation, one on day 6 postpartum and another on day 9 postpartum. The cause of these deaths was not determined. No clinical signs preceded either death and

the only finding at necropsy was discolouration of the lungs. No treatment-related clinical signs were seen in other animals. Transient reductions (less than 10% compared to control; $p < 0.05$) in maternal body weight parameters (correlating with changes in food consumption) occurred in the high-dose group during gestation. Pregnancy rates and parturition were unaffected by treatment. At 200 ppm the mean number of dead pups at day 0 PP was increased (1.1 compared with 0.1 in control; $p < 0.05$), the mean number of live pups at day 4 PP was decreased (12.9 compared with 14.6 in control; $p < 0.05$) and the day 4 PP viability index was reduced (75.5% compared to 98.9% in control; $p < 0.05$). However, after day 4 PP, survival was unaffected by treatment. At 10 ppm and greater female pup body weights were reduced (minus 5–20% compared with control values; $p < 0.05$) over days 0–21 PP. At 200 ppm male body weights were reduced (–9% to –25% compared with control values; $p < 0.05$) over days 0–21 PP At 10 ppm, male body weights were also reduced (about –0.7% of control value; $p < 0.05$) on day 4 PP, but this was considered non-adverse. No statistically significant differences in pup body weights were noted following weaning. A marginal delay in lower incisor eruption, delayed swimming development and reduction in lower maximum voltage eliciting an auditory startle response occurred at 200 ppm on day 21 PP ($p < 0.05$). No neuroanatomic abnormalities were detected. The NOAEL for maternal toxicity, postnatal developmental neurotoxicity and developmental toxicity was 10 ppm (equal to 0.91 mg/kg bw per day) (Mandella, 1995).

Dog

In a repeat daily oral dose study of neurotoxicity, four female dogs were given 20 mg/kg bw per day of fipronil until signs of neurotoxicity were observed (from day 6 onwards). Treatment was then terminated and the dogs retained untreated over a 28-day reversibility period. One female dog was fed no fipronil as a control. Clinical signs of neurotoxicity observed in fipronil-exposed dogs were: underactivity, abnormal gait, tremors, stiffening of the limbs or body, convulsions, head nodding, facial twitches, central blindness; depression of the blink, tactile and visual placing, pupillary, consensual light reflexes; reduced extensor postural thrust, tonic neck, hind limb hopping and flexor responses, together with stance and gait abnormalities; exaggerated flexor, gag and corneal reflexes, hind limb hopping and extensor postural thrust responses. Signs of neurotoxicity persisted for up to 10 days following the cessation of dosing. There was no evidence of neurological abnormalities in the control animal. There were no neuroanatomical abnormalities (Holmes, 1991d).

(b) Phototoxicity

Fipronil at concentrations up to 215.4 µg/mL was not phototoxic in an appropriately validated in vitro 3T3 NRU phototoxicity test (Landsiedel & Cetto, 2015).

(c) In vitro primary pharmacology studies

Based on ion channel studies using whole cell and single channel recordings made from HEK 293 cells transiently expressing rat α1β2γ2L $GABA_A$ receptors, fipronil induced the accumulation of receptors in a novel, long-lived, blocked state. This process proceeds in parallel with, and independently of, channel desensitization. The lower potency of fipronil-sulfone (M&B 46136) indicates that conversion to this metabolite may serve as a detoxifying process in mammalian brain (Li & Akk, 2008).

(d) Secondary and safety pharmacology studies

The general secondary and safety pharmacology of fipronil was investigated male Wistar rats and male ICR mice following single oral (gavage) dosing; details are summarized in Table 7. The overall NOAEL for secondary and safety pharmacological effects was 3 mg/kg bw (Horri 1994).

Table 7. Secondary and safety pharmacology properties of fipronil in rodents

Test	Species & group size	Fipronil dose mg/kg bw	Positive control	Observations	Key findings
Central nervous system					
General signs	3 mice	0,10, 30, 100, 300	None	1, 2, 4, 6, 8 and 24 hours; 1, 3, 4, and 7 days	Convulsions 6–8 h post dose at ≥ 30 mg/kg bw; Clinical signs of neurotoxicity (straub tail, tremor, pupil dilation,) at 8 h post dose at ≥ 30 mg/kg bw; Deaths, reduced activity, reduced motor activity, CNS excitation at ≥ 100 mg/kg. NOAEL 10 mg/kg bw
Convulsive effects					
Electro-convulsions	10 mice	0, 3, 10, 30; 6 h prior electrical stimuli	40 mg/kg PTZ (sc); 15 min prior electrical stimuli	After 0.5 s electrical stimulus to the cornea: TF, TE. CL, CO & death	No effect. NOAEL 30 mg/kg bw
Pentylene-tetrazol seizure threshold	10 mice	0, 3, 10, 30; 6 h prior electrical stimuli	40 mg/kg PTZ (sc); 6 h prior electrical stimuli + 150 mg/kg caffeine (oral), 15 min prior PTZ	TE, deaths	Convulsions and convulsion-associated deaths at ≥ 10 mg/kg bw. Fipronil at ≥ 10 mg/kg bw lowered the PTZ seizure threshold; at 30 mg/kg bw the effects were 50% of the positive control. NOAEL 3 mg/kg bw
Body temperature	6 rats	0, 3, 10, 30	None	Rectal temp.: prior & at 1, 2, 4, 6, 8 and 24 h	No effect. NOAEL 30 mg/kg bw
Autonomic nervous system					
Pupil size	6 rats	0, 3, 10, 30	None	Prior & at 1, 2, 4, 6, 8 and 24 h using stereo microscope	Pupil dilation at 6 h post dose at 30 mg/kg bw NOAEL 10 mg/kg bw
Digestive system					
Intestinal tract transport capability	8 mice	0, 3, 10, 30; 6 hours, thereafter: oral 5% charcoal meal solution	None	30 min after charcoal administration: GI length (start of duodenum to charcoal), passage rate (%, charcoal point relative to total length)	30% Inhibition of gastrointestinal transport at 30 mg/kg bw NOAEL 10 mg/kg bw
Skeletal muscle					
Traction test	8 mice	0, 3, 10, 30	None	Hindlimb tractions within 10 s: 1, 2, 4, 6, 8, 24 h	No effect. NOAEL 30 mg/kg bw

Test	Species & group size	Fipronil dose mg/kg bw	Positive control	Observations	Key findings
			Blood		
Haemolytic effect	6 rats	0, 3, 10, 30	None	After 6 hours: absorbance of plasma measured at 540nm	No effect. NOAEL 30 mg/kg bw

sc: Subcutaneous; GI: Gastrointestinal; PTZ = pentylenetetrazol; TF: Tonic flexor;
TE: Tonic extensor; CL: Clonic convulsion; CO: Coma

(e) Summary of ToxCast high throughput screening evaluations

A summary of the ToxCast high throughput screening evaluations of fipronil in relation to its cytotoxic limit of 11.72 µM in HEK 293 and the concentration resulting in mitochondrial depolarization in human hepatocytes (2.02 µM; TOX21_MMP_ratio_down assay) is shown in Fig. 3. Hits on endpoints with well-characterized human-relevant biological effects and AC_{50} values less than 2.02 µM below the mitochondrial depolarization AC_{50} identified by ToxCast, are discussed below. Mitochondrial depolarization was selected as a comparator because mitochondrial membrane potential is a key indicator of mitochondrial activity since it reflects the process of electron transport and oxidative phosphorylation, the driving force behind ATP production. Mitochondrial membrane depolarization results in reduced cellular ATP production and the potential for cell death and thus is a sensitive marker of celluar stress and cytotoxicity. Given that cytotoxicity was not a feature of fipronil in vivo, findings in assays with AC_{50} values below 2.02 µM are not likely to be relevant to the level of human fipronil exposure via the diet. The fipronil purity used in all ToxCast assays was greater than 90%.

Effects consistent with fipronil acting via the constitutive androstane receptor (CAR)

NVS_NR_hCAR_Antagonist assay:
In this nuclear receptor-binding reporter assay, the AC_{50} was 0.49 µM (positive direction), indicating binding to the CAR receptor (NR1I3) and consistent with the in vivo findings (described above), that fipronil induces CYPs via activation of the CAR xenosensor.

CLD_SULT2A_48h assay:
The AC_{50} for activation of SULT2A1 in a human hepatocyte cell line was 0.19 µM (direction positive), consistent with CAR activation (Runge-Morris, Kocarek & Falany, 2013).

NVS_ADME_hCYP2C19 assay:
The AC_{50} for inhibition of human of CYP2C19 was 0.70 µM, with an analysis direction of positive.

CLD_CYP2B6_24hr assay:
The AC_{50} for the induction of CYP2B6 mRNA in an inducible reporter assay using the Quantitative Nuclease Protection Assay (qNPA) in a human hepatocyte cell line was 0.78 µM, consistent with a phenobarbital-like, CAR-mediated mode of action (Kawamoto et al., 1999).

CLD_CYP2B6_48hr assay:
The results of this assay were consistent with those of the CLD_CYP2B6_24hr assay, with an AC_{50} of 1.64 µM.

NVS_ADME_hCYP2C9 assay:
The AC_{50} for induction of CYP2C9-dependent activity was 1.04 µM, consistent with the CAR activation since this receptor is responsible for the transcriptional activation of CYP2C9 observed in human primary hepatocytes and HepG2 cells (Chen & Goldstein, 2009).

CLD_CYP3A4_24hr:
The AC_{50} for the induction of CYP3A4 mRNA is an inducible reporter assay using qNPA in human hepatocytes was 1.71 µM, consistent with CYP3A4 induction in human hepatocytes (Das et al., 2006) and supporting a phenobarbital-like mode of action for fipronil (Martínez-Jiménez et al., 2007).

CLD_CYP3A4_48hr:
The results of this assay were consistent with those of the CLD_CYP3A4_24hr assay, with an AC_{50} of 1.35 µM.

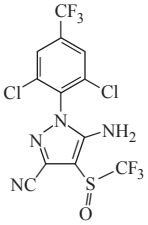
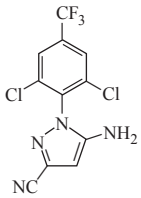
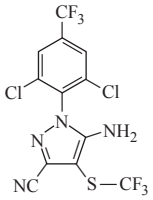
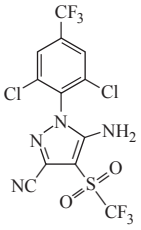
Effects on deiodinases

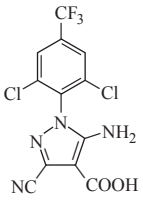
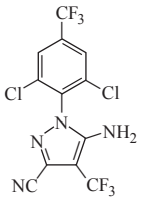
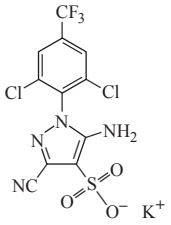
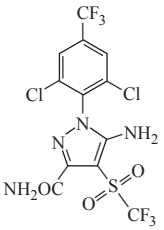
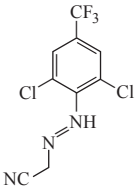
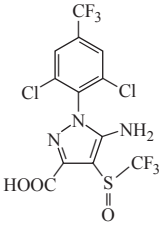
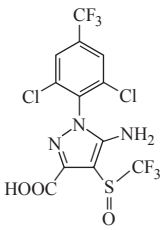
NHEERL_MED_hDIO3_dn assay:
Fipronil downregulated the expression of the DIO3 gene, which codes for human Type III iodothyronine deiodinase (a member of the selenodeiodinase enzyme family). The AC_{50} was 1.16 μM (scaled top approximately 5; compare with the AC_{50} for mitochondrial membrane depolarization of 2.02 μM). DIO3 is the gene that codes for Type 3 iodothyronine deiodinase. Type 3 iodothyronine deiodinase is the physiological inactivator of thyroid hormones, catalyzing the inner ring deiodination of thyroxine to reverse triiodothyronine (rT3), and T3 to 3,3′-diiodothyronine (T2), both of which products are biologically inactive. Downregulating the production of Type 3 iodothyronine deiodinase would, in theory, have a thyroid hormone conservation effect since it would decrease their inactivation. The relevance of this finding to humans exposed to fipronil via the diet is uncertain.

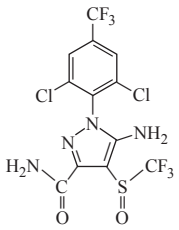
2.8 Studies on metabolites and degradates of fipronil

The chemical structures and toxicological properties of fipronil, its metabolites and environmental degradation products are shown in Table 8.

Table 8. Chemical structures of fipronil, its metabolites and environmental degradation products

Metabolite code	Chemical name	Chemical structure	Major residue in food or feeds?*	Detected in rat metabolism study?	Tox studies available addressing genotoxicology?	Rat brain binding assays?	Tox studies available addressing general toxicology?
M&B 46030 (parent)	Fipronil 5-amino-1-[2,6-dichloro-4-(trifluoromethyl) phenyl]-4-[(trifluoromethyl) sulfinyl]-1H-pyrazole-3-carbonitrile		Yes	Not relevant (parent molecule)	Yes (parent molecule)	Yes	Yes (parent molecule)
M&B 45897	5-amino-1-[2,6-dichloro-4-(trifluoromethyl) phenyl]-1H-pyrazole-3-carbonitrile		No	Identified as conjugate in urine and in bile	Yes	Yes (low binding)	Yes
M&B 45950	Fipronil-sulfide Fipronil-thioether 5-amino-1-[2,6-dichloro-4-(trifluoromethyl) phenyl]-4-[(trifluoromethyl) thio]-1H-pyrazole-3-carbonitrile		Yes	Yes Faeces (maximum 3% of dose) Identified as conjugate in urine	Yes	Yes	Yes
M&B 46136	Fipronil-sulfone 5-amino-1-[2,6-dichloro-4-(trifluoromethyl) phenyl]-4-[(trifluoromethyl) sulfonyl]-1H-pyrazole-3-carbonitrile		Yes	Major metabolite found in all metabolism study species after dosing with parent (including in vitro comparison)	Yes	Yes	Yes

Metabolite code	Chemical name	Chemical structure	Major residue in food or feeds?*	Detected in rat metabolism study?	Tox studies available addressing genotoxicology?	Rat brain binding assays?	Tox studies available addressing general toxicology?
M&B 46400	5-amino-3-cyano-1-(2,6-dichloro-4-trifluoromethylphenyl)-1H-pyrazole-4-carboxylic acid		Yes	Yes Urine, after dosing with M&B 46513	Metabolite of M&B 46513	No	No
M&B 46513	Fipronl-desulfinyl 5-amino-1-(2,6-dichloro-4-(trifluoromethyl) phenyl)-4-trifluoromethylpyrazole-3-carbonitrile		Plants: no Animals: after dosing with M&B 46513	Separate rat metabolism study available	Yes	Yes	Yes
RPA 104615	Potasium 5-amino-3-cyano-1-(2,6-dichloro-4-trifluoromethylphenyl)-1H-pyrazole-4-sulfonate		Yes	Not reported	Yes	Yes (low binding)	Yes
RPA 105320	5-amino-1-(2,6-dichloro-4-trifluoromethylphenyl)-4-(trifluoromethylsulfonyl)-1H-pyrazole-3-carboxamide		Yes	Not reported	Yes (bacterial reverese mutation assay)	Yes (low binding)	No**
RPA 105048	5-amino-1-(2,6-dichloro-4-trifluoromethylphenyl)-4-(trifluoromethyl)-1H-pyrazole-3-carboxamide		Yes	Not reported	Yes (bacterial reverese mutation assay)	No	No**
RPA 108058			Yes	Yes Urine	No Positive QSAR alerts for DNA biding, in vitro bacterial reverse mutagenicity, carcinogenicity	No	No
RPA 200761	5-amino-1-[2,6-dichloro-4-(trifluoromethyl) phenyl]-4-[(trifluoromethyl) sulfinyl]-1H-pyrazole-3-carboxylic acid		Yes	Yes Bile	Yes (bacterial reverese mutation assay)	Yes (low binding)	No**

Meta-bolite code	Chemical name	Chemical structure	Major residue in food or feeds?*	Detected in rat metabolism study?	Tox studies available addressing genotoxicology?	Rat brain binding assays?	Tox studies available addressing general toxicology?
RPA 200766	5-amino-1-[2,6-dichloro-4-(trifluoromethyl) phenyl]-4-[(trifluoromethyl) sulfinyl]-1H-pyrazole-3-carboxamide		Yes	Yes Faeces max. 0.8% of dose; Urine: max. 1.9% of dose	Yes	Yes (low binding)	Yes

* Equal to or greater than 10% total radioactive residue; ** Acute oral study available

(a) Metabolites: biochemical aspects

M&B 45897 (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-1H-pyrazole-3-carbonitrile; (de-trifluoromethyl)sulfinylated fipronil))

Following oral gavage dosing of male SD rats with a single dose of 4 mg/kg bw of [^{14}C]M&B 45897, the majority (about 52%) of the administered radioactivity was excreted in the urine over a 168-hour post dose period. About 37% of the dose was eliminated in the faeces over the same time interval. Orally dosed [^{14}C]M&B 45897 was rapidly absorbed and had a terminal elimination half-life ($t_{½}$) of about 115 hours. Little radioactivity was present in the tissues at 168 hours post dosing, with the highest concentrations in fat, liver and kidneys. The major chemical form present in urine was unchanged [^{14}C]M&B 45897 (about 27% of the urine radioactivity). A multicomponent chromatographic peak named UMET/4 was responsible for about 21% of the radioactivity present in urine. Other metabolites were present at less than 1% of AD. Up to ten [^{14}C]M&B 45897-derived metabolites were present in faeces with the major metabolite being FMET/1, accounting for about 12% of the administered dose (Fisher, 1996).

M&B 45950 (Fipronil-sulfide, fipronil-thioether)

In adult female SD rats given a single oral gavage dose of [^{14}C]M&B 45950 of 50 mg/kg bw or repeated oral gavage doses of 50 mg/kg bw per day for seven days, the major route of elimination was via the faeces which accounted for 66% or more of the administered radioactivity. Repeated dosing was associated with an approximately three-fold increase in the C_{max} for blood radioactivity, however repeated dosing had little effect on elimination rate from the blood. Radioactivity was widely distributed in the tissues but was highest in the fat (Lowden & Fisher, 1989).

M&B 46136 (Fipronil-sulfone)

Fipronil-sulfone (M&B 46136) is the dominant metabolite in all tissues and all mammalian species thus far evaluated. In adult SD rats that received an oral gavage dose of approximately 3 mg/kg bw of uniformly phenyl ring-labelled [^{14}C]M&B 46136, it was slowly excreted with approximately 56% (males) and 71% (females) of the radioactivity being excreted over a seven-day period. Oral bioavailability, based on mass balance, was estimated to be 84% or greater. Fipronil sulfone-derived radioactivity was widely distributed in the tissues with the highest concentrations in fat, adrenals, liver, pancreas and skin and fur. The predominant chemical species in the tissues was M&B 46136, which represented greater than 97% of tissue radioactivity (Odin-Feurtet, 2001). More than 35% of the administered radioactivity remained in the tissues 168 hours post dose. The dominant route of excretion was in faeces with about 45% (males) and 63% (females,) of the radioactivity being eliminated by this pathway. Urinary excretion accounted for 10% or less of excreted radioactivity. Up to 13 different metabolites of M&B 46136 were detected in urine and up to 11 metabolites were detected in faeces. The dominant metabolite in urine was the 5-amino-glucuronide conjugate UMET/3 which represented about 7% and 4% of the dose in males and females respectively. The remaining urinary metabolites were present at less than 1% of AD. The dominant chemical species present in faeces was M&B 46136 which accounted for about 41 and 57%

of AD in males and females respectively. The second most abundant metabolite was FMET/6 which accounted for about 2% of the dose in males and females. The remaining faecal metabolites were present at less tha 1% of AD (Odin-Feurtet, 2001).

A study of M&B 46136 was performed using adult male SD rats which were orally gavage dosed at 4 mg/kg bw, or administered 3 mg/kg bw via infusion into the bile duct over 24 hours, or intravenously dosed at 4 mg/kg bw. This study demonstrated an oral bioavailability for M&B 46136 of about 40%. M&B 46136 was mainly eliminated via the bile following oral and intravenous dosing. Some direct diffusion of M&B 46136 directly into the GI tract occurred. A high degree of enterohepatic circulation was evident (Kemp, 1999b).

M&B 46513 (fipronil-desulfinyl; a photometabolite)

Adult SD rats were orally gavaged with either a single low (1 mg/kg bw) dose of [^{14}C](M&B 46513), repeated low doses (1 mg/kg bw per day for 14 days) of unlabelled M&B 46513) followed by a single oral dose of 1 mg/kg bw of [^{14}C](M&B 46513), or with a single 10 mg/kg bw dose of [^{14}C](M&B 46513). The ^{14}C-labelled M&B 46513 was relatively well absorbed and extensively metabolized by phase I (oxidations) and phase II (sulfate and amino acid conjugation) reactions. Most of the administered radioactivity was eliminated in the faeces. Radioactivity was widely distributed in the tissues with a predominance in fatty tissues and a significant fraction of the radioactivity (8–17%) still present in the carcass at 168 hours post dose. These results and the relatively long elimination half-life suggest the presence of a deep kinetic compartment such as fat. The main metabolic reactions observed included oxidation, sulfonic acid and amino acid conjugation (Totis, 1996). The chemical identification of the various polar urinary and faecal metabolites (sulfate, glucuronide, cysteine, and cysteine–glycine conjugates) is incompletely understood. The cysteine conjugate, the amino sulfate conjugate, the amino glucuronic acid conjugate and M&B 46400 are highly plausible and formed by similar reactions, as seen in the rat studies after dosing with parent fipronil. Less plausible are metabolites which are formed by a replacement of the trifluoromethyl group with a cyano group (Jones & Hardy, 1996).

Acute toxicity and its structure-activity relationships and correlation with rat brain binding

The acute oral toxicity of fipronil and selected metabolites was evaluated in male and female SD rats (see Table 9). Summary data (full study not evaluated) was submitted regarding competitive GABA-gated chloride channel binding assays for fipronil (and its metabolites) with *t*-[^{35}S]butylbicyclophosphorothionate (TBPS) and [^{3}H]1-[(4-ethynylphenyl]-4-*n*-propyl-2,6,7-trioxabicyclo[2.2.2]octane (EBOB).

Overall the brain-binding activity of fipronil and selected metabolites correlates with their acute oral toxicity in SD rats. Metabolites that have an equal or lower acute oral LD_{50} in rats (and associated equal or lower brain-binding half-maximal inhibitory concentration [IC_{50}] values) compared with fipronil include fipronil-sulfide (M&B 45950) and fipronil-desulfinyl (M&B 46513). The acute oral LD_{50} for the photometabolite fipronil-desulfinyl was about five-fold to seven-fold lower than that of fipronil. Despite differences between fipronil and fipronil-sulfone (M&B 46136) regarding brain binding and acute oral toxicity in rats, fipronil-sulfone is likely the main driver of neurotoxicity given that it is rapidly formed during first-pass metabolism and is by far the predominant circulating and tissue chemical form.

Fipronil binding to the GABA-gated chloride channel depends on the presence of a cyanide group and a side chain with the formula $–SOCF_3$, $–SO_2CF_3$, $–SCF_3$ or $–CF_3$. As shown in Table 9 there is a structure–activity relationship between brain-binding activity, acute toxicity and the presence of a cyanide group accompanied by one of the specified side chains. Loss of either the cyanide group or the side chain reduced brain binding (a surrogate measure of binding to brain GABA-gated chloride channels) and substantially reduced acute oral toxicity (as well as a propensity for fipronil-like neurotoxicity).

Table 9. Comparative rat brain binding and acute toxicity of fipronil and its metabolites

Compound	Structural characteristics present	Rat brain binding IC_{50} (nM)		SD Rat oral LD_{50} (mg/kg bw)		SD Rat dermal LD_{50} (occlusive) (mg/kg bw)	Acute toxicity references
		[^{35}S] TBPS	[^{3}H] EBOB				
Fipronil (M&B 46030)	CN group + $–SOCF_3$ side chain	483	772	Males Females	92 103	>2000	Gardner, 1988a, b
Fipronil-sulfide[a] (M&B 45950, fipronil thioether)	CN group + $–SCF_3$ side chain	155	157	Males Females	69 100	>500 < 4000	Dange 1994a Haynes, 1988b
Fipronil-sulfone (M&B 46136)[b]	CN group + $–SO_2CF_3$ side chain	84	315	Males Females	184 257	>2000	Gardner 1988c Gardner 1988d
Fipronil-desulfinyl a photometabolite (M&B 46513)[c]	CN group + CF_3 side chain	36	213	Males Females	18 15	>2000	Dange 1993a, d
M&B 45897	CN group but lacks side chain	>10 000	>10 000	Males	>2000	>2000	Haynes 1988a, b
RPA 104615 a photometabolite	CN group but lacks CF_3 on the side chain	>10 000	>10 000	Males Females	>2000	No data	Dange, 1993b
RPA 105320	Lacks CN group $–SO_2CF_3$ side chain	3943	>10 000	Males Females	>2000	No data	Dange, 1994b
RPA 200766	Lacks CN group $–SOCF_3$ side chain	>10 000	>10 000	Males Females	>2000	No data	Dange, 1993c
RPA 200761 (metabolite of RPA 200766)	Lacks CN group $–SOCF_3$ side chain	>10 000	>10 000	Males Females	>2000	No data	Katchadourian, 1995

TBPS: *t*-[^{35}S]butylbicyclophosphorothionate

EBOB: [^{3}H]1-[4-ethynylphenyl]-4-*n*-propyl-2,6,7-trioxabicyclo[2.2.2]octane

[a] Clinical signs of neurotoxicity (excessive jumps, increased motor activity, tremor, convulsions) occurred at ≥ 50 mg/kg bw

[b] Major systemic metabolite; clinical signs consistent with neurotoxicity (gait abnormalities) occurred at ≥ 64 mg/kg bw

[c] Clinical signs consistent with neurotoxicity (increased reactivity to noise at ≥ 3 mg/kg bw, reduced motor activity at ≥ 10 mg/kg bw, convulsions at 30 mg/kg bw) occurred at ≥ 3 mg/kg bw

(b) Metabolites: acute dermal irritation

M&B 45897

Occlusive dermal exposure to 0.5 g of M&B 45897 (moistened with water) of 6 female New Zealand white rabbits for 4 h did not result in any skin or systemic toxicity reactions (Haynes 1988c).

M&B 45950 (fipronil-sulfide, fipronil-thioether)

Occlusive dermal exposure to 0.5 g of M&B 45950, (moistened with water) of six female New Zealand White rabbits for four hours did not result in any skin reactions or evidence of systemic toxicity (Haynes, 1987a).

M&B 46136 (fipronil-sulfone)

Occlusive dermal exposure to 0.5 g of M&B 46136 (moistened with water) of three female New Zealand White rabbits for four hours did not result in any skin reactions or evidence of systemic toxicity (Liggett, 1988c).

(c) Metabolites: acute eye irritation

M&B 45897

Administration of 63 mg of M&B 45897 (in 0.1 mL) into the lower conjunctival sac of three female New Zealand White rabbits followed by rinsing with distilled water resulted in well-defined conjunctival redness (grade 2) and chemosis within one hour of exposure in all animals (grade 2 in 2/3 animals; grade 4 with blistering of the conjunctival membrane in 1/3 rabbits) . These effects were fully reversed by 48 hours post exposure.

Administration of 63 mg of M&B 45897 (in 0.1 mL) into the lower conjunctival sac of six female New Zealand White rabbits resulted in conjunctival redness (grade 1–2) in all animals and chemosis (grade 1–2) in 3/6 animals within one hour of exposure. These effects were fully reversed by 48 hours post exposure. It was concluded that M&B 45897 was a slight eye irritant in rabbits, and post exposure rinsing increased the severity of the irritation (Haynes, 1988d).

M&B 45950 (fipronil-sulfide, fipronil-thioether)

Administration of 97 mg (in 0.1 mL) of M&B 45950 into the lower conjunctival sac of three female New Zealand White rabbits followed by rinsing with distilled water resulted in mild transient (at 1 h time point only) conjunctival redness (grade 1) in 2/3 animals. Administration of 97 mg (in 0.1 mL) of M&B 45950 into the lower conjunctival sac of six female New Zealand White rabbits resulted in conjunctival redness (grade 1–2) in 5/6 animals and chemosis (grade 1–2) in 5/6 animals within one hour of exposure. These effects were fully reversed by 72 hours post exposure. It was concluded that M&B 45950 was a slight eye irritant in rabbits (Haynes, 1987b).

M&B 46136 (fipronil-sulfone)

Administration of 60 mg of M&B 46136 (in 0.1 mL) into the lower conjunctival sac of three male New Zealand White rabbits resulted in transient (fully reversed by 72 hours post dose) conjunctival redness (mean irritation score ≤ 1) and chemosis (mean irritation score ≤ 1). It was concluded that M&B 46136 was a mild eye irritant in rabbits (Liggett, 1988d).

(d) Metabolites: dermal sensitization

M&B 45897

Metabolite M&B 45897 did not induce delayed hypersensitivity in a Magnusson & Kligman maximisation test conducted using groups of 10 male and 10 female Dunkin Hartley Guinea pigs (five per sex in the control group). The study lacked a positive control or reliability check. (Rees, 1995).

(e) Metabolites: short-term studies of toxicity

M&B 45950 (fipronil-sulfide, fipronil-thioether)

Rat

Five groups each consisting of 10 male and 10 female SD rats were administered M&B 45950 in the diet for 13 weeks at concentrations of 0, 10, 25, 50 or 300 ppm (equal to 0, 0.69, 1.77, 3.54, and 21.49 mg/kg bw per day in males, 0, 0.81, 2.15, 4.14 and 24.55 mg/kg bw per day in females). Feeding at 300 ppm was associated with damaged vibrissae in 7/10 males. This was accompanied by an increased incidence of nasal discharge (5/10 males). Feeding males at 300 ppm was also associated with reduced body weight gain (by about 11%; $p < 0.05$), correlated with lower food consumption. No consistent effects on body weight parameters occurred in females. At all doses in males and at 50 ppm and above in females, non-adverse increases in serum total protein and α1-globulin occurred ($p < 0.05$) at all exposure levels. Increased serum cholesterol and phosphorus occurred ($p < 0.05$) in females dosed at 300 ppm. Increased relative liver weights ($p < 0.05$; without anatomical pathology correlates and regarded as adaptive) occured in males at 50 ppm and above, and at 25 ppm and above in females. Relative thyroid weights were increased in males at 50 ppm and above ($p < 0.05$), and in females at 300 ppm. An increased incidence of thyroid follicular cell hyperplasia and hypertrophy ($p < 0.05$) occurred in males at 300 ppm. In females an increased incidence of thyroid follicular cell hyperplasia occurred at 300 pppm ($p < 0.05$).

The NOAEL was 50 ppm (equal to 3.54 mg/kg bw per day) due to effects on body weight, vibrissae and nasal discharge observed at the highest dose (Broadmeadow, 1991c).

Dog

Four groups each consisting of two male and two female beagle dogs were orally capsule dosed with 0, 1, 5 or 15 mg/kg bw per day of M&B 45950 for 28 days. At 15 mg/kg bw per day, overall body weight gains of both females were lower than controls and small, non-adverse increases (less than two-fold) in serum alkaline phosphatase were noted in both sexes; these lacked anatomic pathology correlates.

The NOAEL was 5 mg/kg bw per day due to the effects on body weight observed at the high dose (Broadmeadow, 1991b).

M&B 46513 (fipronil-desulfinyl; a photometabolite)

Mouse

Four groups each consisting of 10 male and 10 female OF-1 mice were fed diets containing M&B 46513 at 0, 0.5, 2 or 10 ppm (equal to 0, 0.08, 0.32, and 1.74 mg/kg bw per day for males, 0, 0.11, 0.43 and 2.15 mg/kg bw per day for females) for at least 90 days. Unplanned premature mortality occurred in all males and 1/10 females at 10 ppm. Clinical signs consistent with neurotoxicity (excessive jumping, irritability, aggression) occurred in males at 10 ppm. Mild centrilobular hepatocyte hypertrophy occurred in 6/10 males at 10 ppm. The NOAEL was 2 ppm due to unplanned premature mortality and neurotoxicity observed at the highest dose (equal to 0.32 mg/kg bw per day) (Lasserre-Bigot, 1996).

Rat

Five groups each consisting of 10 male and 10 female SD rats were administered M&B 46513 for 28 days in the diet at concentrations of 0, 0.5, 3, 30 or 100 ppm (equal to 0, 0.04, 0.23 and 2.20 mg/kg bw per day in surviving males, 0, 0.04, 0.24 and 2.32 mg/kg bw per day in surviving females). All animals fed at 100 ppm died within 15 days of treatment with one female developing convulsions (Table 10). Feeding at 30 ppm resulted in the death of one male and was associated with clinical signs consistent with neurotoxicity (piloerection, curling up when handled). Reduced body weight occurred at 30 ppm and above ($p < 0.05$). Body weight gain was reduced ($p < 0.05$) over days 1–15 in males at 30 ppm and above, and over days 1–8 in females at 30 ppm and above. These effects were correlated with reduced feed consumption ($p < 0.05$) over the first two weeks at the same dose values. Reduced thymus weights ($p < 0.05$; no anatomical pathology correlates) occurred in females at 30 ppm.

The NOAEL was 3 ppm due to the effects on body weight and neurotoxicity observed at the next highest dose (equal to 0.23 mg/kg bw per day) (Dange, 1995b).

Table 10. Findings in a 28-day study in rats with M&B 46513 (fipronil-desulfinyl)

	Dose level (ppm)									
	Males					**Females**				
	0	**0.5**	**3**	**30**	**100**	**0**	**0.5**	**3**	**30**	**100**
	Number of unplanned deaths									
	0	0	0	1	10	0	0	0	0	10
	Incidence of clinical signs (n = 10)									
Curled up at handling	0	0	0	6	4	0	0	0	8	5
Piloerection	0	0	0	9	4	0	0	0	5	6
Tonic clonic convulsions	0	0	0	0	0	0	0	0	0	1

	Dose level (ppm)									
	Males					Females				
	0	0.5	3	30	100	0	0.5	3	30	100
Day	**Body weight (g)**									
1	244.8	244.9	244.9	237.6	239.7	187.2	186.4	186.5	190.1	188.4
8	309.5	310.1	313.1	258.2*	197.8*	221.1	217.6	216.8	193.2*	163.0*
15	353.6	352.9	355.9	289.7*	PM	239.9	236.9	237.3	215.3*	PM
22	389.0	391.0	399.4	329.2*	PM	255.8	252.5	255.2	233.5*	PM
28	404.3	402.7	409.0	345.6*	PM	263.5	260.6	261.7	239.9*	PM
Day	**Body weight change (g)**									
1–8	9.27	9.33	9.78	3.08*	–6.10*	4.80	4.44	4.35	0.51*	–4.53*
1–15	6.30	6.12	6.11	4.54*	PM	2.71	2.75	2.91	3.14	PM
1–28	2.61	1.96	1.60	2.70	PM	1.29	1.35	1.10	1.09	PM
	Thymus weights									
Mean absolute weight (g)	0.6006	0.6976	0.6327	0.5681	PM	0.5733	0.5713	0.5864	0.4070**	PM
Organ wt/bw (%)	0.1575	0.1835	0.1651	0.1764	PM	0.2341	0.2369	0.2385	0.1829*	PM

PM: No data due to unplanned premature mortality; bw: Body weight; wt: Weight

* $p < 0.05$, ** $p < 0.01$;

Five groups each consisting of 10 male and 10 female SD rats were fed diets for 90 days containing M&B 46513 at 0, 0.5, 3, 10 or 30 ppm (equal to 0, 0.029, 0.177, 0.594 and 1.772 mg/kg bw per day in males, 0, 0.035, 0.210, 0.709 and 2.101 mg/kg bw per day in females). Unplanned premature mortality occurred in four males fed 30 ppm. Clinical signs consistent with neurotoxicity (aggressive behaviour, irritability, excessive vocalization, increased motor activity and curling up on handling) occurred at 3 ppm and greater in males and 10 ppm and more in females (Table 11). Reductions in body weight parameters ($p < 0.05$) occurred at 10 ppm and more. Reduced food consumption occurred at 30 ppm during the first two weeks ($p < 0.05$).

The NOAEL was 0.5 ppm (equal to 0.029 mg/kg bw per day) due to the presence of clinical signs consistent with neurotoxicity at 3 ppm. (Dange, 1994c).

Table 11. Findings in a 90-day study in rats with M&B 46513 (fipronil desulfinyl)

	Dose level (ppm)										
	Males						Females					
	HCD range	0	0.5	3	10	30	HCD range	0	0.5	3	10	30
	Incidence of clinical signs (n = 10)											
Aggressive behaviour	0–1	0	0	1	1	4	0–2	0	0	0	0	0
Irritability to touch	0–4	0	0	1	6	6	1–4	0	0	0	5	8
Excessive vocalization	0	0	0	1	2	0	0	0	0	0	0	1
Increased motor activity	0	0	0	0	0	1	0–1	0	0	0	1	9
Curled up at handling	0	0	0	0	0	5	0	0	0	0	0	1

	Dose level (ppm)										
	Males						Females				
	HCD range	0	0.5	3	10	30	HCD range	0	0.5	3	10	30
	Body weight (g)											
Day 1		274.3	274.7	275.4	276.5	275.2		202.4	202.1	202.0	198.0	199.0
Day 8		333.6	337.7	332.9	327.5	294.2**		226.3	225.5	226.6	219.0	201.1**
Day 43		504.0	511.2	490.4	470.1	452.5**		291.4	287.1	297.1	280.9	266.7
Day 64		562.9	570.9	539.7	516.8*	513.1*		309.8	310.2	317.6	303.3	287.7
Day 90		598.6	606.2	579.3	551.0	557.7		313.4	317.9	327.5	308.6	306.7
	Body weight gain (g/day)											
Week 1		8.47	9.00*	8.21	7.28**	2.71**		3.41	3.34	3.51	3.00	0.30**
Week 6		3.31	3.51	3.27	2.47	3.22		1.20	0.60	1.44	1.08	1.07
Week 12		2.40	2.43	2.61	2.71	2.61		0.61	0.75	0.05	0.73	−0.30

HCD: Historical control data; * $p < 0.05$, ** $p < 0.01$

Dog

Four groups each consisting of two male and two female beagle dogs were fed diets containing M&B 46513 at 0, 27, 80 or 270 ppm for 28 consecutive days. Unplanned premature mortality occurred in all animals fed at 80 ppm and above due to inappetence, poor body condition, reduced body weight and/or severe adverse clinical signs. Dietary intake in the surviving 27 ppm cohort was equal to 1 mg/kg bw per day in both sexes. Clinical signs consistent with neurotoxicity (excessive anxiety, irritability, reduced motor activity, staggering step, increased salivation, convulsions) occurred at doses of 27 ppm and higher. Stress-associated reductions in thymic weight occurred in males at 27 ppm. Thymic atrophy (likely stress-associated) and hepatotoxicity, characterized by diffuse sinusoidal leukocytosis and centrilobular hepatocyte enlargement, occurred in both sexes at 80 ppm. The LOAEL was 27 ppm (equal to 1 mg/kg bw per day) due to signs of neurotoxicity at this dose level and unplanned premature mortality at the higher doses (Dange,1995c).

Four groups each consisting of five male and five female beagle dogs were fed diets for 90 days containing M&B 46513 at 0, 3.5, 9.5 or 35 ppm (equal to 0, 0.1, 0.27 and 0.95 mg/kg bw per day for males, 0, 0.1, 0.29, and 1.05 mg/kg bw per day for females). The only effect noted was a single incidence of excessive barking and aggressive behaviour in one high-dose female and a separate single incidence of excessive salivation, irritable behaviour and tremor in another female dog. The NOAEL was 9.5 ppm (equal to 0.27 mg/kg bw per day) due to clinical signs consistent with neurotoxicity in the high-dose group (Dange, 1996).

RPA 104615 (photometabolite)

Rat

Five groups each consisting of 10 male and 10 female SD rats were fed diets for 28 days containing 0, 50, 500, 5000 or 10 000 ppm of RPA 104615 (equal to 0, 4.5, 45.7, 458.5 and 915.9 mg/kg bw per day in males, 0, 4.7, 50.4, 487.4 and 949.7 mg/kg bw per day in females). Increased prothrombin time occurred in males treated at 5000 ppm and above (Table 12). Mean alkaline phosphatase activity was increased ($p < 0.05$) in both sexes at 5000 and 10 000 ppm. In females, total cholesterol concentration was increased ($p < 0.05$) at 10 000 ppm, whilst triglycerides concentration was increased ($p < 0.05$) at both 5000 and 10 000 ppm. Urinary pH was increased ($p < 0.05$, non-adverse) in males at 500 ppm and above. Increased serum T4 levels ($p < 0.05$) occurred in females at 5000 ppmand above. At 10 000 ppm, absolute liver weight was increased ($p < 0.05$) whilst body weight-relative liver weight was increased ($p < 0.05$) at both 5000 and 10 000 ppm. At 10 000 ppm, findings in the liver of both sexes comprised a slight increase in the incidence of minimal or slight sinusoidal lymphoid aggregations associated with occasional degenerate hepatocytes. Females exhibited a slight increase in the incidence of minimal or slight diffuse fine vacuolation of hepatocytes, mainly in the periportal area. In the male thyroid, an increased incidence of follicular epithelial hypertrophy occurred at 10 000 ppm. The NOAEL was 500 ppm (equal to 45.7 mg/kg bw per day) due to increased prothrombin time and increased serum triglycerides at 5000 ppm (Dange, 1997, 2000).

Table 12. Findings in the RPA 104615 28-day study in rats

	Dose level (ppm)									
	0	50	500	5000	10 000	0	50	500	5000	10 000
	Males					Females				
Mean coagulations parameters										
Prothrombin in time, (s)	13.21	13.21	13.50	14.31*	15.37**	14.16	14.33	14.32	13.73	13.29
Mean clinical chemistry parameters										
Alkaline phosphatase, (IU/L)	163.9	170.2	167.2	220.1*	544.9**	126.5	126.8	122.0	371.3**	674.1**
Cholesterol, (mmol/L)	1.341	1.406	1.471	1.504	1.536	1.314	1.494	1.386	1.520	1.780**
Triglycerides (mmol/L)	0.777	0.985	0.934	0.813	1.152	0.503	0.759*	0.628	1.133**	1.078**
Urinalysis										
Urine pH	6.39	6.83	7.00*	7.33**	7.15**	6.06	6.63	6.56	6.13	6.39
Mean serum thyroid hormone levels										
T_3	0.65	0.98	0.83	0.85	0.75	0.86	0.78	0.85	0.84	0.74
T_4	37.06	42.51	40.30	34.45	31.41	34.52	30.16	31.06	27.49*	26.62**
TSH	0.75	0.83	0.85	0.89	1.04	0.57	0.50	0.56	0.51	0.64
Mean liver weights										
Liver weight, g	8.76	9.49	9.77	9.71	10.08*	6.21	6.56	6.12	6.87	6.95
Liver weight, % bw	2.84	2.97	3.05	3.10*	3.19**	2.92	3.06	2.93	3.27**	3.27**
Incidence of anatomic pathology (n = 10)										
Liver: sinusoidal lymphoid aggregation with occasional degenerate hepatocytes										
	5	4	4	4	7	6	2	6	2	8
Liver: fine vacuolation of hepatocytes (mainly periportal)										
	1	0	0	0	1	5	6	7	5	9
Thyroid: follicular epithelial hypertrophy										
	2	0	1	1	5	0	0	0	0	1

* $p < 0.05$, ** $p < 0.01$

RPA 200766 (fipronil-amide, fipronil-carboxamide)

Rat

Five groups of 10 male and 10 female SD rats were fed diets for 28 days containing RPA 200766 at 0, 50, 500, 5000 or 15 000 ppm (equal to 0, 3.8, 38.16, 385.07 and 1087.05 mg/kg bw per day in males, 0, 4.44, 43.97, 386.75 and 1062.84 mg/kg bw per day in females). Reduced body weight parameters occurred at 5000 ppm and above ($p < 0.05$; Table 13). This effect was correlated with reduced feed consumption ($p < 0.05$) throughout the study in the high-dose males and in females at 5000 ppm and above. Small reductions ($p < 0.05$) in haematological parameters consistent with a reduction in the circulating erythron mass occurred throughout the study at 500 ppm and above. Likewise, increased serum cholesterol ($p < 0.05$) and triglycerides occurred at 500 ppm and above. Serum creatinine was increased in males at 500 ppm and above ($p < 0.05$). Serum urea was increased in females at 5000 ppm and above ($p < 0.05$). A small, non-adverse reduction in urine pH ($p < 0.05$) occurred in high-dose males. Liver weight parameters were increased at 500 ppm and above ($p < 0.05$). There was an increased incidence of enlarged livers and adrenal glands at 5000 ppm. Dark livers were noted in females at 5000 ppm and above, and in males at 500 ppm and above. Adaptive hepatocellular hypertrophy occurred

in all animals dosed at 5000 ppm. An increased incidence of adrenal extramedullary haematopoeisis occurred at 15 000 ppm. The incidence of adrenal zona fasciculata vacuolation was increased in all males exposed to RPA 200766 and in females at 5000 ppm. The incidence, extent and severity of adrenal zona fasciculata vacuolation in males at 50 ppm was considered non-adverse. The NOAEL was 50 ppm (equal to 3.80 mg/kg bw per day) due to the presence of adverse adrenal zona fasciculata vacuolation at 500 ppm (Berthe, 1996).

Table 13. Findings in a 28-day study of RPA 200766 in the rat

	Dose level (ppm)									
	Males					**Females**				
	0	**50**	**500**	**5000**	**15 000**	**0**	**50**	**500**	**5000**	**15 000**
					Mean body weight, (g)					
Day 1	225.7	225.4	222.5	227.1	226.3	160.4	161.7	161.2	160.5	160.4
Day 8	280.7	278.1	272.0	245.2**	215.2**	193.8	197.3	187.7	164.3**	146.8**
Day 15	321.8	319.4	315.4	288.6**	260.5**	222.5	228.0	217.5	188.1**	167.5**
Day 22	351.6	349.7	342.0	322.2**	287.6**	243.6	250.4	237.0	206.4**	178.3**
Day 28	364.2	363.9	352.4	327.6**	281.0**	250.0	258.2	245.4	209.3**	180.6**
					Mean body weight change, (g)					
Week 1	7.85	7.52	7.07	2.58**	−1.58**	4.77	5.08	3.78	0.54**	−1.94**
Week 2	5.87	5.90	6.20	6.20	6.47	4.10	4.38	4.25	3.40	2.95
Week 3	4.25	4.32	3.80	4.80	3.87	3.01	3.20	2.78	2.61	1.54
Week 4	2.10	2.36	1.73	0.90*	−1.10**	1.06	1.30	1.40	0.48	0.57
					Mean food consumption, (g [% of control])					
Week 1	25.7	24.6	23.8	19.6**	12.4**	19.9	19.8	17.7**	11.6**	7.0**
	[100]	[96]	[93]	[76]	[48]	[100]	[99]	[89]	[58]	[35]
Week 2	26.5	25.9	26.3	25.0	23.6**	21.0	21.2	20.2	16.0**	14.0**
	[100]	[98]	[99]	[94]	[89]	[100]	[101]	[96]	[76]	[67]
Week 3	26.3	25.3	25.3	25.1	22.7**	21.9	21.8	20.7	16.9**	14.4**
	[100]	[96]	[96]	[95]	[86]	[100]	[100]	[95]	[77]	[66]
Week 4	23.2	22.5	21.1	20.7	17.3**	18.9	19.3	18.6	14.7**	12.7**
	[100]	[97]	[91]	[89]	[75]	[100]	[102]	[98]	[78]	[67]
					Haematology					
Mean Hb conc. (g/100 mL)	16.48	15.98	15.84*	15.15**	15.11**	15.47	15.26	14.96*	14.40**	14.60
Mean Ht	0.501	0.484	0.480	0.463**	0.467**	0.459	0.450	0.445	0.431**	0.442
MCH (pg)	19.43	19.47	19.04	18.71	18.58*	19.70	19.74	19.84	18.94*	18.72**
					Clinical chemistry					
Mean cholesterol (mmol/L)	1.179	1.242	1.537**	3.255**	4.532**	1.255	1.346	1.875**	4.182**	4.881**
Mean serum triglycerides (mmol/L)	0.533	0.646	0.577	1.602**	3.265**	0.582	0.635	0.631	1.972**	1.822**
Mean creatinine (µmol/l)	60.5	61.3	64.7*	68.9*	72.3*	62.5	61.7	65.4	66.2	66.2
Mean urea (mmol/L)	5.030	5.067	5.490	4.813	4.908	4.617	4.872	5.080	6.180**	6.252**

	Dose level (ppm)									
	Males					Females				
	0	50	500	5000	15 000	0	50	500	5000	15 000
Mean liver weight										
Liver wt (g)	9.41	9.80	10.92*	16.05**	17.85**	6.78	7.35	8.78**	12.55**	12.93**
(% control)	(100)	(104)	(116)	(171)	(190)	(100)	(108)	(129)	(186)	(191)
Liver wt, (% bw)	2.73	2.87	3.30**	5.21**	6.80**	2.87	3.06	3.82**	6.33**	7.63**
(% control)	(100)	(105)	(121)	(191)	(249)	(100)	(107)	(133)	(221)	(266)
Liver wt, % brain wt	484.1	505.8	578.4**	855.4**	1009.2**	380.5	413.1	485.0**	712.2**	740.2**
(% control)	(100)	(104)	(119)	(177)	(208)	(100)	(109)	(127)	(187)	(195)
Incidence of macroscopic anatomic pathology findings										
Enlarged liver	0/10	0/10	0/10	8/10	10/10	0/10	0/10	0/10	10/10	9/9
Dark liver	0/10	0/10	1/10	8/10	10/10	0/10	0/10	0/10	9/10	9/9
Enlarged adrenals	0/10	0/10	0/10	5/10	3/10	0/10	0/10	0/10	3/10	2/9
Incidence of microscopic anatomic pathology findings										
Hepatocellular hypertrophy	0/10	0/10	0/10	10/10	ND	0/10	0/10	0/10	10/10	ND
Adrenal extramedullary haematopoesis	0/10	0/10	0/10	5/10	ND	0/10	0/10	0/10	4/10	ND
Adrenal vacuolation	0/10	2/10	5/10	10/10	ND	0/10	0/10	0/10	7/10	ND

ND: No data; Hb: Haemoglobin; MCH: Mean corpuscular haemoglobin; bw: Body weight

* $p < 0.05$, ** $p < 0.01$

(f) Metabolites: long-term studies of toxicity and carcinogenicity

M&B 46513 (fipronil-desulfinyl; a photometabolite)

Rat

In a combined chronic toxicity and carcinogenicity study four groups each consisting of 70 male and 70 female SD rats were fed diets containing M&B 46513 at 0, 0.5, 2 or 10 ppm for males (equal to 0, 0.025, 0.098 and 0.797 mg/kg bw per day); and 0, 0.5, 2 or 10↓6 ppm in females (dose was reduced from 10 ppm to 6 ppm after 26 weeks in the high-dose female cohort due to excessive mortality; equal to 0, 0.032, 0.127, and 0.733↓0.546 mg/kg bw per day).

After 53 weeks of treatment 10 animals per sex per dose were killed in order to assess chronic toxicity (chronic toxicity cohort). The remaining 60 animals in each group were exposed to M&B 46513 for at least 104 weeks. Unplanned premature mortalities occurred at 10 ppm over weeks 1–26 (see Table 14). An increased incidence of convulsions ($p < 0.05$) occurred in females at 2 ppm and greater, and a trend towards an increased incidence of animals displaying aggressivity and irritability at 2 ppm and above. Plasma total bilirubin and triglycerides levels were reduced ($p < 0.05$) and glucose was slightly increased ($p < 0.05$; non-adverse) in high dose females at week 26. No evidence of treatment-related neoplastic changes were seen in rats from either the chronic toxicity or carcinogenicity phases.

The NOAEL was 0.5 ppm (equal to 0.025 mg/kg bw per day) due to the presence of clinical signs consistent with neurotoxicity at 2 ppm (Lasserre-Bigot, 1998).

Table 14. Findings in a combined chronic toxicity/carcinogenesis study in rats exposed to M&B 46513 (fipronil-desulfinyl)

	Dose level (ppm)							
	0	**0.5**	**2**	**10**	**0**	**0.5**	**2**	**10**
	Males				**Females**			
				Mortality (*n* = 70)				
Mortality, weeks 1–26	1	0	1	3	1	0	0	7
				Incidence of clinical signs (*n* = 70)				
Tonic/clonic convulsion	7	2	9	10	5	8	13 *	20**
Aggressivity	2	0	4	9	0	2	1	20
Irritability to touch	3	3	8	10	0	4	0	25
				Clinical chemistry				
Mean total bilirubin (µmol/L)	1.63	1.60	1.57	1.43	2.32	2.65	2.23	1.86*
Mean triglyceride (mmol/L)	0.973	1.189	1.066	1.227	1.039	0.833	0.868	0.585**
Mean glucose (mmol/L)	9.298	9.752	9.395	9.716	7.562	8.241*	8.058	9.112**

n: Total number of animals in group; * $p < 0.05$, ** $p < 0.01$;

(g) Metabolites: genotoxicity

In vitro

Table 15. Summary of in vitro genotoxicity studies with metabolites of fipronil

Study type	Test system	Concentrations	Purity (%)	Result	Reference
		M&B 45897			
Bacterial reverse mutation test	*S. typhimurium* TA 1535, TA 1537, TA 98 and TA 100	TA100 8–5000 µg/plate (± S9 mix) Others: 4–2500 µg/plate (± S9 mix) Solvent: DMSO	> 99	Negative	Kenelly, 1988
	S. typhimurium TA 1535, TA 1537, TA 102, TA 98 and TA 100	0, 100, 250, 500, 1000 and 2500 µg/plate (two tests) (± S9 mix) 0, 25, 50, 100, 250, 500, 1000 and 2500 µg/plate (two tests) +S9 in first exp.; –S9 in second exp. Solvent: DMSO	99.7	Negative	Percy, 1995a
Chromosome aberration	Human lymphocytes	0, 6.25, 12.5, 25, 50, 100, 200 and 400 µg/mL (± S9 mix) Solvent: DMSO	99.7	Negative	Johnson, 1995

Study type	Test system	Concentrations	Purity (%)	Result	Reference
		M&B 45950 (fipronil-sulfide, fipronil-thioether)			
Bacterial reverse mutation	*S. typhimurium* TA 1535, TA 1537, TA 1538, TA 98 and TA 100	0, 1.6, 8, 40, 200 and 1000 µg/plate (two tests) (± S9 mix) Solvent: DMSO	ND	Negative	Asquith, 1987
	S. typhimurium TA 1535, TA 1537, TA 1538, TA 98 and TA 100	0, 10, 25, 50, 100 and 250 µg/plate (two tests) (± S9 mix) Solvent: DMSO	98.9	Negative	Percy, 1994a
Chromosome aberrations in mammalian cells	Human lymphocytes	0, 6.25, 12.5, 25, 50, 100, 200 and 400 mg/µg/mL (± S9 mix) Solvent: DMSO	>99	Negative	Marshall, 1988b
		M&B 46136 (fipronil-sulfone)			
Bacterial reverse mutation	*S. typhimurium* TA 1535, TA 1537, TA 1538, TA 98 and TA 100	Without S9 mix: 0, 0.32, 1.6, 8, 40, 200 µg/plate (test 1) 0, 12.5, 25, 50, 100, 150 µg/plate (test 2) With S9 mix: 0, 0.8, 4, 20, 100, 500 µg/plate (test 1) 0, 25, 50, 100, 200, 400 µg/plate (test 2) Solvent: DMSO (both tests)	98.7	Negative	Clare, 1988b
Chromosome aberrations in mammalian cells	Human lymphocytes	0, 4.69, 9.38, 18.75, 37.5, 75, 150 , 300 µg/mL in DMSO	98.7	Negative	Marshall, 1989
		M&B 46513 (fipronil-desulfinyl, photometabolite)			
Bacterial reverse mutation	*S. typhimurium* TA 1535, TA 1537, TA 1538, TA 98 and TA 100	0, 10, 25, 50, 100 and 250 µg/plate in (two tests) Solvent: DMSO	98.6	Negative	Percy, 1993a
Chromosome aberrations in mammalian cells	Human lymphocytes	Test 1: 0, 5, 15 and 30 µg/mL (± S9 mix) 0, 5, 10 and 15 µg/mL without S9 mix Test 2: 0, 15, 30 and 40 µg/mL with S9 mix Solvent: DMSO	98.6	Negative	Adams, 1996a
Gene mutation in mammalian cells	Chinese hamster ovary cells (CHO-K1-BH_4)	Without S9 mix: 0, 5, 10, 15, 30, 60, 80, 100 and 125 µg/mL With S9 mix: (test 1): 0, 15, 30, 60, 80, 100, 125, 250 and 625 µg/mL With S9 mix: (test 2): 0, 30, 60, 80, 100, 125, 175, 250 and 625 µ g/mL	98.6	Negative	Adams, 1996b

Study type	Test system	Concentrations	Purity (%)	Result	Reference
		RPA 104615 (photometabolite)			
Bacterial reverse mutation	*S. typhimurium* TA 1535, TA 1537, TA 1538, TA 98 and TA 100	0, 250, 500, 1000, 2500 and 5000 µg/plate (two tests) (± S9 mix) Solvent: DMSO	94	Negative	Percy, 1993b
Chromosome aberrations in mammalian cells	Human lymphocytes	Test 1: (± S9 mix) 0 (DMSO), 1250, 2500, 5000 µg/mL Test 2: (without S9 mix) 0, 625, 1250, 2500, 73 µg/mL Test 2: with S9 mix 0, 1250, 2500, 5000 µg/mL	94	Negative	Allais, 2002a
		RPA 105320			
Bacterial reverse mutation	*S. typhimurium* TA 100, TA 1535, TA 1537, TA 98, TA 102	0, 50, 100, 250, 500, 1000 µg/plate (± S9 mix)	96.4	Negative	Percy, 1994b
		RPA 105048			
Bacterial reverse mutation	*S. typhimurium* TA 1535, TA 1537, TA 98 and TA 100	0, 250, 500, 1000, 2500 and 5000 µg/plate (two tests) Solvent: DMSO	97.5	Negative	Percy, 1994a
		RPA 200761 (metabolite of RPA 200766)			
Bacterial reverse mutation	*S. typhimurium* TA 1535, TA 1537, TA 98, TA 100 and TA 102	Without S9 mix: 0, 250, 500, 1000, 2500 and 5000 µg/plate (two tests) With S9 mix: 0, 100, 250, 500, 1000, 2500 and 5000 µg/plate (two tests) Solvent: DMSO	>98	Negative	Percy, 1995b
		RPA 200766			
Bacterial reverse mutation	*S. typhimurium* TA 1535, TA 1537, TA 1538, TA 98, and TA100	Without S9 mix: 0, 50, 100, 250, 500 and 1000 µg/plate (two tests) With S9 mix 0, 50, 100, 250, 500, 1000 and 2500 µg/plate (two tests) Solvent: DMSO	>98	Negative	Percy, 1993c
Chromosome aberrations in mammalian cells	Human lymphocytes	Test 1: 0, 200, 300 and 400 mg/mL (–S9 mix); 0, 156.25, 312.5 and 625 µg/mL (+S9 mix) Test 2: 0, 31.25, 62.5 and 125 µg/mL (–S9 mix); 0, 312.5, 625 and 800 µg/mL (–S9 mix) Solvent: DMSO Overall regarded as negative since effects only noted at cytotoxic concentrations	>98	Positive with S9 mix at cytotoxic dose levels Negative without S9 mix	Allais, 2002b

Study type	Test system	Concentrations	Purity (%)	Result	Reference
In vitro gene mutation (HPRT)	Chinese hamster ovary cells	Test 1: 0, 31.25, 62.5, 125, 250 µg/mL (–S9 mix); 0, 31.25, 62.5, 125, 250 µg/mL (+S9 mix) Test 2: 0, 12.5, 25, 50, 100, 200, 300, 400 µg/mL (–S9 mix); 0, 25, 50, 100, 200, 300, 400 µg/mL (+S9 mix) Test 3: (+S9 mix) 0, 100, 200, 300, 400 µg/mL Test 4: (+S9 mix) 0, 200, 250, 300, 350, 400, 450, 500 µg/mL	>98	Negative	Engelhardt & Leibold, 2004b

ND: No data; DMSO: Dimethylsulfoxide; HPRT: Hypoxanthine guanine phosphoribosyltransferase

In vivo

Table 16. Summary of in vivo genotoxicity studies with metabolites of fipronil

Study type	Test system	Dose levels	Purity (%)	Result	Reference
		M&B 45897			
Mouse micronucleus	CD-1 mice erythrocyte bone marrow cells	0, 2, 4, 8 and 16 mg/kg bw in corn oil	98.6	Negative	Proudlock, 1996
		RPA 200766			
Micronucleus test	Bone marrow of SD rats	0, 500, 1000 and 2000 mg/kg bw in corn oil		Negative	Mehmood, 2002

Most of the submitted studies are historic and while they were performed in accordance with relevant guidelines available at the time they were conducted, many of the assays are not consistent with current international guidelines and standards. Most of the bacterial reverse mutation assays lacked either *S. typhimurium* TA102 or the *E. coli* strain. In some, but not all cases, a second assay was performed. However, these assays were not performed according to the pre-test protocol. In vivo assays were not performed for many of the metabolites. Despite these limitations the overall weight of evidence is that the fipronil metabolites evaluated are not genotoxic.

(h) Metabolites: developmental toxicity

M&B 46513 (fipronil desulfinyl, a photometabolite)

Rat

Four groups containing 25 mated female SD rats were orally gavage dosed with 0, 0.2, 1.0 or 2.5 mg/kg bw per day of M&B 46513 over days 6–15 PC. An increased incidence of hair loss occurred in the high-dose group (7/24 compared with 1/24 in the controls). Reductions in maternal body weight ($p < 0.05$) occurred at 1.0 mg/kg bw per day and above (Table 17). Reduced food consumption ($p < 0.05$) during the dosing period occurred at 2.5 mg/kg bw per day. There were no treatment-related macroscopic maternal findings and no effects on litter size, embryofetal survival, placental weight or sex ratio at any dose level. Reduced fetal weights ($p < 0.05$), an increased incidence of incompletely ossified pubic bones (within historical control range) and an increased incidence of unossified caudal vertebrae (within historical control range) occurred at 2.5 mg/kg bw per day. The maternal NOAEL was 0.2 mg/kg bw per day due to effects on maternal body weight at 1 mg/kg bw per day. The NOAEL for developmental toxicity was 1 mg/kg bw per day due to the presence of reduced fetal weights at the highest dose.

Table 17. Findings in a developmental toxicity study in rats with M&B 46513 (fipronil-desulfinyl)

	Dose level (mg/kg bw per day)			
	0	**0.2**	**1.0**	**2.5**
Number of dams	**24**	**24**	**25**	**24**
		Mean body weight (g)		
Day 0 PC	263.2	263.9	264.0	263.3
Day 6 PC	294.2	294.1	297.8	297.9
Day 9 PC	302.9	302.9	306.8	300.3
Day 12 PC	317.0	315.5	318.1	304.2
Day 16 PC	341.5	340.3	343.0	325.3
Day 20 PC	405.5	403.3	406.9	391.3
Gravid uterus weight	81.71	79.79	80.56	80.54
Corrected day 20 body weight	323.83	323.46	326.32	310.71
		Mean body weight gain (g)		
Days 0–6 PC	30.96	30.25	33.80	34.63
Days 6–9 PC	8.71	8.75	8.96	2.42**
Days 9–12 PC	14.13	12.58	11.36*	3.92**
Days 12–16 PC	24.46	24.88	24.92	21.13
Days 16–20 PC	64.08	62.92	63.84	65.92
Days 6–16 PC	47.29	46.21	45.24	27.46**
Corrected weight change Days 0–20	60.63	59.58	62.32	47.46*
		Mean food consumption (g/day [% control])		
Days 6–9 PC	25.81 [100]	25.38 [98]	25.99 [101]	23.90 [93]
Days 9–12 PC	25.56 [100]	25.35 [99]	25.64 [100]	21.19 [83]**
Days 12–16 PC	25.92 [100]	25.65 [99]	25.50 [98]	21.82 [84]**
Days 16–20 PC	28.17 [100]	27.88 [99]	27.84 [99]	27.29 [97]
		Mean fetal body weights (g)		
Male fetal weight	3.64	3.57	3.62	3.55*
Female fetal weight	3.43	3.44	3.47	3.35**
		Fetal skeletal observations[a]		
Number of foetuses [litters examined]	187 [24]	183 [24]	192 [25]	186 [24]
Pubic bones incompletely ossified [litters affected]	3 [3]	3 [3]	2 [2]	6 [5]
Unossified 5th and 6th caudal vertebrae [litters affected]	54 [21]	66 [18]	73 [24]	82** [21]

[a] One fetus may have more than one observation; PC: Post coitum

* $p < 0.05$, ** $p < 0.01$

(i) Special studies on metabolites and degradates: acute neurotoxicity

M&B 46513 (fipronil-desulfinyl, a photometabolite)

Rat

Four groups containing 12 male and 12 female SD rats were administered single oral gavage doses of 0, 0.5, 2.0 or 12 mg/kg bw of M&B 46513 in corn oil. There were no deaths or clinical signs attributable to M&B 46513. Reduced body weight gain ($p < 0.05$) and food consumption occurred in the first week following dosing at 12 mg/kg bw (Table 18). Reduced ($p < 0.05$) hind leg splay, body temperature and motor activity occurred at 12 mg/kg bw at six hours post dose. At 7 and 14 days post dosing an increased proportion of males with slow righting reflex occurred at 12 mg/kg bw. The NOAEL for general toxicity and neurotoxicity was 2 mg/kg bw due to effects on body weight gain, hind leg splay, body temperature, motor activity and righting reflex at the highest dose (Hughes, 1996).

Table 18. Findings for an acute neurotoxicity study in rats with M&B 46513 (fipronil-desulfinyl)

	Dose level (mg/kg bw)							
	0	**0.5**	**2.0**	**12**	**0**	**0.5**	**2.0**	**12**
	Males				**Females**			
	Mean body weight (g)							
Week −1	181	183	183	183	159	160	157	158
Week 0	209	215	215	215	163	167	161	168
Week 1	282	292	289	273	209	211	207	202
Week 2	319	328	328	320	228	230	222	221
	Mean body weight gain (g)							
Week 0–1	73	76	74	57**	45	44	45	34**
Week 1–2	37	36	39	48*	20	19	15	19
	Mean food consumption (g/week)							
Week −1[a]	212	218	217	214	146	144	141	141
Week 1	223	230	230	194**	179	177	178	145**
Week 2	230	239	242	237	175	182	179	180
Functional observations at 6 hours post-dose (time of peak effect based on dose-ranging study)								
Mean hind leg splay (cm)	8.3	9.2	7.7	6.9*	8.5	8.8	8.3	6.8**
Mean body temperature (°C)	38.5	38.5	38.7	38.1*	38.7	38.7	38.5	37.9**
Mean motor activity during first 30 min.	288	362	300	133**	283	301	337	121**
Mean motor activity during first hour	309	397	347	199	286	344	376	135**

[a] Only six days recorded for females

* $p < 0.05$, ** $p < 0.01$

(j) Summary of studies on metabolites and degradates of fipronil

A consolidated summary of the toxicological characteristics of fipronil and its metabolites is shown in Table 20. Based on the lowest oral LD_{50} values in rodents, the photometabolite M&B 46513 (fipronil-desulfinyl) is more acutely toxic than fipronil, however their acute oral NOAELs for neurotoxic effects in rats were approximately equivalent. Parent fipronil, M&B 45897, M&B 45950 (fipronil sulfide) and M&B 46136 (fipronil-sulfone) displayed similar acute irritancy properties. Neither fipronil nor M&B 45897 were skin sensitizers. Overall, the short-term oral toxicity NOAELs for fipronil and M&B 46513 (fipronil-desulfinyl) were broadly similar following exposure for 28–90 days in rats and after 90 days of exposure in dogs. This is consistent with the findings of the JMPR 2000 evaluation which concluded that fipronil and M&B 46513 (fipronil-desulfinyl) were equitoxic. Of note was the finding that M&B 46513 (fipronil-desulfinyl) did not produce thyroid tumours in rats, unlike the parent compound fipronil. Furthermore, the short-term studies of M&B 46513 (fipronil-desulfinyl) provided no evidence of disruption of thyroid homeostasis, as was found with fipronil. This implies that the photoconversion of the $–SOCF_3$ side chain present in fipronil to the CF_3 side chain present in M&B 46513 is associated with the loss of the effects on the hypothalamic–piuitary–thyroid axis in rats. The NOAELs for short-term oral toxicity of M&B 45950 (fipronil-sulfide), RPA 104615 and RPA 200766 were higher than that of fipronil. Since RPA 200761 (fipronil-amide) is a metabolite of RPA 200766, this finding also applies to the amide. The NOAELs for chronic and developmental toxicity for fipronil and M&B 46513 (fipronil-desulfinyl) were broadly similar.

In practical terms and when all of the data is considered, the toxicological dose thresholds for fipronil and M&B 46513 (fipronil-desulfinyl) are essentially equivalent. As far as has been currently ellucidated, the toxicological dose thresholds for M&B 45897, M&B 45950 (fipronil-sulfide), RPA 104615, RPA 200766 and likely RPA 200761 (fipronil-amide) are higher than those of fipronil.

able 19. Summary of toxicological properties of fipronil and its metabolites present at ≥10% of the total radioactive residue in foods and food commodities

Brain binding lowest IC_{50} (nM)	Acute oral lowest LD_{50} (mg/kg bw)	Acute dermal lowest LD_{50} (mg/kg bw)	Short-term oral toxicity lowest NOAEL/ LOAEL (mg/kg bw/day)	Long term oral toxicity and carcinogenicity lowest NOAEL/ LOAEL (mg/kg bw/day)	Genotoxicity	Reproductive toxicity lowest NOAEL/ LOAEL (mg/kg bw/day)	Developmental toxicity lowest NOAEL/ LOAEL (mg/kg bw/day)	Acute neurotoxicity NOAEL/ LOAEL (mg/kg bw)	Post-natal developmental neurotoxicity NOALE/LOAEL (mg/kg bw/day)	Comments
Fipronil (M&B 46030)										
483	92 (rat)	345 (rabbit)	28-day: 1/10 (dog) 90-day: 0.5/2 (dog) 1-year: 0.2/1 (dog)[a]	0.02/0.6 (rat)	Negative	Maternal, offspring and reproductive: 2.5/26 (rat)	Maternal: 0.2/0.5 Developmental: 1[b] (rabbit)	0.5/5[c], 2.5/7.5[d] Overall NOAEL 2.5 (rat)	Maternal, postnatal developmental neurotoxicity & developmental toxicity: 0.9/15.2	–
M&B 46400										
	No data Detected in rat urine (after dosing with M&B 46513) Positive QSAR alerts for DNA binding and in vivo micronucleus test									
M&B 45950 (fipronil-sulfide, fipronil-thioether)										
155	69 (rat)	>500<4000 (rat)	28 day: 5/15 (dog) 90 day: 3.5/21.5, (rat)	-	Negative	-	-	-	-	-
M&B 46513 (fipronil-desulfinyl, a photometabolite)										
36	15 (rat)	> 2000, (rat)	28 day: 0.2/2.2 (rat) 90 day: 0.2/0.6 (rat)	0.025/0.1 (rat)	Negative	-	Maternal: 0.2/1 Developmental: 2.5[b] (rat)	2/12 (rat)	-	Short-term repeat dose NOAELs were similar in mice, rats and dogs.
M&B 46136 (fipronil-sulfone)										
84	184 (rat)	>2000 (rat)	-	-	Negative	-	-	-	-	-

Brain binding lowest IC_{50} (nM)	Acute oral lowest LD_{50} (mg/kg bw)	Acute dermal lowest LD_{50} (mg/kg bw)	Short-term oral toxicity lowest NOAEL/ LOAEL (mg/kg bw/day)	Long term oral toxicity and carcinogenicity lowest NOAEL/ LOAEL (mg/kg bw/day)	Genotoxicity	Reproductive toxicity lowest NOAEL/ LOAEL (mg/kg bw/day)	Developmental toxicity lowest NOAEL/ LOAEL (mg/kg bw/day)	Acute neurotoxicity NOAEL/ LOAEL (mg/kg bw)	Post-natal developmental neurotoxicity NOALE/LOAEL (mg/kg bw/day)	Comments
M&B 45897										
>10000	>2000 (rat)	>2000 (rat)	-	-	Negative	-	-	-	-	-
RPA 104615, a photometabolite										
>10 000	>2000 (rat)	-	28-day: 46/459 (rat)	-	Negative	-	-	-	-	-
RPA 105320										
3943	>2000 (rat)	-	-	-	-	-	-	-	-	-
RPA 105048										
	Negative bacterial reverse mutation assay Found in rat urine following dosing with M&B 465413									
RPA 108058										
	No data Positive QSAR alerts for DNA biding, in vitro bacterial reverse mutagenicity, carcinogenicity									
RPA 200761 (metabolite of RPA 200766)										
>10 000	>2000 (rat)	-	-	-	Negative	-	-	-	-	-
RPA 200766										
>10 000	>2000 (rat)	-	28-day: 4/38 (rat)	-	Negative	-	-	-	-	-

[a] NOAEL from Holmes, 1992; LOAEL from Holmes, 1993; [b] Highest dose tested;

[c] NOAEL was based on Gill et al., 1993 [d] Hughes ,1997; - No data;

3. Observations in humans

Manufacturing and production

No cases of human occupational disease associated with the production, transportation, formulation or packaging of fipronil-containing materials have been reported. While fipronil exposure was inversely correlated with blood thyroid hormones in people with occupational exposure to fipronil during veterinary drug manufacture, there were no effects on blood TSH concentrations detected (Herin et al. 2011).

Human poisoning incidents

Mohamed et al. (2004) reported seven documented cases of fipronil poisoning following the suicidal ideation-associated ingestion of a formulated agricultural product. Two of these cases involved ingestion of about 75 mg/kg bw of fipronil resulting in excessive sweating, nausea and vomiting. One asymptomatic patient had a peak measured plasma concentration of 1040 µg/L. Three of the seven cases developed clinical signs of central neurotoxicity including non-sustained generalized tonic-clonic seizures. In two of these patients peak measured plasma fipronil concentrations were 1600 and 3744 µg/L; seizures were responsive to benzodiazepines or barbiturates. Consistent with the toxicokinetics of fipronil in rats, high plasma levels of total fipronil (fipronil plus its sulfone, M&B 46136) following acute oral poisoning occurred for at least 3–4 days.

Fung et al. (2003) reported mild subjective sensory impairment in a patient following the ingestion of an ant bait containing about 0.14 mg fipronil. After spraying his field with a dilute fipronil solution a 50-year-old man complained of headache, nausea, vertigo and weakness, all symptoms resolving spontaneously after five hours. The French Antipoison Centres reported 410 cases of human exposure to fipronil-containing insecticide formulations from 1994 to 2004. Symptoms, if present at all, were considered mild and ascribed to associated solvents or adjuvants. The Dublin, Edinburgh, Göttingen, Zürich, and Perth Antipoison Centres collected information on 138 human exposures. In general, these reports described only minor symptoms such as vomiting and dizziness after ingestion, and irritation after inhalation and eye contact. There have been reports from China of 14 poisoning incidents possibly related to fipronil. Symptoms associated with these poisonings have been nausea, vomiting, stomach pain, diarrhoea, twitching and unconsciousness. In most cases, follow-up information was not available.

Despite the mild effects and good clinical outcomes reported in the above cases, serious cases of neurotoxicity that are poorly responsive to benzodiazepines have been reported. Bharathraj et al. (2015) reported on a non-fatal case of fipronil poisoning (dose not stated) in which the patient developed status epilepticus that was poorly responsive to repeated doses of lorazepam, although the seizures were eventually controlled with benzodiazepines.

4 Microbial aspects

4.1 Mechanism and type of antimicrobial action

No information was available.

4.2 Microbiome of the human gastrointestinal tract

There is no available information on the impact of fipronil or its metabolites on the human gut microbiome. However, a few studies have investigated the effects of fipronil on non-pathogenic *Escherichia coli* and the honeybee gut microbiota.

Bhatti et al. (2019) conducted a study to understand the impact of fipronil on non-pathogenic *E. coli*, which are important commensals of the intestinal tract of humans and animals and are also indicator organisms in the environment. In the study, exposure of *E. coli* to fipronil (100 µM concentration) led to significant production of reactive oxygen species and loss of membrane potential, also a 20% loss in viability. Furthermore, fipronil was bioaccumulated and biotransformed by *E. coli* at non-lethal concentrations such as 5 µM (equal to 2.1 ppm, or near to the concentration detected in vegetable and fruits) and 50 µM (21 ppm). The biotransformed products (fipronil-sulfone and fipronil-sulfide) are also

the major metabolites (along with the amide) reported in the faeces of mammals exposed to fipronil. Thus the authors suggested that there is a possibility that in the gut *E. coli* might play a role in degrading fipronil, and thus removing it. In addition, the bioaccumulation of fipronil in bacteria is of concern and needs to be further explored as the process could lead to biomagnification in the higher trophic level and can disturb the ecological balance.

Rouzé et al. (2019) invesitigated the honeybee gut microbiota when chronically exposured to low doses of neurotoxic insecticides including fipronil. Treatment with 1.0 µg/kg fipronil produced no stronger effect on lactic acid bacteria than a 0.25 µg/kg treatment, but induced an increase in *Gilliamella apicola* and *Snodgrasella alvi* in winter.

Paris et al. (2020) examined and quantified the effects of a parasite, *Nosema ceranae*, the neonicotinoid thiamethoxam, the phenylpyrazole fipronil and the carboxamide boscalid, alone and in combination, on the honeybee gut microbiota. Chronic exposures (0.5 µg/L) to fipronil and thiamethoxam alone or combined with *N. ceranae* infection significantly decreased honeybee survival, whereas the fungicide boscalid had no effect on uninfected bees. Regarding gut microbiota composition, co-exposure to the parasite and each pesticide led to a decreased abundance of Alphaproteobacteria, and increased abundance of Gammaproteobacteria. The parasite also induced an increase of bacterial alpha-diversity (species richness). The study demonstrated that exposure of honeybees to *N. ceranae* and/or pesticides plays a significant role in colony health and is associated with the establishment of a dysbiotic gut microbiota.

4.3 Antimicrobial spectrum of activity

No information was available.

4.4 Antimicrobial resistance mechanisms and genetics

No information was available.

Comments

Biochemical aspects

Fipronil exhibits dose- and dose regimen-dependent, but sex-independent, oral absorption in rats; oral systemic bioavailability is estimated at 39–53%. Following oral absorption, fipronil undergoes rapid and extensive first-pass metabolism. As a result fipronil-sulfone (M&B 46136) is the predominant systemically circulating and tissue chemical form found, representing >90% of systemic exposure following oral dosing. Fipronil and its first-pass metabolites were extensively distributed and persistent in lipophilic tissues (up to 55% of the administered radioactivity at 168 hours post dosing). The terminal elimination half-life ($t_{½}$) for fipronil-derived radioactivity was up to 175 hours. The major route of elimination was via faeco-biliary excretion. Enterohepatic cycling is extensive.

Mammalian metabolism of fipronil has four main features:

- the dominant metabolic pattern is rapid first-pass hepatic metabolism to fipronil-sulfone (M&B 46136), the predominant in vivo circulating and tissue metabolite;
- rats convert fipronil to fipronil-sulfone (M&B 46136) more quickly than humans;
- the metabolism of fipronil by rats is more extensive compared with metabolism in other species, including humans;
- a large number of minor metabolites are also generated in vivo.

Several of these minor metabolites, and the metabolic pathways that generate them, are not fully characterized. Enzymes of the CYP3A family (CYP3A4 in humans, CYP3A2 and CYP3A1 in rats) predominantly catalyse the conversion of fipronil to fipronil-sulfone. Fipronil-sulfone is an inhibitor of the CYP2C subfamily. Enantioselective metabolism of fipronil was not toxicologically significant in laboratory mammals.

Toxicological data

The acute oral median lethal dose (LD_{50}) of fipronil was ≥ 49 mg/kg bw (Gardner, 1988a; Oba, 1994; Dange, 1995a) and the dermal LD_{50} was ≥ 354 mg/kg bw (Gardner, 1988b; Myers, 1992). The inhalation median lethal concentration (LC_{50}) of fipronil was ≥ 0.36 mg/L (Cracknell, 1991; Nachreinner, 1995). Fipronil was not irritating to skin under aqueous conditions but was slightly irritating to the skin under lipophilic conditions (Liggett, 1988a; Myers & Cristopher, 1993). Fipronil was slightly irritating to the eyes in rabbits (Liggett, 1988b; Myers & Cristopher, 1993) and was not skin sensitizing in the Guinea pig (Rees, 1993, 2007; Smith, 1990).

In a battery of secondary and safety pharmacology studies in mice, oral gavage dosing of fipronil induced convulsions and clinical signs of neurotoxicity at ≥ 30 mg/kg bw (NOAEL 10 mg/kg bw), reduced pentylenetetrazol seizure threshold at ≥ 10 mg/kg bw (NOAEL 3 mg/kg bw), and pupillary dilation and inhibition of gastrointestinal transport at 30 mg/kg bw (NOAEL 10 mg/kg bw). No effects on the electroconvulsion threshold, body temperature, or hind limb traction were noted at doses up to 30 mg/kg bw. Fipronil at doses up to 30 mg/kg bw was not haemolytic (Horri, 1994).

Fipronil at concentrations up to 215.4 µg/mL was not phototoxic in an appropriately validated in vitro 3T3 NRU phototoxicity test (Landsiedal & Cetto, 2015).

In repeat-dose toxicity studies on mice, rats and dogs, the key effects were on the central nervous system, the liver and body weight parameters. Oral short-term toxicity studies with fipronil were conducted in the rat and the dog and consisted of four-week studies, ninety-day studies and, in the dog, two 1-year studies. Based on a human-relevance mode of action (MOA) evaluation, the effects of fipronil on the hypothalamic–pituitary–thyroid axis appeared secondary to its properties as a CAR and PXR receptor agonist. These effects are not, however, relevant to humans exposed to fipronil-derived residues in the diet.

No short-term studies of toxicity in the mouse were supplied. In a four-week oral toxicity study rats were fed diets incorporating fipronil at 0, 25, 50, 100, 200, or 400 ppm (equal to 0, 3.4, 6.9, 13, 24, and 45 mg/kg bw per day for males, 0, 3.5, 6.7, 13, 25, and 55 mg/kg bw per day for females). The NOAEL was 50 ppm (equal to 6.7 mg/kg bw per day) due to reduced body weight gain during the first week of treatment at ≥ 100 ppm (equal to 13 mg/kg bw per day) (Peters et al., 1990).

In a 13-week oral toxicity study rats were fed diets incorporating fipronil at 0, 1, 5, 30 or 300 ppm (equal to 0, 0.07, 0.33, 1.9 and 20 mg/kg bw per day for males, 0, 0.07, 0.37, 2.3 and 24 mg/kg bw per day for females). The NOAEL was 30 ppm (equal to 1.9 mg/kg bw per day) due to increased body fat deposition occurring in males dosed at 300 ppm (equal to 20 mg/kg bw per day) (Holmes, 1991a).

In a 4–6 week oral repeat-exposure toxicity study, dogs were capsule dosed with fipronil at 1 or 20 mg/kg bw per day for four weeks; other dogs were dosed at 0 or 10 mg/kg bw per day for six weeks. The NOAEL was 1 mg/kg bw per day due to clinical signs consistent with neurotoxicity at 10 mg/kg bw per day (Holmes, 1991b).

In a 13-week oral repeat-exposure toxicity study, dogs were capsule dosed with fipronil at 0, 0.5, 2 or 10 mg/kg bw per day. The NOAEL was 0.5 mg/kg bw per day due to reduced body weight gain at 2 mg/kg bw per day (Holmes, 1991c).

In a one-year oral repeat-exposure toxicity study, dogs were capsule dosed with fipronil at 0, 0.2, 2.0 or 5.0 mg/kg bw per day. The NOAEL was 0.2 mg/kg bw per day due to clinical signs consistent with neurotoxicity at 2.0 mg.kg bw per day (Holmes, 1992).

In a one-year dietary exposure toxicity study, dogs were fed fipronil in food at concentrations that achieved doses equal to 0, 0.075, 0.3, 1 or 3 mg/kg bw per day; the highest dose was reduced to 2 mg/kg bw per day after 38 days. The NOAEL was 0.3 mg/kg bw per day due to clinical signs consistent with neurotoxicity at 1 mg/kg bw per day (Holmes, 1993).

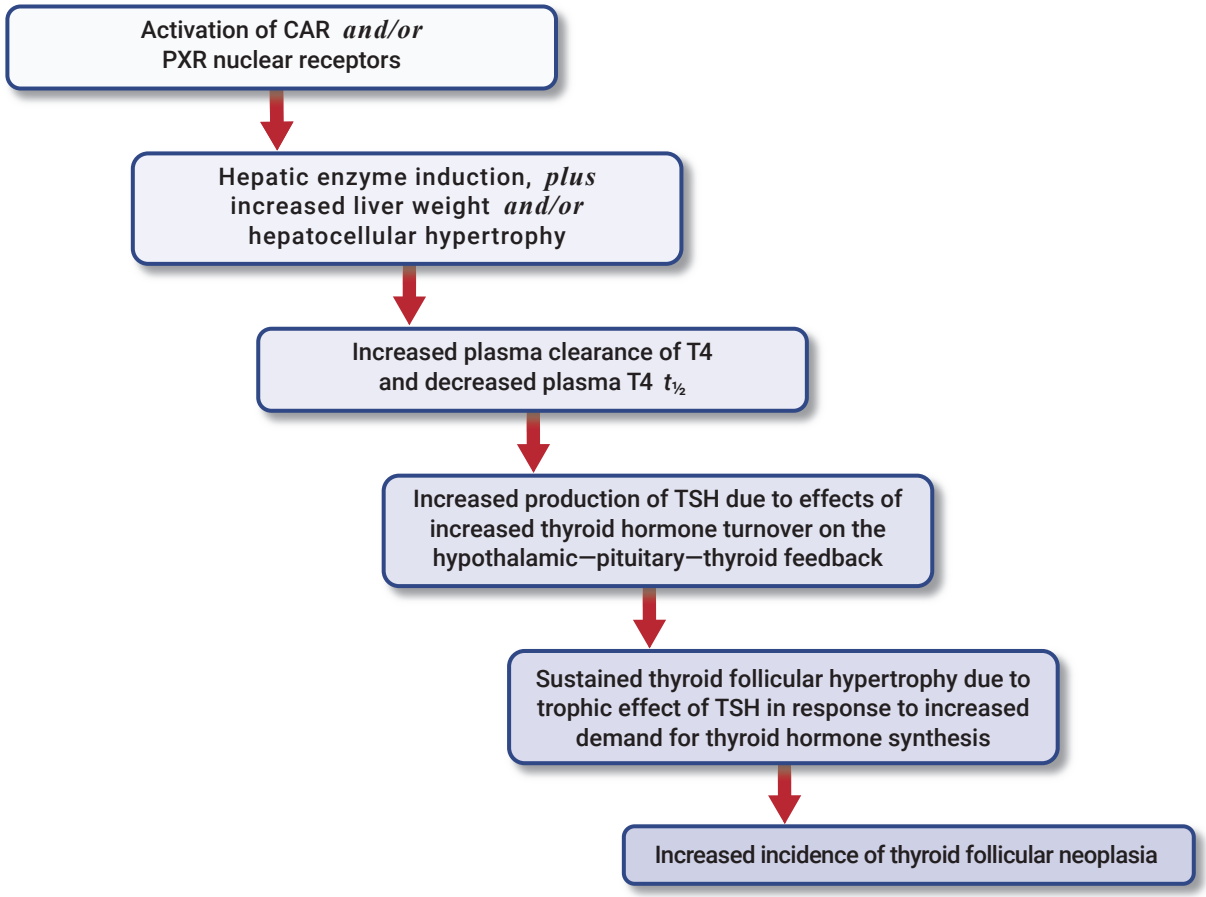
Based on the combined findings of the 13-week and one-year studies the overall NOAEL for repeat-dose toxicity in the dog was 0.3 mg/kg bw per day based on the occurrence of clinical signs of neurotoxicity occurring at doses of ≥ 1 mg/kg bw per day.

In a 78-week combined chronic toxicity and carcinogenicity study, mice were fed diets containing fipronil at 0, 0.1, 0.5, 10 or 30 ppm (equal to 0, 0.011, 0.055, 1.2 and 3.4 mg/kg bw per day

in males, 0, 0.012, 0.063, 1.2 and 3.6 mg/kg bw per day in females). An additional cohort was fed a diet containing 60 ppm. The dose in mg/kg bw per day was not determined because the animals in this last cohort either died during the first nine weeks of exposure or were euthanized after 10 weeks of treatment. The NOAEL for the study was 0.5 ppm (equal to 0.055 mg/kg bw per day) due to an increased incidence of hepatic microvesicular periacinar vacuolation in males and decreased body weight gain over the first 13 weeks of the study, both at 10 ppm (equal to 1.2 mg/kg bw per day). Fipronil was not carcinogenic in mice.

In an 89/91 week combined chronic toxicity and carcinogenicity study, rats were given dietary concentrations of 0, 0.5, 1.5, 30 or 300 ppm of fipronil (equal to 0, 0.019, 0.059, 1.3 and 13 mg/kg bw per week in males, 0, 0.025, 0.078, 1.6 and 17 mg/kg bw per day in females). The NOAEL for toxicity was 0.5 ppm (equal to 0.019 mg/kg bw per day) due to clinical signs consistent with neurotoxicity at 1.5 ppm (equal to 0.059 mg/kg bw per day). The NOAEL for carcinogenicity was 30 ppm (equal to 1.3 mg/kg bw per day), based on an increase in the incidence of thyroid follicular cell neoplasia at 300 ppm (equal to 13 mg/kg bw per day) (Aughton, 1993).

Fipronil had phenobarbital-like CAR and PXR receptor agonist properties with oral exposure in rats inducing hepatic CYP1A1, CYP2B1, CYP2B2, CYP2E1, CYP3A1, CYP3A4, the carboxylesterases Ces2 and Ces6, the phase II enzymes UDP-glucuronosyltransferase 1A1 (UGT1A1), SULT1B1 and glutathione *S*-transferase α2 (GSTA2), and the membrane transporters ATP-binding cassette subfamily C Member 2 (ABCC2), ABCC3, ABCG5, ABCG8, and the solute carrier organic anion transporters 1a1 (Slco1a1) and Slco1a4. ToxCast high throughput screening evaluation confirmed that subcytotoxic concentrations of fipronil showed CAR-receptor agonist behaviour. Based on a human relevance MOA evaluation, the effects of fipronil on the rat hypothalamic–pituitary–thyroid axis and induction of thyroid follicular epithelial neoplasia are consistent with the following mode of action:



Evaluating this from a human relevance standpoint, compared to rats humans are insensitive to this MOA. Thus, the effects of fipronil on the rat hypothalamic–pituitary–thyroid axis (and associated thyroid follicular epithelial cell neoplasia) have no relevance to humans exposed to fipronil at the levels that occur in the diet.

The Meeting concluded that fipronil is carcinogenic in rats due to a mode of action that is not relevant to humans. Fipronil is not carcinogenic in mice.

Fipronil was tested in an adequate range of validated in vitro and in vivo genotoxicity assays (Clare, 1988a; Edwards, 1993, 1995; Engelhardt & Leibold, 2004a, 2005a; Knight et al., 2009; Kurita 1994; Lloyd, 1993; Marshall, 1988a; Schulz, 2011; Schulz & Hellwig, 2006; Wright, 1995). No evidence of genotoxicity was found.

The Meeting concluded that fipronil is unlikely to be genotoxic.

In view of the lack of genotoxicity, the absence of carcinogenicity in mice, and the fact that the thyroid tumours observed in rats occurred via a MOA considered not relevant to humans, the Meeting concluded that fipronil is unlikely to pose a carcinogenic risk to humans following exposure in the diet.

In a rat multigenerational reproductive toxicity study, rats were fed fipronil at dietary concentrations of 0, 3, 30 or 300 ppm (equal to 0, 0.25, 2.5 and 26 mg/kg bw per day in males, 0, 0.27, 2.7 and 28 mg/kg bw per day in females) continuously for two generations. The NOAEL for parental toxicity, offspring toxicity and reproductive toxicity was 30 ppm (equal to 2.5 mg/kg bw per day) due to the presence of parental effects (deaths, convulsions, hepatotoxicity, reduced body weight gain, reduced food consumption), reproductive effects (reduced mating performance, reduced fertility index) and developmental effects (reduced pup body weight gain, delayed tooth eruption, delayed pinna unfolding and reduced prenatal and postnatal viability) at 300 ppm (equal to 26 mg/kg bw per day) (King, 1992).

In a developmental toxicity study in rats, dose levels of 0, 1, 4 or 20 mg/kg bw per day of fipronil were administered by oral gavage from days 6 to 15 post coitum (PC). The NOAEL for maternal toxicity was 4 mg/kg bw per day due to reduced body weight gain at 20 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 20 mg/kg bw per day, the highest dose tested (Brooker & David, 1991).

In a prenatal toxicity study in rabbits, dose levels of 0, 0.1, 0.2, 0.5 or 1.0 mg/kg bw per day were administered by oral gavage from days 6–19 PC. NOAEL for maternal toxicity was 0.2 mg/kg bw per day due to reduced body weight gain at 0.5 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 1.0 mg/kg bw per day, the highest dose tested (King, 1990).

In an acute neurotoxicity study rats were given a single oral gavage dose of 0, 0.5, 5.0 or 50.0 mg/kg bw. The NOAEL for the study was 0.5 mg/kg bw due to the presence of clinical signs consistent with neurotoxicity at 5.0 mg/kg bw (Gill, Wagner & Driscoll, 1993).

In a second acute neurotoxicity study rats were administered a single oral gavage dose of 0, 2.5, 7.5 or 25 mg/kg bw of fipronil. The NOAEL for the study was 2.5 mg/kg bw due to decreased hind leg splay in males at 7.5 mg/kg bw (Hughes, 1997). Taking into account the different dose ranges evaluated in the two studies of acute neurotoxicity (Gill et al., 1993; Hughes, 1997) the overall NOAEL for the two studies was 2.5 mg/kg bw.

In a 90-day neurotoxicity study rats were administered fipronil in the diet at concentrations of 0, 0.5, 5.0 or 150 ppm (equal to 0, 0.03, 0.3 and 8.9 mg/kg bw per day in males, 0, 0.04, 0.35 and 11 mg/kg bw per day in females). The NOAEL was 150 ppm (equal to 8.9 mg/kg bw per day), the highest dose tested (Driscoll & Hurley, 1993).

In a postnatal developmental neurotoxicity study in rats, fipronil was administered continuously via the diet at dietary concentrations of 0, 0.5, 10 or 200 ppm (equal to 0, 0.05, 0.91 and 15 mg/kg bw per day) from day 6 PC to day 10 of lactation. The NOAEL for maternal toxicity, postnatal developmental neurotoxicity and developmental toxicity was 10 ppm (equal to 0.91 mg/kg bw per day) (Mandella, 1995).

In an oral, daily repeat-dose study of neurotoxicity, four female dogs were given 20 mg/kg bw per day of fipronil until signs of neurotoxicity were observed. Clinical signs consistent with neurotoxicity were observed after ≥ 6 days of dosing and signs of neurotoxicity persisted for up to 10 days following the cessation of dosing. There were no neuroanatomical abnormalities (Holmes, 1991d).

Toxicological data on metabolites and degradates

M&B 45950 (Fipronil-sulfide, fipronil-thioether)

Fipronil-sulfide (M&B 45950) is present in plants, livestock, milk and the environment.

Following oral dosing of rats with fipronil-sulphide, the major route of excretion of radioactivity was the faeces (≥66% of the administered radioactivity). Absorbed radioactivity was widely distributed to the tissues but was highest in the fat (Lowden & Fisher, 1989).

Fipronil-sulfide retains the –CN group present on fipronil, but a $–SCF_3$ side chain replaces the parent's $–SOCF_3$ side chain. These features were associated with an approximately three- to five-fold reduction in the half-maximal inhibitory concentration (IC_{50}) for rat brain $GABA_A$-gated chloride channel binding compared to that of fipronil. Notably, and in contrast to fipronil, clinical signs consistent with neurotoxicity were not observed in short-term repeated oral exposure studies in rats and dogs treated with fipronil-sulfide.

The acute oral median lethal dose (LD_{50}) for fipronil-sulfide was ≥69 mg/kg bw (similar to fipronil; Dange, 1994a) and its dermal LD_{50} was >500 < 4000 mg/kg bw (Haynes, 1988b). Fipronil-sulfide was not irritant to the skin of rabbits but was a slight eye irritant in this species (Haynes, 1987a, b).

In a 13-week toxicity study rats were administered fipronil-sulfide in the diet at concentrations of 0, 10, 25, 50 or 300 ppm (equal to 0, 0.69, 1.8, 3.5 and 21 mg/kg bw per day in males, 0, 0.81, 2.2, 4.1 and 25 mg/kg bw per day in females). The NOAEL was 3.5 mg/kg bw per day due to effects on body weight, damaged vibrissae and nasal discharge observed at 21 mg/kg bw per day (Broadmeadow, 1991c).

In a 28-day oral toxicity study dogs were capsule dosed with 0, 1, 5 or 15 mg/kg bw per day of fipronil-sulfide. The NOAEL was 5 mg/kg bw per day due to reduced body weight gain at 15 mg/kg bw per day. Fipronil-sulfide did not induce bacterial reverse mutations or chromosomal aberrations in human lymphoctyes in vitro (Asquith, 1987; Marshall, 1988b; Percy, 1994a).

The Meeting concluded that fipronil-sulfide (M&B 45950) is unlikely to be genotoxic.

Overall, the available data indicates that fipronil-sulfide (M&B 45950) has similar acute toxicity to fipronil, has similar toxicity following repeated oral exposure (in rats 13-week oral LOAELs for fipronil-sulfide and fipronil were 21 and 20 mg/kg bw per day, respectively) and a lower propensity to induce fipronil-like neurotoxicity. Fipronil sulfide has toxicological relevance in relation to dietary exposure to fipronil residues in the diet, is regarded as being equitoxic with fipronil (although likely having reduced neurotoxic potency) and is covered by the health-based guidance values for fipronil.

M&B 46136 (fipronil-sulfone)

Fipronil-sulfone (M&B 46136), the dominant systemic and tissue metabolite of fipronil in all evaluated mammalian species, is found in plants, livestock and the environment.

Oral bioavailability in rats was estimated to be ≥84%. Fipronil-sulfone is widely distributed and persists in the tissues (>35% of fipronil-sulfone and its metabolites remaining at 168 hours post dose). Up to 63% of fipronil-sulfone and its metabolites are excreted via the faeco-biliary route. The dominant chemical species present in faeces was fipronil-sulfone (up to 57% of the administered dose). Up to 10% of fipronil-sulfone and its metabolites were excreted in urine. The dominant metabolite in urine was the 5-amino-glucuronide conjugate UMET/3 (up to 7% of the dose; Kemp, 1999b).

In fipronil-sulfone the $–SOCF_3$ side chain of fipronil is replaced with $–SO_2CF_3$. This is associated with a rat brain $GABA_A$-gated chloride binding IC_{50} about 2.5-fold to 5.8-fold lower than that of fipronil. Being the dominant chemical form in all tissues, fipronil-sulfone is likely the main systemic driver of the neurotoxic effects seen with fipronil.

The acute oral LD_{50} of fipronil-sulfone was ≥184 mg/kg bw (similar to fipronil; Dange, 1994a) and the dermal LD_{50} was > 500 < 4000 mg/kg bw (Dange, 1993d). Fipronil-sulfone was not irritant to the skin of rabbits but was a mild eye irritant in this species (Liggett, 1988c, d).

Fipronil-sulfone did not induce bacterial reverse mutations or chromosomal aberrations in mammalian cells in vitro (Clare, 1988b; Marshall, 1989).

The Meeting concluded that fipronil-sulfone (M&B 46136) is unlikely to be genotoxic.

Overall, the available data indicates that fipronil-sulfone is likely responsible for the neurotoxic effects associated with the exposure of mammals to fipronil. Accordingly, based on this and the fact that it accounts for most of the dose (>90%) in rats, it is toxicologically relevant and is covered by the health-based guidance values for fipronil.

M&B 46513 (fipronil-desulfinyl), 5-amino-1-(2,6-dichloro-4-(trifluoromethyl)phenyl)-4-trifluoromethylpyrazole-3-carbonitrile, a photometabolite

Fipronil-desulfinyl (M&B 46513) is found in plants and livestock. Fipronil-desulfinyl is also an environmental metabolite produced by soil metabolism as well as soil and aqueous photolysis.

Following oral dosing of rats, fipronil-desulfinyl was relatively well absorbed and extensively metabolized by oxidations followed by sulfate and amino acid conjugations. Fipronil-desulfinyl was widely distributed, particularly to lipophilic tissues, and was persistent in the carcass (up to 17% of the administered radioactivity remaining at 168 hours post dose). Elimination was primarily in the faeces. The chemical identification of its various polar urinary and faecal metabolites (sulfate, glucuronide, cysteine and cysteine–glycine conjugates) is incomplete (Jones & Hardy, 1996).

Fipronil-desulfinyl retains fipronil′s –CN group, but the $-SOCF_3$ sidechain present in fipronil is replaced with a $-CF_3$ group. This change results in a three- to five-fold decrease in the rat brain $GABA_A$-gated chloride binding IC_{50} when compared with that of fipronil.

The acute oral LD_{50} of fipronil-desulfinyl was ≥15 mg/kg bw (about six-fold lower than fipronil; Dange, 1993a) and the dermal LD_{50} was >2000 mg/kg bw (Dange, 1993d). The key clinical signs observed in the rat acute oral toxicity studies for fipronyl-desulfinyl were consistent with neurotoxicity: increased reactivity to noise at the LOAEL of ≥3 mg/kg bw, reduced motor activity at ≥10 mg/kg bw, convulsions at 30 mg/kg bw.

In a 90-day toxicity study mice were fed diets containing fipronil-desulfinyl at 0, 0.5, 2 or 10 ppm (equal to 0, 0.08, 0.32, and 1.7 mg/kg bw per day in males, 0, 0.11, 0.43 and 2.2 mg/kg bw per day in females). The NOAEL was 2 ppm (equal to 0.32 mg/kg bw per day) due to mortality and neurotoxicity observed at 1.7 mg/kg bw per day (Lasserre-Bigot, 1996).

In a 28-day toxicity study rats were administered fipronil-desulfinyl in their diet at concentrations of 0, 0.5, 3, 30 or 100 ppm (equal to 0, 0.04, 0.23 and 2.2 mg/kg bw per day in males, 0, 0.04, 0.24 and 2.3 mg/kg bw per day in females). All animals fed at 100 ppm died before the completion of the study. The NOAEL was 3 ppm (equal to 0.23 mg/kg bw per day) due to the effects on body weight and neurotoxicity observed at 30 ppm (equal to 2.2 mg/kg bw per day) (Dange, 1995b).

In a 90-day toxicity study rats were fed diets containing fipronil-desulfinyl at 0, 0.5, 3, 10 or 30 ppm (equal to 0, 0.029, 0.18, 0.59 and 1.8 mg/kg bw per day in males, 0, 0.035, 0.21, 0.71 and 2.1 mg/kg bw per day in females). The NOAEL was 0.5 ppm (equal to 0.029 mg/kg bw per day) due to the presence of clinical signs consistent with neurotoxicity at 3 ppm (equal to 0.18 mg/kg bw per day) (Dange, 1995c).

In a 28-day toxicity study dogs were fed diets containing fipronil-desulfinyl at 0, 27, 80 or 270 ppm. All animals dosed at ≥80 ppm died before the completion of the study. The LOAEL was 27 ppm (equal to 1 mg/kg bw per day; the lowest dose tested) due to the evidence of neurotoxicity at this dose level (Dange, 1995c).

In a 90-day toxicity study dogs were fed diets containing 0, 3.5, 9.5 or 35 ppm (equal to 0, 0.1, 0.27 and 0.95 mg/kg bw per day in males, 0, 0.1, 0.29 and 1.05 mg/kg bw per day in females) of fipronil-desulfinyl for 90 days. The NOAEL was 9.5 ppm (equal to 0.27 mg/kg bw per day) due to clinical signs consistent with neurotoxicity at 35 ppm (equal to 0.95 mg/kg bw per day) (Dange, 1996).

In a 24-month combined chronic toxicity and carcinogenicity study, rats were fed diets containing fipronil-desulfinyl at 0, 0.5, 2 or 10 ppm, equal to 0, 0.025, 0.098 and 0.797 mg/kg bw per day in males.

In females this was equal to 0, 0.032, 0.127, and 0.733 mg/kg bw per day. However, due to excessive mortality amongst high-dose females over the first 26 weeks their dose was reduced to 6 ppm (equal to 0.546 mg/kg bw per day) for the remainder of the study. The NOAEL for toxicity was 0.5 ppm (equal to 0.025 mg/kg bw per day) due to the presence of clinical signs consistent with neurotoxicity at 2 ppm (equal to 0.098 mg/kg bw per day) (Lasserre-Bigot, 1998). No evidence of treatment-related neoplastic changes was seen.

Fipronil-desulfinyl did not induce bacterial reverse mutations or forward mutations in mammalian cells or chromosomal aberrations in mammalian cells in vitro (Adams, 1996a, b; Percy, 1993a).

The Meeting concluded that fipronil-desulfinyl (M&B 46513) is unlikely to be genotoxic.

In a developmental toxicity study rats were orally gavage dosed with 0, 0.2, 1.0 or 2.5 mg/kg bw per day of fipronil-desulfinyl over days 6–15 PC. The maternal NOAEL was 0.2 mg/kg bw per day due to effects on maternal body weight at 1.0 mg/kg bw per day. The NOAEL for developmental toxicity was 1.0 mg/kg bw per day due to the presence of reduced fetal weights at 2.5 mg/kg bw per day.

In an acute oral neurotoxicity study, rats were administered single oral gavage doses of 0, 0.5, 2.0 or 12 mg/kg bw of fipronil-desulfinyl in corn oil. The NOAEL for general toxicity and neurotoxicity was 2 mg/kg bw due to effects on body weight gain, hind leg splay, body temperature, motor activity and righting reflex at 12 mg/kg bw (Hughes, 1996).

Overall the available database on fipronil-desulfinyl (M&B 46513) is very extensive and indicated that it is approximately equitoxic with fipronil, has a similar toxicological profile to fipronil and is toxicologically relevant to exposure of fipronil-derived residues in the diet. It is covered by the health-based guidance values for fipronil.

M&B 45897
5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-1H-pyrazole-3-carbonitrile

Metabolite M&B 45897 is not a food or feed residue. It is an aerobic metabolite in soil.

Following oral dosing of rats, radioactivity derived from M&B 45897 was rapidly absorbed, and widely distributed to the tissues, with a terminal elimination half-life of about 115 hours. It was eliminated in the urine (about 52% over 168 h) and in the faeces (37% over 168 h). The major chemical form present in urine was unchanged M&B 45897 (about 27% of the urine radioactivity). A multi-component chromatographic peak designated UMET/4 was responsible for about 21% of the radioactivity present in urine. Other metabolites were present at < 1% of the administered dose. Up to ten M&B 45897-derived metabolites were present in faeces, the most significant being FMET/1, which accounted for about 12% of the administered dose (Fisher, 1996).

M&B 45897 lacks the $-SOCF_3$ side chain present on fipronil, but retains the $-CN$ group. This is consistent with its high IC_{50} for rat brain $GABA_A$-gated chloride binding, its low acute oral toxicity in rats, and a low propensity for fipronil-like neurotoxicity.

The acute oral (LD_{50}) of M&B 45897 was > 2000 mg/kg bw (Haynes, 1988a) and its dermal LD_{50} was > 2000 mg/kg bw (Haynes, 1988b). M&B 45897 was not irritant to the skin of rabbits but was a slight eye irritant in this species (Haynes, 1988c, d).

M&B 45897 did not induce bacterial reverse mutations, did not induce chromosomal aberrations in human lymphocytes in vitro and did not induce erythrocyte bone marrow micronuclei in mice in vivo (Johnson, 1995; Kenelly, 1988; Percy, 1995a).

The Meeting concluded that M&B 45897 is unlikely to be genotoxic.

As M&B 45897 lacks the neurotoxic potential of fipronil, exposure in the diet should be compared with the Cramer class III threshold of toxicological concern, 1.5 µg/kg bw per day.

M&B 46400
5-amino-3-cyano-1-(2,6-dichloro-4-trifluoromethylphenyl)-1H-pyrazole-4-carboxylic acid

Metabolite M&B 46400 is found after the heat treatment of food at 120°C, as well as in livestock. It is also a soil and water hydrolysis product of the photometabolite fipronil desulfinyl (M&B 46513).

No data was submitted for this metabolite. M&B 46400 retains the –CN group of fipronil, but replaces fipronil′s $-SOCF_3$ side chain with a $-CO_2H$ group. Although definitive data is lacking, the known neurotoxicity structure activity relationships of this family of compounds predict that M&B 46400 is likely to have a low propensity for neurotoxicity.

M&B 46400 is a major metabolite in urine (> 5% of the administered dose) after exposure of rats to M&B 46513 (Jones & Hardy, 1996). Therefore it can be assumed that M&B 46400 is also formed in significant amounts in the S9 mix reactions performed as part of the in vitro genotoxicity studies with M&B 46513. Consequently, the genotoxicity data for M&B 46513 is also applicable for M&B 46400. On this basis the Meeting concluded that M&B 46400 is unlikely to be genotoxic.

As M&B 46400 lacks the neurotoxic potential of fipronil, exposure in the diet should be compared with the Cramer class III threshold of toxicological concern, 1.5 µg/kg bw per day.

RPA 104615
potassium 5-amino-3-cyano-1-(2,6-dichloro-4-trifluoromethylphenyl)-1H-pyrazole-4-sulfonate; a photometabolite

RPA 104615 is found in plants and is an environmental metabolite produced by soil and aqueous photolysis.

RPA 104615 retains the –CN group of fipronil, but replaces fipronil′s $-CF_3$ moiety on the side chain with a $-SO_3^-$. This is associated with high IC_{50} value for rat brain $GABA_A$-gated chloride binding, and a high acute oral LD_{50}.

The acute oral LD_{50} of RPA 104615 was > 2000 mg/kg bw (Dange, 1993b).

In a 28-day toxicity study rats were fed diets containing 0, 50, 500, 5000 or 10 000 ppm of RPA 104615 (equal to 0, 4.5, 45.7, 459 and 916 mg/kg bw per day in males, 0, 4.7, 50.4, 487 and 950 mg/kg bw per day in females). The NOAEL was 500 ppm (equal to 45.7 mg/kg bw per day) due to increased prothrombin time and increased serum triglycerides at 5000 ppm (equal to 459 mg/kg bw per day) (Dange, 1997, 2000)

RPA 104615 did not induce bacterial reverse mutations and did not induce chromosome aberrations in human lymphocytes in vitro (Allais, 2002a; Percy, 1993b).

The Meeting concluded that RPA 104615 is unlikely to be genotoxic.

Due to the minimal toxicity of RPA 104615 at doses up to 459 mg/kg bw per day, exposure to RPA 104615 in the diet is not of toxicological concern.

RPA 105048
5-amino-1-(2,6-dichloro-4-trifluoromethylphenyl)-4-(trifluoromethyl)-1H-pyrazole-3-carboxamide

RPA 105048 is a major (present at ≥ 10%) residue in food following heating at 120°C, as well as an environmental hydrolysis product of fipronil-desulfinyl (M&B 46513).

RPA 105048 substitutes a $-CF_3$ side-chain for the $-SOCF_3$ side chain present in fipronil, and replaces the –CN moiety with a $-CONH_2$ group. Although definitive data is lacking, the known neurotoxicity structure activity relationships of this family of compounds predict that RPA 105048 is likely to have a low propensity for neurotoxicity.

RPA 105048 did not induce bacterial reverse mutations in vitro (Percy, 1994a).

The Meeting concluded that RPA 105048 is unlikely to be genotoxic.

As RPA 105048 lacks the neurotoxic potential of fipronil, exposure in the diet should be compared with the Cramer class III threshold of toxicological concern, 1.5 µg/kg bw per day.

RPA 105320
5-amino-1-(2,6-dichloro-4-trifluoromethylphenyl)-4-(trifluoromethylsulfonyl)-1H-pyrazole-3-carboxamide

RPA 105320 is found in plants and is also an environmental residue produced by soil hydrolysis.

RPA 105320, lacks fipronil's –CN group and has a $-SOCF_3$ side chain. This is associated with high IC_{50} values for rat brain $GABA_A$-gated chloride binding (> 10 000 nM) and a high acute oral LD_{50} in rats. RPA 105320 is unlikley to cause fipronil-like neurotoxicity.

The acute oral LD_{50} of RPA 105320 was > 2000 mg/kg bw (Dange, 1994b).

RPA 105320 did not induce bacterial reverse mutations in vitro (Percy, 1994b).

The Meeting concluded that RPA 105320 is unlikely to be genotoxic.

As RPA 105320 lacks the neurotoxic potential of fipronil, exposure in the diet should be compared with the Cramer class III threshold of toxicological concern, 1.5 µg/kg bw per day.

RPA 108058

RPA 108058 is found in livestock that have been dosed with fipronil-desulfinyl (M&B 46513).

No data was submitted for this metabolite. RPA 108058 has substantial structural differences from fipronil and is unlikely to produce fipronil-like neurotoxicity.

Based on QSAR evaluation, RPA 108058 is not predicted to be genotoxic. RPA 108058 is also a metabolite of fipronil-amide (RPA 200766), which did not induce bacterial reverse mutations or chromosomal aberrations in mammalian cells in vitro (Allais, 2002b; Percy, 1993c).

The Meeting concluded that RPA 105320 is unlikely to be genotoxic at levels likely to occur in the diet.

As RPA 108058 is considered to lack the neurotoxic potential of fipronil, dietary exposure should be compared with the Cramer class III threshold of toxicological concern per day, 1.5 µg/kg bw per day.

RPA 200761
5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfinyl]-1H-pyrazole-3-carboxylic acid

RPA 200761 is found in plants.

RPA 200761 was detected in the bile of rats orally dosed with fipronil; the fraction of administered radioactivity was not reported. Compared with fipronil it lacks the –CN group but posesses a $-SOCF_3$ side chain. This is associated with high IC_{50} values for rat brain GABA-gated chloride channel binding (> 10 000 nM) and a high acute oral LD_{50} in rats).

The acute oral LD_{50} of RPA 200761 was > 2000 mg/kg bw (Katchadourian, 1995).

RPA 200761 is a metabolite of fipronil-amide (RPA 200766). Fipronil-amide did not induce bacterial reverse mutations, or chromosomal aberrations in mammalian cells in vitro (Allais, 2002b; Percy, 1993c).

The Meeting concluded that RPA 200761 is unlikely to be genotoxic at levels likely to occur in the diet.

As RPA 200761 lacks the neurotoxic potential of fipronil, exposure in the diet should be compared with the Cramer class III threshold of toxicological concern, 1.5 µg/kg bw per day.

RPA 200766, (fipronil-amide, fipronil-carboxamide) 5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfinyl]-1H-pyrazole-3-carboxamide

Fipronil amide (RPA 200766) is found in plants and livestock. It is an environmental metabolite formed by soil metabolism and hydrolysis.

Fipronil-amide was detected in the faeces and urine (approximately 2%) of rats orally dosed with fipronil. Compared with fipronil it replaces the –CN group with a $-CONH_2$ group, and also has a $-SOCF_3$ side chain. This is associated with high IC_{50} values for rat brain $GABA_A$-gated chloride binding (> 10 000 nM) and a high acute oral LD_{50} in rats.

The acute oral LD_{50} of fipronil-amide (RPA 200766) was > 2000 mg/kg bw (Dange, 1993c).

In a 28-day toxicity study, rats were fed diets containing 0, 50, 500, 5000 or 15 000 ppm (equal to 0, 3.8, 38, 385 and 1087 mg/kg bw per day in males, 0, 4.4, 44, 387 and 1063 mg/kg bw per day in females) of fipronil-amide. The NOAEL was 50 ppm (equal to 3.8 mg/kg bw per day) due to the presence of adverse adrenal zona fasciculata vacuolation at 500 ppm (equal to 38 mg/kg bw per day) (Berthe, 1996).

Fipronil-amide did not induce bacterial reverse mutations, or chromosomal aberrations in mammalian cells in vitro (Allais, 2002b; Percy, 1993c).

The Meeting concluded that fipronil-amide (RPA 200766) is unlikely to be genotoxic.

Overall, the available data indicates that fipronil amide (RPA 200766) is likely to be less toxic than fipronil, but has toxicological relevance to exposure of fipronil-derived residues in the diet. It is covered by the health-based guidance values for fipronil.

Overall conclusions on metabolites

The Meeting concluded that fipronil-sulfide (M&B 45950), fipronil-sulfone (M&B 46136), fipronil-desulfinyl (M&B 46513) and fipronil amide (RPA 200766) are toxicologically relevant to exposure to fipronil-derived residues in the diet and are of equal or less potency than the parent. Risk assessment should be performed using the health-based guidance values for fipronil.

RPA 104615 is of considerably lower toxicity than fipronil and other metabolites and was considered not to be toxicologically relevant.

Exposure in the diet to the remaining fipronil-derived metabolites should be compared with the Cramer class III threshold for toxicological concern, 1.5 µg/kg bw per day.

Microbiological data

Fipronil, at the level of exposure encountered in food, is unlikely to result in adverse effects on the human gut microbiome.

Human data

No cases of human occupational disease associated with the production, transportation, formulation and packaging of fipronil-containing materials have been reported.

In terms of occupational exposure, one study examined thyroid function among workers involved in production of fipronil-containing veterinary drugs (Herin et al., 2011). Fipronil was detected in serum of around 20% of workers while fipronil-sulfone was detected in all participants. Eighteen workers had abnormal serum thyroid levels of which seven had elevated thyroid-stimulating hormone (TSH). Serum fipronil and fipronil-sulfone were not associated with thyroid hormone levels, with the exception of a modest inverse relationship between fipronil-sulfone and THS (Spearman′s $\rho \sim -0.2$). Taken together this study found no suggestion for a link between occupational exposure to fipronil and thyroid function.

In terms of background exposed population, one small study ($n = 59$) from South Korea quantified fipronil-sulfone in maternal serum and cord blood sampled at delivery (Kim et al., 2019). The concentrations in these samples were highly correlated (Pearson′s $r \sim 0.75$), suggesting efficient placental transfer. An inverse correlation of fipronil-sulfone with T3 and free T3 concentrations in cord blood was observed, but no association was found for other thyroid hormones. Fipronil-sulfone in cord blood was also inversely associated with Apgar score five minutes after birth. Given the cross-sectional nature of these analyses and the small sample size, no conclusion on possible associations with newborn thyroid hormones or Apgar score can be drawn.

The human clinical toxidrome associated with suicidality-associated ingestion of fipronil is consistent with its neurotoxicological MOA (clinical signs of central neurotoxicity including tonic-clonic convulsions). In general, such poisonings are responsive to $GABA_A$ agonist drugs (benzodiazepines, barbiturates).

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

Toxicological evaluation

The Meeting established an ADI of 0–0.000 2 mg/kg bw per day based on the NOAEL of 0.019 mg/kg bw per day in the combined chronic toxicity and carcinogenicity study in rats and using a safety factor of 100.

The Meeting established an ARfD (for the general population) of 0.03 mg/kg bw, on the basis of the group NOAEL of 3 mg/kg bw per day in the acute neurotoxicity study in rats and the safety pharmacology studies (pentylenetetrazol seizure threshold) in mice. A safety factor of 100 was used.

Because of the long elimination half-lives of fipronil and fipronil-sulfone (the major chemical form in the body) intermittent and repeated exposures to fipronil dietary residues over any duration should not exceed the ADI.

Levels relevant to risk assessment of fipronil

Species	Study	Effect	NOAEL	LOAEL
Mouse	78-week study of toxicity and carcinogenicity [a]	Toxicity	0.5 ppm, equal to 0.6 mg/kg bw per day	10 ppm, equal to 1.2 mg/kg bw per day
		Carcinogenicity	30 ppm, equal to 3.6 mg/kg bw per day [c]	-
	Acute pentylenetetrazol seizure threshold [b]	Neurotoxicity	3 mg/kg bw	10 mg/kg bw
Rat	Acute neurotoxicity study [b]	Neurotoxicity	2.5 mg/kg bw	5.0 mg/kg bw
	Two-year studies of toxicity and carcinogenicity [a, d]	Toxicity	0.5 ppm, equal to 0.019 mg/kg bw per day	1.5 ppm, equal to 0.06 mg/kg bw per day
		Carcinogenicity	300 ppm, equal to 16.8 mg/kg bw per day [c]	-
	Two-generation study of reproductive toxicity [a]	Reproductive toxicity	30 ppm, equal to 2.5 mg/kg bw per day	300 ppm, equal to 26 mg/kg bw per day
		Parental toxicity	30 ppm, equal to 2.5 mg/kg bw per day	300 ppm, equal to 26 mg/kg bw per day
		Offspring toxicity	30 ppm, equal to 2.5 mg/kg bw per day	300 ppm, equal to 26 mg/kg bw per day
	Developmental toxicity study [b]	Maternal toxicity	4 mg/kg bw per day	20 mg/kg bw per day
		Embryo and fetal toxicity	20 mg/kg bw per day [c]	-
Rabbit	Developmental toxicity study [b]	Maternal toxicity	0.2 mg/kg bw per day	0.5 mg/kg bw per day
		Embryo and fetal toxicity	1.0 mg/kg bw per day [c]	-
Dog	13-week and one-year studies of toxicity [d, f]	Toxicity	0.3 mg/kg bw per day	1 mg/kg bw per day
Fipronil-sulfide (M&B 45950)				
Rat	13-week study of toxicity [a]	Toxicity	3.5 mg/kg bw per day	21.5 mg/kg bw per day
Fipronil-sulfone (M&B 46136)				
Major systemic active metabolite that is responsible for > 90% of systemic exposure following oral dosing				

(Continued on next page)

Species	Study	Effect	NOAEL	LOAEL
Fipronil-desulfinyl (M&B 46513, photometabolite)				
Rat	Two-year studies of toxicity and carcinogenicity [a, d]	Toxicity	0.5 ppm, equal to 0.03 mg/kg bw per day	2 ppm, equal to 0.1 mg/kg bw per day
		Carcinogenicity	10 ppm, equal to 0.8 mg/kg bw per day [c]	-
Fipronil-amide (RPA 200766)				
Rat	28-day study of toxicity [a]	Toxicity	3.8 mg/kg bw per day	38.2 mg/kg bw per day

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 fipronil, fipronil-sulfide (M&B 45950), fipronil-sulfone (M&B 46136), fipronil-amide (RPA 200766) and fipronil-desulfinyl (M&B 46513) expressed as fipronil

0–0.000 2 mg/kg bw

Acute reference dose (ARfD) applies to fipronil, fipronil-sulfide (M&B 45950), fipronil-sulfone (M&B 46136), fipronil-amide (RPA 200766) and fipronil-desulfinyl (M&B 46513) expressed as fipronil

0.03 mg/kg bw

Because of the long elimination half-lives of fipronil and fipronil-sulfone (M&B 46136) the major chemical form in the body, intermittent and repeated exposures to fipronil dietary residues over any duration should not exceed the ADI.

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

No additional data is required.

Critical end-points for setting guidance values for exposure to [compound]

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rats: low to high (estimated 34 to 90%) and was dose- and dose regimen-dependent (but sex-independent)
	Rabbits: high (estimated 80%)
Dermal absorption	Rat: low (< 0.4% to < 3%); dose-dependent
Distribution	Rat: rapid and extensive with accumulation in lipophilic tissues
	Rabbit: rapid and extensive with accumulation in lipophilic tissues
Potential for accumulation	Significant potential for accumulation.
	Rat: present in the tissues for at least 99 days post dose; mean adipose tissue elimination $t_{½}$ of up to 545 ± 56 h following repeated oral dosing. Retention of administered radioactivity in the tissues was dose-dependent and was higher with lower doses
	Rabbit: present in the tissues for at least 75 days days post dose; blood elimination $t_{½}$ up to about 49.5 days

Rate and extent of excretion	Rat: following a single oral dose the radioactivity mean terminal elimination $t_{½}$ was about 175 h, likely due to the slow release of fipronil-sulfone from lipophilic tissues; considerable enterohepatic recirculation occurs Rabbit: excretion via urine and faeces was protracted, likely reflecting the slow release of radioactivity from lipophilic tissues
Metabolism in animals	Rat: fipronil underwent rapid and extensive first-pass metabolism to fipronil-sulfone (major systemic metabolite); minor metabolites included fipronil conjugates (mostly glucuronides); the conjugates were rapidly excreted in urine whereas fipronil-sulfone was the major component (90% of radiolabel) in tissues (especially in lipophilic tissues) Humans: based on in vitro data, conversion from fipronil to fipronil-sulfone is slower in humans than in rats
Toxicologically significant compounds in animals and plants	Fipronil, fipronil-sulfone, fipronil-sulfide, fipronil-amide and fipronil-desulfinyl
Acute toxicity	
Rat, LD_{50}, oral	92 mg/kg bw
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rabbit, LD_{50}, dermal	354 mg/kg bw
Rat, LC_{50}, inhalation	0.36 mg/L
Rabbit, dermal irritation	Aqueous conditions: not irritating Lipophilic conditions: slightly irritating
Rabbit, ocular irritation	Slightly irritating
Guinea pig, dermal sensitization	Not sensitizing (Buehler three-fold induction)
Short-term studies of toxicity	
Target/critical effect	CNS neurotoxicity
Lowest relevant oral NOAEL	0.3 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	5 mg/kg bw per day (rabbit; occlusive exposure)
Lowest relevant inhalation NOAEC	No data.
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	CNS neurotoxicity
Lowest relevant NOAEL	0.2 mg/kg bw per day (rat)
Carcinogenicity	Carcinogenic in rats [a] Thyroid carcinogen in rats secondary to CAR xenosensor activation-associated increased plasma clearance of T4 and T4 $t_{½}$: this mode of action is not relevant to humans
Genotoxicity	Unlikely to be genotoxic
Reproductive toxicity	
Target/critical effect	Increased mortality, convulsions, reduced body weight gain and food consumption, reduced mating index, reduced fertility index, reduced viability index, delayed tooth eruption, delayed pinna unfolding, and hepatoxicity
Lowest relevant parental NOAEL	2.5 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	2.5 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	2.5 mg/kg bw per day (rat)

Developmental toxicity	
Target/critical effect	Maternal: reduced body weight gain Embryo/fetal: no adverse effects
Lowest relevant maternal NOAEL	0.1 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	1 mg/kg bw per day; highest dose tested (rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	3 mg/kg bw (rat)
Subchronic neurotoxicity NOAEL	9 mg/kg bw per day; highest dose tested (rabbit)
Developmental neurotoxicity NOAEL	0.9 mg/kg bw per day (rat)
Immunotoxicity	No direct data, but no evidence of immunotoxicity in the general toxicity studies
Studies on toxicologically relevant metabolites	
Fipronil-desulfinyl (M&B 46513)	Acute oral LD_{50}: 18 mg/kg bw (rat) 28-day NOAEL: 0.2 mg/kg bw per day (rat) 90-day NOAEL: 0.03 mg/kg bw per day (rat) 53/104-week combined chronic toxicity/carcinogenicity NOAEL: 0.025 mg/kg bw per day; not carcinogenic (rat) Not genotoxic (bacterial reverse mutation, chromosomal abberation and mammalian cell gene mutation assays, in vitro) Developmental toxicity (rat): Maternal NOAEL: 0.2 mg/kg bw per day Developmental NOAEL: 1 mg/kg bw per day; highest dose tested Acute neurotoxicity NOAEL: 2 mg/kg bw
Microbiological data	Not data relevant to humans
Human data	No deaths attributable to fipronil exposure alone have been reported. No cases of human occupational disease associated with the production, transportation, formulation and packaging of fipronil containing materials have been reported. While fipronil exposure was inversely correlated with blood thyroid hormones in people with occupational exposure to fipronil during veterinary drug manufacture, there were no effects on blood TSH concentrations detected. While most cases of fipronil acute neurotoxicity associated with suicidal ideation are minor and have generally good clinical outcomes, serious (non-fatal) cases of convulsive/status epilepticus disorders have been reported which in some cases have responded poorly to treatment with benzodiazepines. While deaths due to suicidal ideation-associated ingestion of fipronil alone have not been reported, a death due to fipronil plus endosulfan ingestion has been reported.

[a] Does not pose a carcinogenic risk to humans via exposure from the diet

Summary

	Value	Study	Safety factor
ADI	0–0.000 2 mg/kg bw per day [a]	Two-year studies of toxicity (rat)	100
ARfD	0.03 mg/kg bw	Studies of the acute neurotoxicity of fipronil (rat) Acute pentylenetetrazol seizure threshold (rat)	100

[a] Applies to fipronil, fipronil-sulfide (M&B 45950), fipronil-sulfone (M&B 46136), fipronil-amide (RPA 200766) and fipronil-desulfinyl (M&B 46513), expressed as fipronil

Appendix 1
Human relevance of the effects of fipronil on the rat hypothalamic–pituitary–thyroid axis

A1. Carcinogenicity data

Human epidemiological data on the carcinogenicity of fipronil are not available. In a combined chronic toxicity and oncogenicity study, groups of Sprague Dawley CD rats were given dietary concentrations of 0, 0.5, 1.5, 30 or 300 ppm of fipronil (equal to 0, 0.019, 0.059, 1.27, 12.68 mg/kg bw per week in males, 0, 0.025, 0.078, 1.61, 16.75 mg/kg bw per day in females; measured over study weeks 0–90). Dosing continued for 89/91 weeks for animals in the oncogenicity study arm (50/sex per dose) or for 52 weeks for those animals in the chronic toxicity study arm (15/sex per dose) The latter arm included the satellite groups used for evaluation of the effects of a 13-week 'off-dose′ recovery period following 52 weeks of treatment. Satellite animals were used to measure thyroid hormone levels during treatment and in the recovery period. The duration of the study was intended to be 104 weeks, however males from the oncogenicity phase were killed after 89 weeks and females after 91 weeks when survival reached 25% (in high-dose males and the 30 ppm females).

Serum thyroxine (T4) levels were reduced in all fipronil-treated groups, but reductions were particularly marked following dosing at 300 ppm (T4 was undetectable during the first week of treatment; see Table A1). Serum T4 remained low in males dosed at 30 ppm and higher for two weeks post dose, but the effect was reversed by week 11 of the recovery phase. The effects of fipronil on serum triiodothyronine (T3) were less consistent although an increase in serum T_3 occurred in females dosed at ≥ 30 ppm during the recovery phase. Increased serum thyroid-stimulating hormone (TSH) occurred in males dosed at 30 ppm or more, and in females dosed at 300 ppm. Elevated serum TSH was still present at the end of the recovery phase in males dosed at 30 ppm. The effects on serum thyroid-associated hormones were accompanied by increased thyroid weights ($p < 0.05$) following dosing at 30 ppm and above and in the high-dose group did not fully resolve by the end of the recovery phase. No treatment-related neoplastic lesions were found in rats from the chronic toxicity phase. A significantly increased incidence of thyroid follicular neoplasia ($p < 0.05$)occurred in males fed fipronil at 1.5 ppm and above, and in females fed at 300 ppm (Aughton, 1993).

Table A1. Thyroid-relevant findings in the rat combined chronic toxicity and oncogenicity study.

	Dose level (ppm)									
	Males[a]					Females[a]				
	0	**0.5**	**1.5**	**30**	**300**	**0**	**0.5**	**1.5**	**30**	**300**
	Group mean serum thyroid hormone concentrations									
Triiodothyronine, T3 (ng/mL)										
Treatment week										
1	0.61	0.59	0.61	0.58	0.53*	0.78	0.75	0.77	0.75	0.80
4	0.75	0.83	0.85	0.84	0.72	0.77	0.83	0.75	0.70	0.81
12	0.92	0.91	0.95	1.10*	1.01	1.17	1.17	1.11	1.03	1.17
24	0.69	0.73	0.74	0.70	0.64	0.93	0.92	0.84	0.82	0.84
50	0.70	0.67	0.84*	0.82*	0.69	0.87	0.88	0.83	0.84	0.87
2R[b]	0.64	0.58	0.64	0.69	0.62	0.98	0.87	0.95	0.89	1.10
4R	0.70	0.59	0.68	0.74	0.77	0.88	0.91	0.88	0.95	1.13***
7R	0.80	0.77	0.78	0.81	0.89	0.91	1.03	0.87	1.10*	1.19**
11R	0.56	0.52	0.59	0.62	0.64	0.75	0.81	0.83	0.95**	1.11***

	Dose level (ppm)									
	Males[a]					Females[a]				
	0	0.5	1.5	30	300	0	0.5	1.5	30	300
Thyroxine, T4 (μg/dL)										
Treatment week										
1	2.93	3.02	2.23*	1.16***	0.00***	2.32	1.86	2.58	1.26**	0.00***
4	3.14	2.70*	2.56**	1.84***	0.39***	3.03	2.48*	2.36*	1.46***	0.79***
12	5.18	4.74	3.96**	3.50***	1.22***	3.62	2.85**	2.87*	2.05***	1.10***
24	4.58	3.81*	3.35***	2.43***	0.76***	2.85	3.09	3.49**	2.98	1.46***
50	5.95	5.51	4.83**	3.90***	2.07***	3.31	3.46	3.00	2.06***	1.38***
2R	3.97	3.71	3.45	3.05**	2.56***	1.67	1.70	1.27	1.39	1.59
4R	4.10	3.45*	2.79***	2.64***	3.21**	2.54	2.53	2.05	2.04	2.21
7R	3.80	3.31	2.94*	2.66**	3.12	2.16	2.30	1.71*	2.04	1.85
11R	3.70	3.58	3.25	3.29	3.52	2.95	3.60*	3.27	3.65*	3.09
Thyroid-stimulating hormone, TSH (ng/mL)										
Treatment week										
1	4.7	7.1	6.2	11.8***	20.3***	3.5	3.5	3.2	3.6	7.6***
4	5.2	8.0	6.5	11.2**	22.9***	3.8	3.9	3.3	3.9	7.5***
12	5.7	7.2	5.8	6.1	18.4***	3.4	3.4	2.9	3.5	8.7***
24	7.2	10.0	6.9	8.6	21.0***	3.2	3.7	3.2	3.9	6.6***
50	13.0	17.1	12.4	26.6*	57.3***	6.2	8.0	5.5	6.1	13.5***
2R	7.2	7.1	5.7	7.4	12.7**	3.5	3.6	3.5	3.5	3.8
4R	5.6	6.4	6.0	7.3	10.8**	3.7	3.5	3.6	3.3	3.7
7R	5.9	6.3	4.4	5.2	9.1*	3.0	3.2	3.1	2.7	3.1
11R	3.8	4.6	4.6	5.1	8.4**	2.7	3.1	2.9	2.7	3.1
	Group mean organ weights									
Liver: absolute weight (g)										
Treatment week										
52	29.5	30.0	29.1	34.3	40.5**	16.4	17.3	17.1	19.1	23.4**
89/91	28.3	32.4	32.1	33.9	39.4**	23.0	22.0	21.5	25.0	27.9*
13R	30.3	30.3	32.9	30.9	30.6	18.5	19.4	17.8	21.0	19.7
Liver: relative weight (% bw)										
Treatment week										
52	3.53	3.44	3.40	4.32**	5.26**	3.56	3.62	3.70	4.31**	5.49**
89/91	3.30	3.24	3.48	4.40**	5.41**	3.88	3.98	3.82	5.12**	6.14**
13R	3.50	3.70	3.44	3.69	4.55*	3.42	3.60	3.54	3.81*	4.68**
Thyroid: absolute weight (g)										
Treatment week										
52	0.039	0.035	0.042	0.047	0.056**	0.027	0.031	0.030	0.032	0.045**
89/91	0.042	0.051*	0.053*	0.063**	0.094**	0.036	0.038	0.036	0.044	0.072*
13R	0.038	0.039	0.043	0.045	0.045	0.031	0.031	0.030	0.034	0.035
Thyroid: relative weight (% bw)										
Treatment week										
52	0.0047	0.0040	0.0050	0.0058*	0.0073**	0.0059	0.0064	0.0065	0.0073*	0.0107**
89/91	0.0049	0.0052	0.0056	0.0082**	0.0129**	0.0060	0.0070	0.0065	0.0090**	0.0156*
13R	0.0045	0.0047	0.0045	0.0054	0.0067*	0.0059	0.0058	0.0061	0.0063	0.0080**

	Dose level (ppm)									
	Males[a]					Females[a]				
	0	0.5	1.5	30	300	0	0.5	1.5	30	300
Neoplastic findings										
Malignant follicular cell carcinoma (affected animals/total in group, [%])										
Week 89/91	0/49 [0]	0/48 [0]	0/50 [0]	0/50 [0]	5/50* [10]	0/50 [0]	1/50 [2.0]	0/50 [0]	1/50 [2.0]	2/50 [10]
Reversibility period	0/15 [0]	0/15 [0]	0/15 [0]	1/14 [7.1]	1/12 [8.3]	0/14 [0]	0/13 [0]	0/15 [0]	0/15 [0]	0/14 [0]
Benign follicular cell adenoma (affected animals/total in group, [%])										
Week 89/91	0/49 [0]	1/48 [2.1]	5/50* [10]	3/50 [6]	12/50*** [24]	0/50 [0]	0/50 [0]	0/50 [0]	0/50 [0]	8/50** [16]
Reversibility period	0/15 [0]	0/15 [0]	0/15 [0]	0/14 [0]	1/12 [8.3]	0/14 [0]	0/13 [0]	1/15 [6.7]	0/15 [0]	2/14 [14.3]
Total thyroid tumours, incidence (affected animals/total in group, [%])										
Week 89/91	0/49 [0]	1/48 [2.1]	5/50* [10]	3/50 [6]	17/50*** [34]	0/50 [0]	1/50 [2]	0/50 [0]	1/50 [2]	10/50*** [20]
Reversibility period	0/15 [0]	0/15 [0]	0/15 [0]	1/14 [7.1]	2/12 [16.7]	0/14 [0]	0/13 [0]	1/15 [6.7]	0/15 [0]	2/14 [14.3]
Rat thyroid tumour historical control range										
Malignant follicular cell carcinoma %	0 to 6					0 to 10				
Benign follicular cell adenoma %	2 to 10					0 to 4				

[a] Male values from weeks 0–88, female values from weeks 0–90

* $p \leq 0.05$, ** $p \leq 0.01$ *** $p \leq 0.001$

A2. Postulated mode of action for thyroid follicular cell tumours in rats

Increased hepatic catabolism of thyroid hormones and associated reduced serum T4 and increased serum TSH is well described in rats that have been exposed to certain xenobiotic receptors (for example, phenobarbital, some PCBs such as Aroclor 1254), pregnenolone-16α-carbonitrile or 3-methylcholanthrene) that induce hepatic enzymes, particularly enhanced phase II thyroid hormone glucuronidation and sulfation catalysed by uridine diphosphate glucuronosyltransferases (UDPGTs) and sulfotransferases (SULTs) (Barter & Klaassen, 1994; Haines et al., 2018; Hood & Klassen, 2000; Kato et al., 2010; Klaassen & Hood, 2001; Vansell & Klaassen, 2002; Wong et al., 2005; Yu, Liu & Jin, 2009; Zhou et al., 2002).

Chronic stimulation of the rat thyroid follicular epithelium by TSH leads to a predictable series of stereotypical tissue responses involving thyroid follicular hypertrophy, hyperplasia, and subsequently thyroid follicular adenoma and carcinoma formation via a receptor-mediated (constitutive androstane receptor (CAR); pregnane X receptor (PXR); aryl hydrocarbon receptor (AhR)), hormonally driven (TSH), non-genotoxic carcinogenic mode of action (Bartsch et al. 2018).

A3. Key events in receptor-mediated, hormonally driven (TSH), non-genotoxic thyroid follicular neoplasia in rats

The current evidence regarding xenobiotic-mediated hepatic enzyme induction (increased UDPGT and SULT activity)-associated disruption of the hypothalamic–pituitary–thyroid axis homeostasis in rats, involves the following key events (Noyes et al., 2019):

Key events according to Noyes et al, 2019

1 Activation of CAR and/or PXR nuclear receptors.

2 Chemical up-regulation in the expression and activity of hepatic UDPGTs (particularly UGT1A1 and UGT1A6) and SULTs (particularly SULT2A1). This is typically accompanied by increased liver weights, and/or hepatocellular hypertrophy.

3 Increased plasma clearance of T4 and decreased plasma T4 $t_{½}$.

4 Increased production of TSH due to effects of increased thyroid hormone turnover on hypothalamic–pituitary–thyroid feedback.

5 Sustained thyroid follicular hypertrophy due to trophic effect of TSH in response to increased demand for thyroid hormone synthesis. Typically the incidence and/or severity is higher in males than in females.

6 Increased incidence of thyroid follicular neoplasia (males are typically more susceptible), typically with progression from adenoma to carcinoma.

A4. Key events in experimental animals

Table A2. Summary of the key events associated with fipronil's effects on the rat thyroid and rat hypothalamic–pituitary–thyroid axis

Key events necessary for hepatic enzyme induction-associated rat thyroid follicular neoplasia	
Molecular initiating event/ key event	**Findings in rats with fipronil**
Activation of CAR and/or PXR nuclear receptors	Micro-array screening of the liver of SD rats treated with fipronil demonstrated the upregulation of gene expression for CYP2B1, CYP2B2 and CYP3A1, the carboxylesterases Ces2 and Ces6, and GSTA2 and the membrane transporters Abcc2, Abcc3, Abcg5, Abcg8, Slco1a1 and Slco1a4. These findings in rats are consisted with activation of both CAR and PXR, a finding that was confirmed in CAR- and PXR-deficient mice. In vitro treatment of rat (hepatocytes) and in vivo treatment of rats with fipronil results in a pattern of hepatic enzyme induction that is consistent with CAR/PXR activation (CYP2B6, CYP2C19, CYP2C9, and CYP3A4 activity).
Chemical up-regulation in the expression and activity of UDPGTs (particularly UGT1A1 and UGT1A6) and SULTs (particularly SULT2A1).	Based on in vitro hepatocellular-based assays and in vivo studies in SD rats, fipronil upregulates the production of mRNA coding for SULT2A1 and UGT1A1.
Increased liver weights, and/or hepatocellular hypertrophy	Repeated daily oral dosing of SD rats with fipronil for ≥4 weeks consistently resulted in hepatocellular hypertrophy and/or increased hepatic weights. Repeated daily dermal exposure of SD rats to fipronil for 28 days and repeated daily inhalation exposure of SD rats to fipronil for 10 days also resulted in increased liver weights.

Key events necessary for hepatic enzyme induction-associated rat thyroid follicular neoplasia	
Molecular initiating event/ key event	**Findings in rats with fipronil**
Increased plasma clearance of T4 and decreased plasma T4 $t_{½}$.	Repeated oral daily dosing of male SD rats with fipronil resulted in increased plasma clearance of T4 (by a factor of about 2.6-fold compared to control), increased biliary T4 excretion (by a factor of about 4-fold compared to control) and reduced T4 plasma terminal $t_{½}$ (by a factor of about 1.9-fold compared to control). Oral dosing of fipronil at 10 mg/kg bw per day for 10 days resulted in an increase in plasma T4 clearance that was quantitatively higher than that of the positive control treatment (80 mg/kg bw per day of phenobarbital for 10 days).
Increased production of TSH due to effects of increased thyroid hormone turnover on hypothalamic–pituitary–thyroid feedback.	Chronic oral dosing of SD rats with fipronil resulted in reduced serum T4 and increased serum TSH.
Thyroid follicular hypertrophy due to increased demand for thyroid hormone synthesis.	Repeated daily oral dosing of SD rats with fipronil for ≥4 weeks consistently resulted in thyroid follicular hypertrophy.
Increased incidence of thyroid follicular neoplasia, typically with progression from adenoma to carcinoma.	Chronic (near life-time) oral dosing of SD rats with fipronil resulted in an increased incidence of thyroid follicular neoplasia (total incidence of thyroid follicular adenomas and thyroid follicular carcinomas).

A5. The dose–response relationship, concordance, temporal relationship, strength, consistency and specificity of association of the tumour response with the key events

A summary of the NOAELs and LOAELs for the key effects in fipronil′s proposed MOA are provided in Table A3.

Sufficient information is available to establish the concordance, temporal relationship, strength, consistency and specificity of the association of fipronil exposure with key events leading to thyroid follicular neoplasia in the rat. Overall the data are concordant and consistent with a thyroid neoplasia MOA, consisting of the following dose-related key events (also shown in Fig. 3) :

> Activation of CAR and/or PXR nuclear receptors ⟶ hepatic enzyme induction and increased liver weight and/or hepatoce llular hypertrophy ⟶ increased plasma clearance of T4 and decreased plasma T4 $t_{½}$ ⟶ increased production of TSH due to effects of increased thyroid hormone turnover on hypothalamic–pituitary–thyroid feedback ⟶ sustained thyroid follicular hypertrophy due to trophic effect of TSH in response to increased demand for thyroid hormone synthesis ⟶ increased incidence of thyroid follicular neoplasia.

The effects of fipronil were dose-dependent and consistent across different durations of exposure and different studies (that is, the findings were repeatable).

Fipronil exposure was required before the appearance of the key events in the proposed mode of action (thus temporality was established). Stop/recovery studies showed that cessation of dosing with fipronil was associated with a reversal of the following key events: increased liver weights, and/or hepatocellular hypertrophy, decreased plasma T4, increased production of TSH and thyroid follicular hypertrophy. This is consistent with the proposed MOA since cessation of exposure would be expected to result in a reversal/reduced progression of this non-genotoxic MOA.

Table A3. NOAELs and LOAELs of the key events associated with fipronil's effects on the rat thyroid and rat hypothalamic-pituitary-thyroid axis

Key event	Findings in rodents treated with fipronil	
	Exposure duration: oral NOAEL	**Exposure duration: oral LOAEL**
Activation of CAR and/or PXR nuclear receptors	No data	14 days: 5 mg/kg bw per day (mouse).
Chemical upregulation in the expression and activity of UDPGTs (particularly UGT1A1 and UGT1A6) and SULTs (particularly SULT2A1)	No data	3 mg/kg bw per day for 14 days in rats (Roques et al., 2013)
Increased liver weights, and/ or hepatocellular hypertrophy	4 weeks: 7 mg /kg bw per day 13 weeks: 0.3 mg/kg bw per day 52 weeks: 0.06 mg/kg bw per day **Reversibility:** depending on dose this effect was partially to fully reversible at 13 weeks following the cessation of dosing.	4 weeks: 13 mg/kg bw per day 13 weeks: 1.9 mg/kg bw per day 52 weeks: 1.3 mg/kg bw per day
Increased plasma clearance of T4 and decreased plasma T4 $t_{½}$	1 day: 10 mg/kg bw per day	14 days: 10 mg/kg bw per day
Decreased plasma T4*	1 week: 0.02 mg/kg bw per day 4 weeks: 0.02 mg/kg bw per day 12 weeks: 0.02 mg/kg bw per day 24 weeks: 0.02 mg/kg bw per day 50 weeks: 0.02 mg/kg bw per day **Reversibility:** effects in males were partially reversible at 4 weeks following the cessation of dosing; effects in females were fully reversible at 2 weeks following the cessation of dosing.	1 week: 0.06 mg/kg bw per day 4 weeks: 0.06 mg/kg bw per day 12 weeks: 0.06 mg/kg bw per day 24 weeks: 0.06 mg/kg bw per day 50 weeks: 0.06 mg/kg bw per day
Increased production of TSH due to effects of increased thyroid hormone turnover on hypothalamic–pituitary–thyroid feedback*	1 week: 0.06 mg/kg bw per day 4 weeks: 0.06 mg/kg bw per day 12 weeks: 0.06 mg/kg bw per day 24 weeks: 0.06 mg/kg bw per day 50 weeks: 0.06 mg/kg bw per day **Reversibility:** effects in males were reversible at two weeks following the cessation of dosing except for the high-dose group where the effects were partially reversible at 11 weeks following the cessation of dosing; effects in females were fully reversible at two weeks following the cessation of dosing.	1 week: 1.3 mg/kg bw per day 4 weeks: 1.3 mg/kg bw per day 12 weeks: 1.3 mg/kg bw per day 24 weeks: 1.3 mg/kg bw per day 50 weeks: 1.3 mg/kg bw per day

Key event	Findings in rodents treated with fipronil	
	Exposure duration: oral NOAEL	**Exposure duration: oral LOAEL**
Thyroid follicular hypertrophy due to increased demand for thyroid hormone synthesis.	4 weeks: no data 13 weeks: 1.9 mg/kg bw per day 52 weeks: 1.3 mg/kg bw per day 89 weeks: < 0.02 mg/kg bw per day **Reversibility:** effects in males were reversible at two weeks following the cessation of dosing except for the high-dose group where the effects were partially reversible at 11 weeks following the cessation of dosing. Effects in females were fully reversible at two weeks following the cessation of dosing.	4 weeks: 3.4 mg/kg bw per day 13 weeks: 20 mg/kg bw per day 52 weeks: 12.7 mg/kg bw per day 89 weeks: 0.02 mg/kg bw per day
Increased incidence of thyroid follicular neoplasia, typically with progression from adenoma to carcinoma.	89 weeks: 0.02 mg/kg bw per day	89 weeks: 0.06 mg/kg bw per day
Alternative key events that can result in reduced thyroid hormone synthesis		
Interference with distributer protein binding resulting in increased thyroid hormone catabolism	No direct data, however there is little evidence of biologically-relevant disturbances of serum proteins in rats from treatment with fipronil	
Effects on the TSH receptor	No data	
Effects on thyroperoxidase (TPO)	Repeated oral daily dosing of male SD rats with fipronil had no effect on iodine organification in a perchlorate discharge test	
Effects on the sodium iodide symporter	Repeated oral daily dosing of male SD rats with fipronil was associated with increased thyroid ^{125}I accumulation in a perchlorate discharge test (associated with stimulated thyroid follicular activity). This demonstrates the presence of functional sodium iodide symporter activity There was no anatomic pathology indicative of thiocyanate-like inhibition of sodium iodide symporter activity in fipronil-exposed rats	
Deiodinase inhibition	Fipronil reduced human DIO3 gene expression in on the NHEERL_MED_hDIO3_dn screening assay (AC_{50} = 1.16 µM); there is no evidence that this occurs in rats The relationship of this finding to the health of humans exposed to fipronil via the diet is uncertain	

A6. Biological plausibility and coherence

There are considerable data from studies in laboratory rats demonstrating the key events and MOA proposed for the effects of fipronil on thyroid follicular neoplasia in the rat (Dellarco et al., 2007; Noyes, 2019). Increased secretion of TSH may result via several mechanisms, including increased hepatic clearance of T4, as is the case with fipronil. Circulating levels of T4 are monitored by the thyrotropic cells of the pituitary gland that are responsible for the synthesis of TSH. In the pituitary gland, T4 is metabolized by 5′-deiodinase type II to T3, which then binds to specific receptors in the cell nucleus. A decrease in T3 receptor occupancy results in stimulation of pituitary TSH synthesis and secretion. TSH then stimulates thyroid follicular epithelial cell hypertrophy and hyperplasia. Thus the hypothesis that fipronil induces a sustained increase in hepatic clearance of T4 resulting in a sustained TSH driven hypertrophy and hyperplasia of the rat thyroid follicular epithelium is fully consistent with the known physiology of the rat hypothalamic–pituitary–thyroid axis.

The rat thyroid follicular neoplasic response elicited by fipronil is also typical of a rodent thyroid follicular epithelial cell carcinogen in that male rats are more sensitive to the effect than females, or mice. In keeping with this, TSH levels are typically higher in male rats than in females, a feature which is seen in the case of fipronil.

A7. Other modes of action

Based on the evaluation presented in this monograph, fipronil and its metabolites are not genotoxic. Furthermore there is no evidence that exposure of rats to fipronil results in an obstruction of thyroid hormone synthesis that could result in increased TSH production (see Table A3).

A8. Uncertainties, inconsistencies and data gaps

Direct evidence of the activation of CAR and/or PXR nuclear receptors in rats is lacking since the studies discussed here were performed in mice. No direct data on the TSH receptor, the thyroid distributor proteins or the hypothalamic–pituitary feedback mechanism are available. Fipronil reduced human DIO3 gene expression in on the NHEERL_MED_hDIO3_dn screening assay (AC_{50} = 1.16 μM). The effect of fipronil on deiodinase gene expression and enzyme function has not been assessed in the rat.

A9. Assessment of proposed mode of action

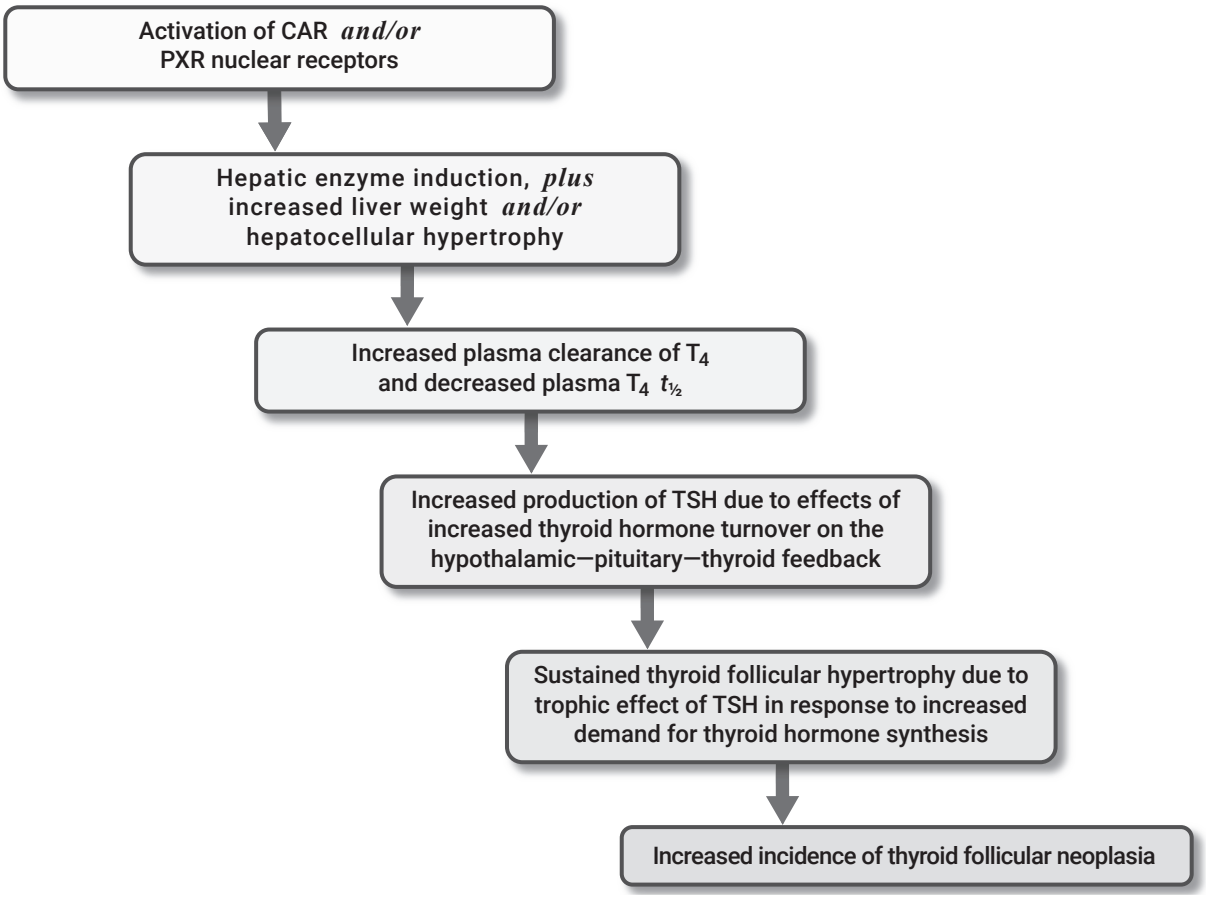
The data presented provide a moderate to high degree of confidence in the proposed mode of action for the development of thyroid follicular cell tumours in rats following chronic dietary exposure to fipronil.

A10. Human applicability of the proposed mode of action

Is the weight of evidence sufficient to establish a mode of action (MOA) in animals?

As described in detail above, there is evidence that fipronil alters hypothalamic–piuitary–thyroid homeostasis via the sequence of events shown in the diagram below:

Summary of events associated with fipronil effect on the hypothalamic–pituitary–thyroid axis



Can human relevance of the MOA be reasonably excluded on the basis of fundamental, qualitative differences in key events between experimental animals and humans?

Can human relevance of the MOA be reasonably excluded on the basis of quantitative differences in either kinetic or dynamic factors between experimental animals and humans?

Human thyroid hormone catabolism is also potentially affected by xenobiotic receptor-mediated hepatic enzyme induction (Benedetti et al., 2005; Christensen et al., 1989; Curran & DeGroot, 1991; Ohnhaus et al., 1981; Rootwelt et al., 1978; Yeo et al., 1978).

However, in humans, such reductions in plasma T4 are often not accompanied by concurrent increases in plasma TSH, that is, the human hypothalamic–pituitary–thyroid axis appears to have a lower response (increased plasma TSH following hypothalamic–pituitary feedback does not occur, or is lower) to xenobiotic receptor-mediated hepatic enzyme induction when compared to that of rodents. For example, studies in healthy human test subjects receiving therapeutic doses of the human CAR activator phenobarbital or other antiepileptic drugs show unchanged or decreased serum thyroid hormones, with generally no alterations in TSH (Benedetti et al., 2005; Curran & DeGroot, 1991). Similarly, healthy human subjects receiving therapeutic doses of the antibiotic and human PXR agonist rifampicin exhibit reduced serum T4, as well as increased T4 and reverse T3 clearance, with no change in TSH (Christensen et al., 1989; Ohnhaus et al., 1981). In this regard it is notable that high human occupational exposures to fipronil during veterinary drug manufacture were not associated with effects on blood TSH concentrations (Herin et al., 2011).

Thyroid hormone metabolic clearance responses in rats may also be less relevant to humans because thyroxine-binding globulin, the major serum thyroid hormone distributor protein chaperone in humans, is less prominent in the adult rat (although it is important in thyroid homeostasis in newborn

and neonatal rat pups; Savu et al., 1991; Young et al., 1988). In adult rodents the major serum thyroid hormone distributor protein chaperone is transthyretin (TTR or thyroxine-binding prealbumin; Dickson, Howlet & Schreiber, 1985; Harms et al., 1991; Power et al., 2000). Transthyretin has a lower binding affinity for T4 compared to thyroxine-binding globulin, resulting in a shorter plasma T4 $t_{½}$ in rats (about 12–24 h) compared to humans (about five days according to Lewandowski, Seeley & Beck, 2004). The higher plasma clearance of T4 in rats has been interpreted to mean that rats have a lower "thyroid efficiency" compared to humans, that is there is a relatively higher basal demand for T4 production by the thyroid follicular epithelium in order to maintain hypothalamic–pituitary–thyroid axis homeostasis in rats. Accordingly, rats are regarded as more sensitive than humans to the effects of increased T4 catabolism on the hypothalamic–pituitary–thyroid axis.

Based on the above findings the current scientific consensus is that exposure to xenobiotics that result in thyroid follicular neoplasia in rats secondary to increased clearance of thyroid hormones is either not relevant to humans and/or that the rat thyroid is more sensitive to a carcinogenic stimulus than the human thyroid (Bartsch et al., 2018).

Conclusion: statement of confidence, analysis and implications.

There is sufficient experimental evidence to establish a thyroid disruption MOA for fipronil-induced thyroid follicular cell neoplasia in rats. Although high levels of fipronil exposure may potentially result in reduced blood T4 in humans, there is sufficient quantitative evidence to conclude that thyroid tumours induced by a process involving increased hepatic clearance of thyroid hormone and altered homeostasis of the pituitary–thyroid axis in rodents is not likely to lead to an increase in susceptibility to tumour development in humans.

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Flutianil

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Explanation

Flutianil is the ISO-approved common name for [(*Z*)-2-[2-fluoro-5-(trifluoromethyl)phenylthio]-2-[3-(2-methoxyphenyl)-1,3-thiazolidin-2-ylidene]acetonitrile (IUPAC), with the Chemical Abstracts Service number 958647-10-4.

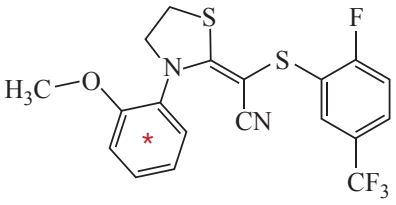
Flutianil (OK-5203) is a novel fungicide that belongs to the thiazolidine chemical class exhibiting both fungicidal and fungistatic contact action. Flutianil is a systemic fungicide for use in controlling *Rhizoctonia solani* (black scurf) and some other basidiomycetes in rice, turf, potato, vegetables and peanuts. Its proposed mode of action (MOA) is the inhibition of haustorium formation in the fungus, thus inhibiting sporulation and spore isolation and preventing disease expansion. This MOA is different from existing fungicides. Flutianil is classified by the Fungicide Resistance Action Committee (FRAC) as having an unknown MOA, placing it in group U13. Flutianil 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.

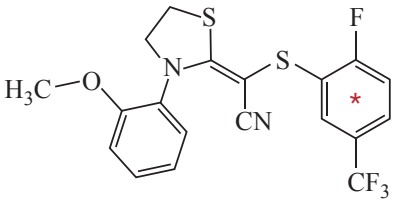
Evaluation for acceptable daily intake

1. Biochemical aspects

Figure 1. Structure of flutianil including position of radiolabels



[*Methoxyphenyl-ring-U*-^{14}C]flutianil
(OK-5203)



[*Fluorotolyl-ring-U*-^{14}C]flutianil
(OK-5203)

* indicates position of ^{14}C label

The absorption, distribution, metabolism and excretion (ADME) of flutianil were studied in rats which were administered labelled flutianil as a single oral low dose, a single oral high dose, or as a radiolabelled oral dose that followed 14 unlabelled daily oral low doses.

1.1 Absorption, distribution and excretion

(a) ***Oral route***

The preliminary, (not fully GLP guideline-compliant) study was designed to investigate the absorption in the rat of radioactivity following oral administration of [^{14}C]flutianil at different dose levels and to characterize the routes and rates of excretion of that radioactivity.

Two male and two female rats each received a single oral dose of flutianil (OK-5203), labelled with ^{14}C within either the fluorotolyl ring, [*CF3Ph-U*-^{14}C]OK-5203, or the methoxyphenyl ring, [*MeOPh-U*-^{14}C]OK-5203, at either 10 or 250 mg/kg body weight. Urine and faeces were collected over the168 hours following dosing, while expired carbon dioxide was measured over just the first 24 hours.

In all groups, radioactivity was mainly eliminated in faeces within 24 hours of dosing. Elimination of radioactivity as exhaled carbon dioxide accounted for ≤ 0.02% of the dose. Carbon dioxide collection was terminated after 24 hours, as exhalation was not a significant route of excretion. Faecal

elimination of a low dose of 10 mg/kg body weight (bw) [*CF3Ph-U*-^{14}C]flutianil was similar in fasted and fed rats (89–95%) and there was no sex difference. Urinary elimination in rats receiving the high dose of 250 mg/kg bw [*CF3Ph-U*-^{14}C]flutianil at 4.1% and 5.9% in males and females respectively, was lower than in rats given 10 mg/kg bw of the same labelled form (6.9% and 11.1% in males and females respectively). Male rats given 10 mg/kg bw of [*MeOPh-U*-^{14}C]flutianil appeared to excrete a greater proportion of the dose in urine than did females, 18.6% compared with 6.0%. However, this was influenced by a single male animal in the group (29.8% urinary excretion). Since no other male in this study excreted urinary radioactivity above 10%, it seemed likely that there was no significant sex difference in excretion. Residual radioactivity in the carcass at 168 h was < 0.25% of the administered dose in all groups.

Based upon the results of this preliminary study, oral absorption was estimated to be 7% (males) to 11% (females) at 10 mg/kg bw [*CF3Ph-U*-^{14}C]flutianil, and 6% (females) to 18% (males) at 10 mg/kg bw [MeOPh-U-^{14}C]flutianil. A dose of 250 mg/kg bw of [*CF3Ph-U*-^{14}C]flutianil resulted in a mean value of 5% oral absorption; 4% for males and 6% for females (Needham, 2006).

Study 1

In a metabolism study with radiolabelled material, groups of four Wistar [Cr1:WI(Han)] rats per sex were given a single dose by gavage of either [*MeOPh-U*-^{14}C]flutianil or [*CF3PhU*-^{14}C]flutianil at doses of 10 mg/kg bw (low dose) or 1000 mg/kg bw (high dose). Blood samples were taken at intervals up to 96 hours after dosing and urine and faeces were collected up to 120 hours post dose, at which time animals were sacrificed and tissues removed. Separate experiments were conducted in bile duct-cannulated rats in which urine, faeces and bile were collected for 48 hours from rats given a single oral dose of 10 mg/kg bw. Six rats per sex and group were cannulated. Five males and five females were dosed with [*CF3Ph*-^{14}C]flutianil while five males and four females were dosed with [*MeOPh-U*-^{14}C]flutianil. Radioactive concentration in all samples was determined by liquid scintillation counting. Pooled urine, faeces and bile samples were analysed by liquid chromatography (LC) to determine the number of metabolites present, some of which were identified by liquid chromatography–mass spectrometry (LC-MS). Results of metabolite identification are included in section 1.2 Biotransformation.

During the course of the study, there were no notable differences in body weights and no overt signs of pharmacological or toxicological effects attributable to the administration of flutianil.

At the low dose, maximum plasma concentrations were achieved within 13 hours following administration of [*CF3PhU*-^{14}C]flutianil and within three hours following administration of [*MeOPh-U*-^{14}C]flutianil (Table 1). There was no significant sex difference following administration of either radiolabel. Plasma levels were below the limit of quantification 96 hours after dosing. Elimination from plasma was relatively slow, though some of the elimination half-life data were calculated using a limited number of time points and were considered unreliable. At the high dose it was reported that concentrations were generally below the limit of detection and pharmacokinetic parameters could not be calculated.

Table 1. Mean plasma pharmacokinetic parameters

	Low oral dose (10 mg/kg bw)			
	[*CF3Ph-U*-^{14}C]flutianil, 'A'		**[*MeOPh-U*-^{14}C]flutianil, 'B'**	
	Male	**Female**	**Male**	**Female**
C_{max} (ng equiv./mL)	171.1 ± 60.36	205.3 ± 69.72	300.0 ± 43.31	348.6 ± 88.07
T_{max} (h)	12.5 ± 9.0	6.8 ± 3.8	3.0 ± 1.2	1.8 ± 0.5
$t_{½\ elim}$ (h)	22.26 ± 8.319	16.08 ± 2.595	11.24 ± 3.633	26.05 ± 6.693
$AUC_{0–96\ h}$ (ng equiv. × h/g)	4827 ± 3500	4987 ± 2724	3621 ± 1071	6032 ± 1548
$AUC_{0–\infty}$ (ng equiv. × h/g)	6679 ± 3465	5818 ± 2621	4123 ± 1153	7364 ± 1813

C_{max}: Maximum concentration; T_{max}: Time to reach C_{max}; $t_{½\ elim}$: Half-life of elimination;
AUC: Area under the concentration–time curve

In contrast to plasma, maximum whole blood concentrations were achieved at the low dose within eight hours of administering [*CF3Ph-U*-^{14}C]flutianil and within five hours following administration of [*MeOPh-U*-^{14}C]flutianil. At the high dose, maximum blood concentrations were achieved within four hours following the administration of [*CF3Ph-U*-^{14}C]flutianil but could not be determined following administration of [*MeOPh-U*-^{14}C]flutianil. There were no significant sex differences in plasma or blood kinetics of radioactivity following administration of either radiolabelled compound (Table 2).

Table 2. Mean blood pharmacokinetic parameters (low and high dose)

	Low oral dose (10 mg/kg)			
	[*CF3Ph-U*-^{14}C]flutianil, (group A)		**[*MeOPh-U*-^{14}C]flutianil, (group B)**	
	Male	**Female**	**Male**	**Female**
C_{max} (ng equiv./mL)	463.3 ± 145.9	705.1 ± 176.6	277.6 ± 37.18	430.5 ± 84.68
T_{max} (h)	6.1 ± 5.1	8.0 ± 3.3	3.8 ± 1.7	5.0 ± 2.4
$t_{½\ elim}$ (h)	68.59 ± 68.41	67.91 ± 20.18	35.01 ± 2.921	34.12 ± 6.175
$AUC_{0–96\,h}$ (ng equiv. × h/g)	14 078 ± 6730	14 353 ± 3931	8156 ± 2828	14 543 ± 3625
$AUC_{0–\infty}$ (ng equiv. × h/g)	19 355 ± 7447	17 689 ± 3973	9728 ± 3185	17 170 ± 5267
	High oral dose (1000 mg/kg)			
	[*CF3Ph-U*-^{14}C]flutianil, (group C)		**[*MeOPh-U*-^{14}C]flutianil, (group D)**	
	Male	**Female**	**Male**	**Female**
C_{max} (ng equiv./mL)	3115 ± 735.2	3895 ± 795.4	Insufficient data to calculate pharmacokinetic parameters	
T_{max} (h)	3.5 ± 1.7	3.3 ± 2.2		
$t_{½\ elim}$ (h)	13.92 ± 5.236	39.69 ± 32.00		
$AUC_{0–96\,h}$ (ng equiv. × h/g)	29 850 ± 13 466	143 092 ± 100 169		
$AUC_{0–\infty}$ (ng equiv. × h/g)	59 461 ± 16 337	296 139 ± 148 433		

C_{max}: Maximum concentration; T_{max}: Time to reach C_{max}; $t_{½\ elim}$: Half-life of elimination;
AUC: Area under the concentration–time curve

There appears to be a label-specific variation in toxicokinetics with [*MeOPh-U*-^{14}C]flutianil, low-dose animals attaining peak levels (C_{max}) of radioactivity in plasma and blood more rapidly than in low-dose [*CF3Ph-U*-^{14}C]flutianil animals.

Comparison of the plasma and blood concentration data following the administration of [*CF3Ph-U*-^{14}C]flutianil at the low-dose level showed that radioactivity was preferentially located in blood cells. In all cases, elimination half-life ($t_{½\ elim}$) was shorter for plasma than whole blood indicating radioactivity was associated with blood cells. No significant difference between sexes in radiolabel concentration were observed in plasma and blood cell for rats given the low dose of either labelled form. It was not possible to make this comparison for the high-dose group because radioactive concentrations were below or close to the limit of quantitation in the plasma and blood cells of these animals.

Although there was a 100-fold difference in dosing levels, the maximum concentration (C_{max}) and area under the concentration–time curve (AUC) values for blood cells in rats in the high-dose group were usually less than 10-fold higher than for rats in the low-dose group. These results show that absorption was not linear over the dose range 10–1000 mg/kg bw and absorption at the high dose is less than at the low dose, but a quantitative difference is difficult to calculate because data for the high-dose group are often at, or close to, the limit of quantitation (LOQ).

Levels of recovery of radiolabel were acceptable, with the main route of excretion via faeces (70–95%) across all dose groups, irrespective of sex; a greater proportion of faecal excretion was observed in rats given the high dose. The greatest proportion of urinary excretion was observed at the low dose [*MeOPh-U*-^{14}C]flutianil male and female rats (16% and 19%, respectively), whereas the smallest proportion of urinary radioactivity was detected in the two high-dose groups, irrespective of radiolabel (ca 1% of the applied dose; see Table 3).

Table 3. Mean recovery of radioactivity (% applied dose)

Group		E		F		G		H	
Dose [mg/kg bw]		10				1000			
Radiolabelled flutianil		[*CF3Ph-U*-^{14}C]		[*MeOPh-U*-^{14}C]		[*CF3Ph-U*-^{14}C]		[*MeOPh-U*-^{14}C]	
Sex		Male	Female	Male	Female	Male	Female	Male	Female
Urine	0–24 h	1.83	4.70	11.95	13.33	0.23	0.30	0.42	0.44
	24–48 h	1.57	2.34	3.39	4.24	0.26	0.26	0.27	0.22
	48–120 h	0.83	0.91	0.88	1.63	0.36	0.42	0.28	0.16
	Subtotal	**4.2**	**8.0**	**16.2**	**19.2**	**0.9**	**1.0**	**1.0**	**0.8**
Faeces	0–24 h	81.86	76.38	43.89	34.46	73.16	70.36	74.04	77.80
	24–48 h	2.94	3.00	27.00	33.05	12.43	15.14	11.01	9.79
	48–120 h	1.50	1.84	3.24	3.38	4.60	5.63	6.08	6.81
	Subtotal	**86.2**	**81.2**	**74.1**	**70.9**	**90.2**	**91.1**	**91.1**	**94.4**
Expired air	Not collected on the basis of preliminary study								
Cage:									
wash		3.39	1.84	3.40	3.26	0.37	0.78	0.98	0.29
debris		< LOQ	0.07	0.20	0.03	< LOQ	< LOQ	< LOQ	< LOQ
Total excretion[a]		**93.9**	**91.1**	**93.9**	**93.4**	**91.4**	**92.9**	**93.1**	**95.5**
Tissues		0.26	1.50	1.32	0.72	1.07	0.47	0.42	0.12
Carcass		0.05	< LOQ	< LOQ	0.09	< LOQ	< LOQ	< LOQ	< LOQ
Apparent absorption[b]		7.9	11.4	21.1	23.3	2.3	2.2	2.4	1.2
Total recovery		**94.2**	**92.6**	**95.3**	**94.2**	**92.5**	**93.4**	**93.5**	**95.6**

[a] Excretion = urine + faeces + cage wash + cage debris

[b] Apparent absorption = urine + cage wash + cage debris + residual carcass + tissues

<LOQ: Below the limit of quantitation = <twice background decay rate of blank samples at the time of scintillation count

Five days after dosing (at termination), a maximum of 1.5% in total of administered dose (AD) was present in the tissues, including the stomach and intestines, irrespective of dose level, sex or label. At this time, the remaining residual radioactivity was primarily associated with the gastrointestinal tract; the stomach, intestines and their contents. In all groups, levels of both forms of labelled flutianil were below the LOQ in the majority of the tissues, with the exceptions of the liver, kidney, lung, blood, plasma and fat. No information is available from this study on radioactivity in tissues/organs at a timepoint corresponding to C_{max}.

Irrespective of sex, dose level or position of radiolabel, the concentration of radioactivity in tissues 120 hours after dosing was either very low or below the LOQ.

There were no remarkable differences in the biliary excretion of the two radiolabels (Table 4). Biliary excretion accounted for approximately 11% and 8% of the 10 mg/kg bw dose of [*CF3Ph-U*-^{14}C] flutianil in males and females respectively; urinary excretion only accounted for a further 4% and 3%, respectively. In male and female bile duct-cannulated rats given [*MeOPh-U*-^{14}C] flutianil, 6% and 8% respectively of the dose was eliminated in the bile, and 5% and 7% respectively in their urine. The remainder of the dose was present in faeces, indicating that the majority of radiolabel remained unabsorbed. These values generally agree with those for non-cannulated rats. However, it is noted that the proportion of urinary radioactivity is lower in cannulated rats (Groups I and J). Although this could be an indication of enterohepatic circulation of parent compound and/or metabolites, the evidence for this is inconclusive due to the limited extent of absorption and the wide variation in toxicokinetics profile data points, where no significant secondary peaks could be confirmed in the plasma profiles.

Table 4. Mean recovery of radioactivity in biliary cannulated animals (% applied dose)

Group		I		J	
Radiolabel		[*CF3Ph-U*-^{14}C]		[*MeOPh-U*-^{14}C]	
Sex		male	female	male	female
Dose [mg/kg bw]		10	10	10	10
Bile	0–24 h	8.02	6.52	6.10	7.45
	24–48 h	2.79	1.24	0.40	0.46
	Subtotal	**10.8**	**7.7**	**6.5**	**7.9**
Urine	0–24 h	2.83	1.91	4.40	5.58
	24–48 h	1.30	0.92	0.37	1.24
	Subtotal	**4.1**	**2.8**	**4.8**	**6.8**
Faeces	0–24 h	57.92	72.33	84.68	72.72
	24–48 h	16.78	10.51	1.92	6.67
	Subtotal	**74.7**	**82.8**	**86.6**	**79.4**
Cage wash plus debris		0.877	2.28	1.16	2.70
Tissues		NC	NC	NC	NC
Carcass		2.53	0.58	0.32	0.85
Apparent absorption[a]		18.3	13.4	12.7	18.3
Total Recovery		**94.9**	**96.3**	**99.3**	**97.6**

NC: Not collected

[a] Apparent absorption = urine + cage wash + cage debris + residual carcass + tissues

Based on the sum of the radioactivity in urine, bile, cage wash and debris, and the residual carcass, these biliary excretion data show that up to 18% of the oral dose of [^{14}C]flutianil was absorbed at 10 mg/kg bw. This is consistent with the maximum of 19% excretion of radioactivity, observed in the urine of non-cannulated rats (group F, females) dosed at 10 mg/kg bw. Faecal elimination was the major route of excretion and accounted for > 80% of the administered dose (Hardwick, 2009a).

The tissue distribution and clearance study groups, consisting of nine Wistar [Crl:WI(Han)] rats per sex, received a single dose by gavage of either [*MeOPh-U*-^{14}C]flutianil or [*CF3Ph-U*-^{14}C]flutianil at dose levels of 10 or 1000 mg/kg bw. Following administration of [*CF3Ph-U*-^{14}C]flutianil, three animals of each sex were exsanguinated at 8, 24 and 48 hours post dose. Following administration of [*MeOPh-U*-^{14}C]flutianil, three animals of each sex were exsanguinated at 2, 24, and 48 hours post dose. At necropsy selected tissues were excised or sampled and radioactive content was determined.

There were no notable differences in body weights at necropsy, and no reports of overt toxicity or pharmacological effects were recorded. Extraction efficiencies of all samples were less than 50%, and were deemed too poor to allow metabolic profiling.

Flutianil was widely distributed throughout the tissues following oral administration and was rapidly eliminated. Generally, radioactivity seemed to be associated with the organs of metabolism and excretion and with fatty tissues. Liver was the tissue containing the highest amount of radioactivity at all time-points, followed by fat. Tissue residues were not proportional to dose, and, with the exception of residues in the gastrointestinal tract, an increase in dose levels from 10 to 1000 mg/kg bw resulted in an increase in tissue residues of much less than 100-fold. Thus, absorption was saturated at the high dose. At comparable times after dosing (24 and 48 hours) tissue concentrations of radioactivity were generally greater in rats that received [*CF3Ph-U*-^{14}C]flutianil than in rats given [*MeOPh-U*-^{14}C]flutianil. At the low dose, clearance of radioactivity from the tissues was relatively rapid, although slightly greater following administration of [*MeOPh-U*-^{14}C]flutianil. At the high dose many tissues and blood exhibited a relatively slow clearance, with significant levels remaining at 48 hours (Tables 5 and 6).

Table 5. Mean concentrations of radioactivity in rat tissues following oral administration of [CF_3Ph-U-^{14}C]flutianil at 10 and 1000 mg/kg bw

Tissue	*CF3Ph-U-*^{14}C, (ng equiv./g)											
	10 mg/kg bw						1000 mg/kg bw					
	8 hours		24 hours		48 hours		8 hours		24 hours		48 hours	
	M	F	M	F	M	F	M	F	M	F	M	F
Adrenals	1527	1344	220.8	563.0	96.70	101.3	8158	9842	6820	2583	2195	1384
Blood	885.2	1046	173.6	386.8	93.42	105.7	4209	6317	4642	1437	1941	1195
Bone	76.77	60.16	31.50	37.11	12.63	8.494	382.6	728.7	640.6	434.1	321.9	457.1
Brain	308.9	356.2	105.8	183.0	42.13	36.81	1635	2360	2056	1422	974.7	905.9
Eyes	185.0	201.9	70.85	115.1	29.30	24.88	7190	1082	1349	532.6	760.6	361.7
Fat	2432	3318	566.9	1147	172.0	206.8	13 151	17 854	13 957	4713	4351	2930
Heart	383.0	437.1	128.8	252.8	56.36	57.19	3155	4394	3158	2515	2059	2514
Kidney	900.7	792.0	314.6	505.4	104.1	110.4	4935	4987	9865	2275	2448	1320
Liver	1647	1373	485.0	651.2	310.0	253.3	8596	12 024	10 485	3226	5373	2615
Lung	508.8	516.8	162.9	278.2	84.20	99.44	2962	4252	3040	2767	1514	1953
Muscle (Quads)	289.9	323.7	88.23	151.9	34.18	29.21	1511	1970	2058	727.0	958.2	377.7
Ovaries	NA	1609	NA	548.1	NA	133.2	NA	10 984	NA	2886	NA	1618
Pancreas	952.4	790.6	243.3	612.4	90.07	112.9	5231	13 602	4898	3383	2969	2554
Pituitary	1462	900.0	120.4	350.3	145.4	314.0	12 105	9583	12620	8632	11729	6167
Plasma	217.2	222.7	104.8	145.2	43.26	42.05	1093	1688	1872	765.7	751.8	435.6
Skin	427.0	616.4	185.2	424.5	71.55	79.23	4275	5990	3766	3129	1456	3003
Spleen	382.6	481.4	120.2	216.9	61.10	66.19	3086	4841	2975	2673	1740	4108
Testes	265.3	-	95.84	-	40.64	-	1360	-	2943	-	972.9	-
Thymus	286.8	272.4	91.49	188.3	38.55	38.63	2691	3048	2057	6325	1492	2215
Thyroid	849.5	1250.5	105.9	260.9	75.15	88.70	5151	3627	4058	2179	5096	2933
Uterus	-	1615	-	671.1	-	103.9	-	7924	-	4739	-	2568
Carcass	3419	1296	175.2	326.0	69.91	99.53	18 6361	36 541	10 755	7676	3261	1397

Table 6. Mean concentrations of radioactivity in rats tissues following oral administration of [MeOPh-U-^{14}C]flutianil at 10 and 1000 mg/kg bw

Tissue	*MeOPh-U-*^{14}C, (ng equiv./g)											
	10 mg/kg bw						1000 mg/kg bw					
	2 hours		24 hours		48 hours		2 hours		24 hours		48 hours	
	M	F	M	F	M	F	M	F	M	F	M	F
Adrenals	280.3	456.4	26.88	53.06	26.32	31.55	3194	6062	470.6	693.9	519.5	555.0
Blood	331.1	388.1	111.2	240.1	60.77	123.3	2451	2855	765.8	1441	973.0	1903
Bone	56.31	52.77	5.726	10.12	8.110	10.57	420.6	475.4	206.9	225.5	198.4	246.6
Brain	46.69	78.45	12.31	13.69	9.632	9.690	715.0	916.7	588.6	730.0	481.3	658.2
Eyes	42.31	46.82	25.06	8.907	11.63	6.029	643.5	692.6	300.9	346.4	298.3	304.2
Fat	1556	436.4	17.38	42.23	19.03	23.40	2743	2580	367.9	497.3	391.2	461.7
Heart	176.3	271.2	34.68	53.60	26.43	34.38	2160	2895	1125	1377	1092	1944
Kidney	963.2	1339	93.83	141.7	76.84	84.57	12782	8717	1689	1257	1615	1597

Tissue	*MeOPh-U*-^{14}C, (ng equiv./g)											
	10 mg/kg bw						1000 mg/kg bw					
	2 hours		24 hours		48 hours		2 hours		24 hours		48 hours	
	M	F	M	F	M	F	M	F	M	F	M	F
Liver	1796	2049	395.2	399.4	450.1	291.8	14639	15059	3254	3144	4061	4118
Lung	304.5	448.3	90.06	245.3	70.76	170.8	2929	5888	1332	2687	1338	2562
Muscle (quads)	666.6	93.97	9.043	11.62	15.62	15.19	709.0	1166	392.7	406.9	393.3	380.0
Ovaries	-	355.4	-	45.89	-	26.60	-	4266	-	629.7	-	618.1
Pancreas	402.7	590.1	28.94	36.68	21.98	27.78	3656	3409	1433	1348	1842	1178
Pituitary	153.2	192.9	434.2	178.6	814.4	144.8	5829	4933	8809	10332	5723	6101
Plasma	371.1	400.3	41.82	81.45	21.74	32.98	3070	3307	397.3	518.7	388.6	562.8
Skin	148.1	189.4	26.60	40.12	30.90	24.94	2101	2625	1431	1895	917.8	1486
Spleen	154.2	271.0	43.54	75.35	38.92	52.75	2091	2561	2690	2337	2591	2314
Testes	69.25	-	9.504	-	8.400	-	903.2	-	477.7	-	457.4	-
Thymus	278.1	169.7	19.33	39.20	19.04	29.24	1405	2263	1499	1715	1535	2903
Thyroid	391.7	316.9	102.2	385.5	86.69	165.5	2431	4669	4439	3679	2669	4547
Uterus	NA	434.4	NA	50.70	NA	32.96	NA	3721	NA	2038	NA	1731
Carcass	219.4	168.0	51.57	69.08	41.64	94.90	2888	2708	1357	2668	1251	1975

Results show there was minimal sex difference in the distribution or clearance of radioactivity (Hardwick, 2009b, 2012).

Study 2

In a study on bile-duct cannulated rats, a single dose of either [*CF3Ph-U*-^{14}C]flutianil or [*MeOPh-U*-^{14}C]flutianil was administered by gavage to Han Wistar rats. In phase 1 two groups of four intact rats each received a single oral administration of either [*CF3Ph-U*-^{14}C]flutianil or [*MeOPh-U*-^{14}C]flutianil at a target dose level of 1 mg/kg bw. In phase 2 two groups of four bile duct-cannulated rats each received a single oral administration of either [*CF3Ph-U*-^{14}C]flutianil or [*MeOPh-U*-^{14}C]flutianil at a target dose level of 1 mg/kg bw. Metabolite identification was performed and is included under section 1.2 Biotransformation.

Following oral administration of both [*CF3Ph-U*-^{14}C]flutianil and [*MeOPh-U*-^{14}C]flutianil to intact rats, the majority of the administered radioactivity was recovered in the faeces with means for the two labels of approximately 94% and 98%, respectively. Urinary excretion accounted for a means of approximately 5% and 6%, respectively. In bile duct cannulated rats the majority of the administered radioactivity was recovered in the faeces with a mean of around 90% for [*CF3Ph-U*-^{14}C]flutianil) and 92% for [*MeOPh-U*-^{14}C]flutianil, while biliary elimination accounted for means of approximately 10% and 5% respectively.

Excretion was rapid with the majority of the administered radioactivity (91–98%) recovered in the first 24 h post dose. Absorption of the administered radioactivity was low, with means of approximately 12% for [*CF3Ph-U*-^{14}C]flutianil and 8% for [*MeOPh-U*-^{14}C]flutianil (Wicksted, 2012).

Study 3

In a repeated-dose study, flutianil (99.38%, Lot no. 04AF1) was administered by gavage to groups of Wistar [Crl:WI(Han)] rats (four per sex and group). Rats were given 14 daily repeat oral administrations of unlabelled flutianil at 10 mg/kg bw per day, followed by a single oral administration (gavage) on day 15 of either [*CF3Ph-U*-^{14}C]flutianil or [*MeOPh-U*-^{14}C]flutianil, also at 10 mg/kg bw) . Urine and faeces were collected up to 120 hours after the last dose. The rats were then killed and selected tissues were sampled or excised. The radioactive concentration in all samples was determined by liquid scintillation counting (LSC). Metabolites were quantified by LC and identified by chromatographic comparison with reference standards or with metabolites identified after administration of a single dose. Metabolite identification was performed and is included under section 1.2 Biotransformation.

No notable differences in body weights were recorded at necropsy, nor were there reports of overt toxicity or pharmacological effects. Recoveries of radioactivity were essentially quantitative following administration of either radiolabelled form of the test substance. Radioactivity was eliminated primarily in the faeces (85–90% of AD). Renal excretion was a minor route of elimination, accounting for < 11% of the dose (see Table 7).

Table 7. Mean recovery of radioactivity from rats 120 hours after 14 repeat oral administrations of flutianil and one oral administration of [^{14}C]flutianil at a nominal dose level of 10 mg/kg, (% AD)

Sample	[*CF3Ph-U*-^{14}C] flutianil		[*MeOPh-U*-^{14}C] flutianil	
	Males	**Females**	**Males**	**Females**
Urine	7.358 ± 1.986	10.47 ± 0.487	7.410 ± 2.099	10.54 ± 3.981
Faeces	90.29 ± 1.609	85.87 ± 4.206	90.10 ± 4.118	89.03 ± 4.891
Cage wash	0.701 ± 0.127	1.637 ± 0.619	0.520 ± 0.112	0.738 ± 0.328
Cage debris	<LOQ	0.114 ± 0.073	<LOQ	0.005 ± 0.009
Carcass	0.173 ± 0.063	0.229 ± 0.068	0.031 ± 0.062	0.095 ± 0.112
Tissues	0.080 ± 0.025	0.078 ± 0.014	0.079 ± 0.011	0.077 ± 0.036
Total	**98.60 ± 2.222**	**98.39 ± 4.333**	**98.14 ± 2.982**	**100.5 ± 1.475**

<LOQ: Below the level of quantitation Hardwick, 2009c

Excised tissues and residual carcass contained minimal amounts of radioactivity. There were no marked differences between the radiolabelled forms with regard to the rates or routes of elimination of radioactivity, and no apparent sex differences. At termination, excluding the GI tract, the only tissues with a higher radioactivity concentration than blood were the liver, lungs, male kidneys and female thyroid. Radioactive residues in the majority of the tissues were below the LOQ.

Comparing the results of this study with data from a study involving single oral administration of either labelled form of flutianil showed that urinary excretion was comparable for single oral dose as regards [*CF3Ph-U*-^{14}C]flutianil, but was slightly lower for [*MeOPh-U*-^{14}C]flutianil. Repeated administration had no major effect on the absorption, distribution, or excretion of flutianil (Hardwick, 2009c).

(b) ***Dermal route***

In an in vitro dermal absorption study with human skin (split-thickness) samples, an emulsifiable concentrate formulation containing flutianil at 50 g/L and a 0.021 g/L dilution were examined. A total of eight samples of human skin membranes obtained from four different donors were dosed topically. No cells were excluded as outliers (Toner, 2015).

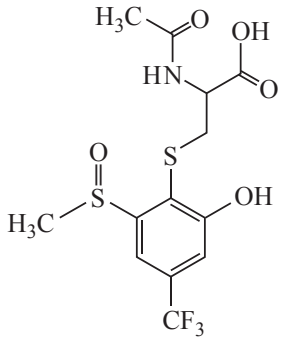
Based on the European Food Safety Authority (EFSA) guidance (EFSA, 2017) the dermal absorption was calculated to be 8.5% for the concentrate and 23% for the 0.021 g/L dilution.

1.2 Biotransformation

In the study by Hardwick (2009a see above under 1.1) samples of urine and faeces were pooled by sex and dose group by taking a percentage of the sample weight. Urine and bile were analysed directly.Faeces was extracted sequentially with ethyl acetate, acetonitrile and 1% methanoic acid in acetonitrile. Treatment with β-glucuronidase and acid hydrolysis of some pooled samples were procedures used to determine whether conjugates were present. Samples were analysed by LC with radiodetection and by LC-MS.

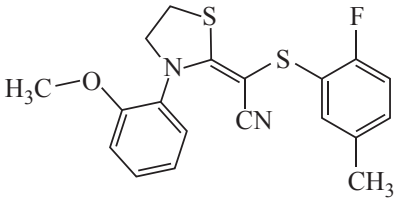
In urine, no parent flutianil was detected in any of the dosed groups, irrespective of cannulation status or label position. The major metabolite (Met 6; see Fig. 2) in the urine of rats dosed with [*CF3Ph-U*-^{14}C]flutianil was identified as a mercapturate conjugate of a hydroxylated methylsulphoxy trifluoromethyl ring structure; it accounted for up to 5.5% of the AD. Five other minor metabolites (Met 1–Met 5) were identified which appeared to have resulted from cleavage of the parent compound between the methoxyphenyl and fluorotolyl ring structures (Table 8).

Figure 2. Structure of Met 6

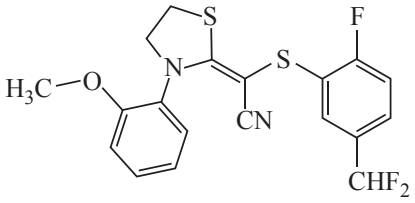


The major component in faeces was the parent compound (94–98% of faecal elimination) and two minor tentative metabolites, OC 53429 and OC 53982 (Table 8), the results of sequential defluorination of the parent compound without cleavage of the two ring structures (Fig. 3).

Figure 3. Structure of metabolites OC 53429 and OC 53429



OC 53429



OC 53982

The residues in bile represented 6.5–10.8% of the total applied radiolabel. Due to ion suppression no metabolites could be identified in the bile of animals dosed with either radiolabelled form, or in the urine after rats had been dosed with [*MeOPh-U*-14C]flutianil. None of the metabolites in these samples were considered significant, accounting for less than 2.5% of the dose (Hardwick, 2009a).

Table 8. Summary of metabolites following administration of [CF_3Ph-U-^{14}C]flutianil, (% of AD)

Metabolite	Low dose		High dose
	Males	Females	Males
		Urine	
Met 1	0.08	0.08	0.08
Mets 2, 3 and 4	0.33	0.08	0.33
Met 5	0.10	0.11	0.10
Met 6	2.58	5.45	2.58
		Faeces	
OC 53982	1.40	0.99	1.40
OC 53429	2.65	3.00	2.65
Flutianil	76.50	70.15	76.50
Total identified	**83.64**	**79.86**	**83.64**

In the study by Wicksted (2012; for study design see section 1.1), selected urine and bile samples, containing 95% of the recovered radioactivity, were pooled and analysed by LC-MS.

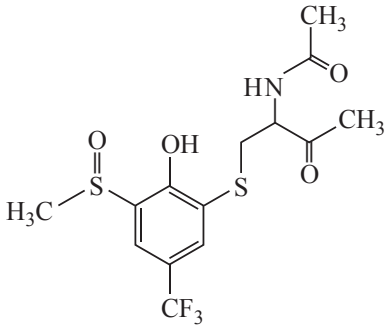
Unchanged flutianil was detected in urine and bile samples, accounting for 0.04–0.45% of AD.

The radio profiles of animals dosed with [*CF3Ph-U*-^{14}C]flutianil and [*MeOPh-U*-^{14}C]flutianil were qualitatively different.

[*CF3Ph-U*-^{14}C] flutianil metabolites

The major peak (Met 11) in urine from animals dosed with [*CF3Ph-U*-^{14}C] flutianil accounted for 3.72% and 0.78% of the AD in intact and bile duct-cannulated animals respectively. Met 11 appears to be a mercapturate conjugate of a hydroxylated methylsulfoxy trifluoromethyl ring structure and a structural isomer of Met 6, proposed in an earlier metabolism study (Hardwick, 2009a). Met 11 was also found at 0.24% in bile.

Figure 4. Structure of metabolite Met 11



Other urinary metabolites (Met 7, Met 8, Met 10 and Met 12) were detected in both intact and bile duct-cannulated animals.

These metabolites accounted for 0.23, 0.67, 0.27 and 0.13% of the AD in intact animals, compared with 0.06, 0.18, 0.25 and 0.10% of AD in bile duct-cannulated ones. Flutianil (designated Met 15 here) was not detected in the urine of intact rats.

In the bile, the major metabolite was Met 8, present at 2.03% of AD. The second most abundant metabolite was Met 9, present at 1.21% of AD. Five other metabolites were named as Met 10, Met 11, Met 12, Met 13 and Met 14. An estimated 27 other unresolved peaks were present in the bile, accounting together for 3.99% of AD.

[*MeOPh-U*-^{14}C] flutianil metabolites

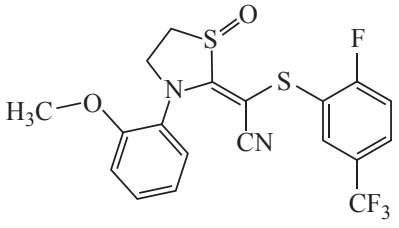
The significant urinary metabolites detected in intact and bile duct-cannulated animals were Met 1, Met 2, Met 3 and Met 7. In intact animals these metabolites accounted for 0.28, 0.20, 0.22 and 0.56% respectively of the AD. In bile duct-cannulated animals they were 0.06, 0.06, 0.10 and 0.18% of AD.

Urinary [*MeOPh-U*-^{14}C] flutianil was also significant, at 0.45% or 0.34% in intact and cannulated animals, respectively. It was detected at 0.04% administered dose in the bile

Other unidentified urinary metabolites were Met 8, Met 9, Met 10, Met 12 and Met 14. In intact animals these metabolites were detected at 0.27, 0.13, 0.15, 0.10 and 0.17% respectively of the AD. In bile duct-cannulated animals values were 0.11, 0.08, 0.05, 0.03 and 0.05% respectively of the AD. Met 14 was tentatively identified as OC 56574.

Met 14 (OC 56574)

Figure 5. Structure of metabolite M14 (OC 56574)



Despite extensive efforts, the majority of the minor metabolites remained unidentified; it was only possible to identify the parent flutianil and OC 56574 by co-chromatography with authentic standards. The most prominent metabolite Met 11 was the only metabolite for which successful tandem liquid chromatography/mass spectrometry (LC–MS/MS) analysis was achieved and a structure proposed.

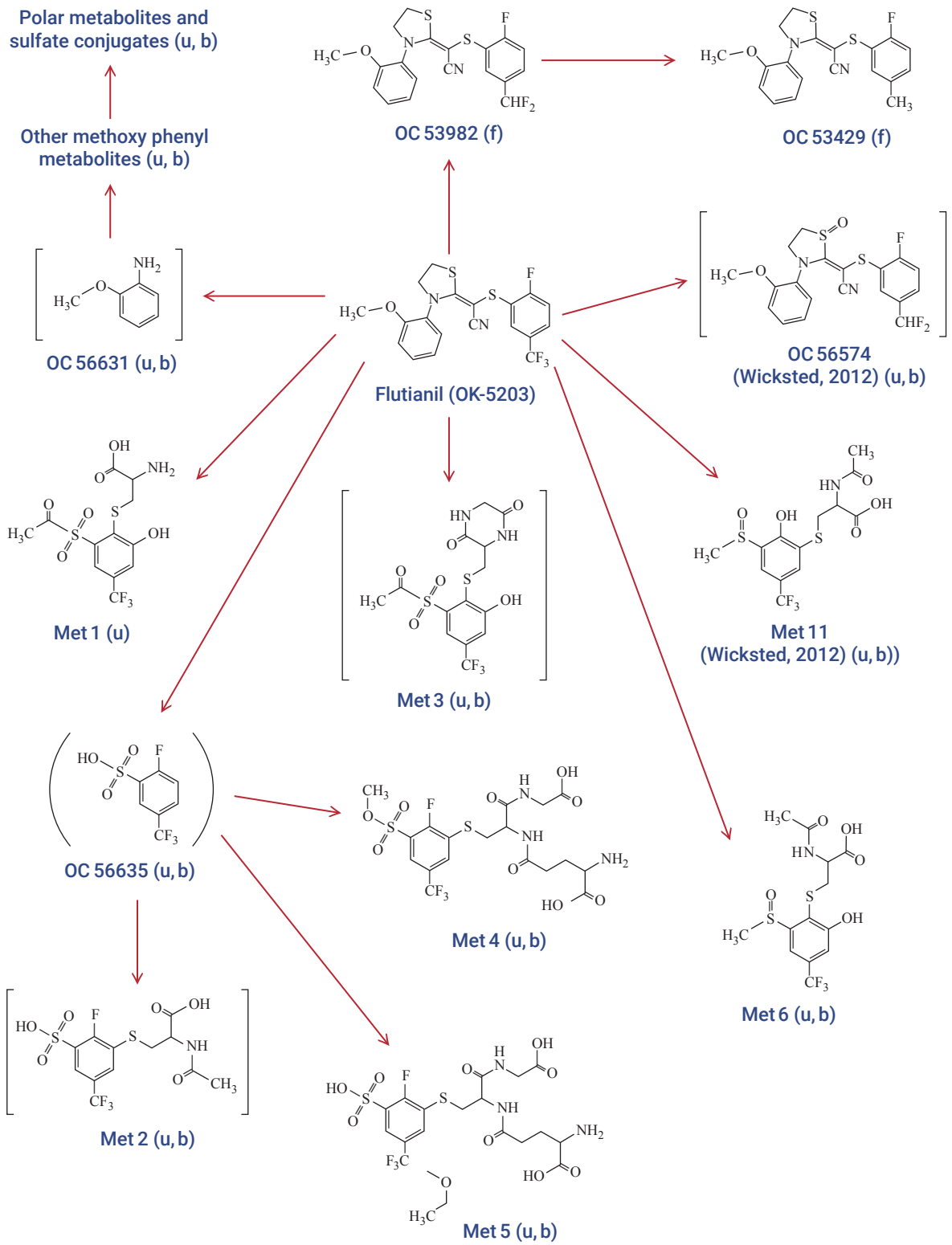
The number and nature of metabolites and difference in radio profiles for animals dosed with [*CF3Ph-U*-^{14}C] and [*MeOPh-U*-^{14}C]labelled flutianil suggested that cleavage of the molecule was a significant process in flutianil's metabolic processing (Wicksted, 2012).

In the repeated-dose study by Hardwick (2009c; for study design see section 1.1) the metabolite profile for the faeces was essentially the same after administration of either labelled form of flutianil, with the major component showing similar chromatographic properties to the parent compound and accounting for up to 81% of AD. There was however a difference in the metabolite profile seen in urine. Following administration of [*CF3Ph-U*-^{14}C]flutianil there was one major region of interest which accounted for up to 11% of AD and was identified as Met 6. Following administration of [*MeOPh-U*-^{14}C]flutianil there was no major region, all minor regions accounting for less than 2% of AD each.

Comparison of the results from this repeated-dose study with data from a single oral administration of either [*CF3Ph-U*-^{14}C]flutianil or [*MeOPh-U*-^{14}C]flutianil showed that urinary excretion was slightly lower with repeated doses compared with a single oral dose, but repeated administration had no major effect on the metabolism of flutianil (Hardwick, 2009c).

A proposed metabolic pathway for flutianil is presented in Fig. 6.

Figure 6. Proposed pathway for the metabolism of flutianil, as provided by the sponsor



1.3 Effects on enzymes and other biochemical parameters

No studies were provided.

2. Toxicological studies

2.1 Acute toxicity

The results of acute oral, dermal and inhalation toxicity studies with flutianil, along with the results of dermal and eye irritation and skin sensitization studies, are summarized in Table 9.

Table 9. Studies of acute toxicity due to flutianil

Species	Strain	Sex	Route	Purity	Result	Reference
Rat	Sprague Dawley	F	Oral	99%	LD_{50} > 5000 mg/kg bw	Lowe, 2015a
Rat	Sprague Dawley	M+F	Dermal	99%	LD_{50} > 5000 mg/kg bw	Lowe , 2015b
Rat	Han Wistar	M+F	Inhalation	99.22%	LC_{50} > 5.17 mg/L (MMAD 3–4 µm)	Dreher, 2007
Rabbit	Japanese white	F	Skin irritation	99.22%	Not irritating	Suzuki, 2006a
Rabbit	Japanese white	F	Eye irritation	99.22%	Transient irritation, resolved within 24 h	Suzuki, 2006b
Guinea pig	Hartley	F	Skin sensitization	99.22%	Not sensitizing	Suzuki, 2006c

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

(a) Lethal doses

In an acute oral study (up-and-down procedure), flutianil (purity 99%) was administered first to one female Wistar rat via gavage at 5000 mg/kg bw as a 40% by weight (w/w) mixture in corn oil. Due to the absence of mortality, two additional females then received the same dose level. Mortality and clinical signs were recorded at 30 minutes and during the first several hours post dosing, then at least once daily thereafter for 14 days following dosing. Individual body weights were recorded prior to test substance administration (initial weight) and again on days 7 and 14 (terminal) following dosing.

No mortality was observed. No signs of gross toxicity, abnormal behaviour or adverse clinical effects were noted. The acute oral median lethal dose (LD_{50}) of flutianil in female rats was greater than 5000 mg/kg bw (Lowe, 2015a).

In an acute dermal study groups of five female and five male Sprague Dawley rats were exposed to flutianil (99%) in water (moistened) over approximately 10% of their body surface at a single limit dose of 5000 mg/kg bw. Test sites were covered with a semi-occlusive dressing for 24 hours and animals observed for 14 days. The animals were observed for mortality, signs of gross toxicity, and behavioural changes during the first several hours after application, after patch removal, and then at least once daily thereafter for 14 days.

No mortality was observed. No signs of gross toxicity, abnormal behaviour or adverse clinical effects were noted. The LD_{50} of flutianil in female and male rats was greater than 5000 mg/kg bw (Lowe, 2015b).

In an acute inhalation study, groups of young adult Han Wistar rats, five male, five female, were exposed (nose only) to an aerosol of flutianil (99.22%) for four hours at a concentration of 5.17 mg/L. Animals were observed for 14 days post dosing. Body weights were recorded and rats were given a gross necropsy at termination.

There were no mortalities. Clinical signs of a reaction to treatment during the exposure period were confined to staining of the head. Clinical signs observed post exposure were wet fur, staining of the head snout and dorsal region, unkempt appearance and vocalisation. The acute median lethal concentration (LC_{50}) of the test article was found to exceed 5 mg/L following a four hour exposure period (Dreher, 2007).

(b) Dermal irritation

In a dermal irritation study, three female Japanese White rabbits were exposed to 0.5 g of pulverized flutianil (purity 99.22%), moistened uniformly with 0.5 mL of distilled water and applied to a piece of lint sheet, patches 5 × 7.5 cm for males and 3 × 4 cm for females. This was then applied to the clipped dorsal skin. Skin irritation reactions were evaluated in accordance with Draize′s criteria 1, 24, 48 and 72 hours after removal of the test article.

No clinical signs or skin irritation were observed on any animal during the 72-hour observation period following the removal of patches. The primary irritation index of flutianil was 0 and therefore flutianil was considered to be non-irritant to rabbit skin (Suzuki, 2006a).

(c) Ocular irritation

In an eye irritation study, pulverized flutianil (purity 99.22 %) was instilled into the conjunctival sac of the left eyes of three young adult female Japanese White rabbits (the unwashed eye group). A second group of three rabbits was left for 30 seconds in contact with the test article, then their eyes were washed for 30 seconds with distilled water. All animals were monitored for ocular lesions using the Draize scale at 1, 24, 48 and 72 hours following application.

In the unwashed eye group conjunctival redness and discharge were observed in all animals and conjunctival chemosis was observed in one of the three animals at one hour after application. These conjunctival changes appeared reversible when examined 24 hours after application. No other ocular changes were observed in any animal during the observation period. No effects on body weight gain or clinical signs were noted. Flutianil was considered to be non-irritant to the eyes of rabbits (Suzuki, 2006b).

(d) Dermal sensitization

In a dermal sensitization study (maximisation test of Magnusson & Kligmann) with flutianil (technical grade; purity 99.22%) in olive oil, ten young adult Hartley strain female Guinea pigs were tested. The control group of five animals received olive oil alone for intradermal and topical induction. In the treatment group, 2% flutianil (mixed 1:1 with Freund's complete adjuvant) was used in the induction phase (intradermal injection) followed by treatment with 50% test formulation by topical application. The animals were then challenged topically with a 25% test formulation. The application site was observed for skin reactions at 24 and 48 hours following injection, and at 24 and 48 hours following topical applications. A positive control group was not provided in this study. However, skin sensitization studies with the positive control substance 2,4-dinitrochlorobenzene (DNCB) have been conducted within six months of this study to ensure the test had been sufficiently sensitive.

There were no flutianil-related effects on general condition or body weight. No dermal response was observed in any animal in the flutianil-treated group.

Based upon the available information it was concluded that flutianil technical is not a skin sensitizer (Suzuki, 2006c).

2.2 Short-term studies of toxicity

(a) Oral administration

Mouse

Study 1

In a 28-day oral toxicity study, flutianil technical (99.38%, Lot no. 04AF1) was administered in feed to six CD1 mice per group of each sex at dose levels of 0, 100, 1000, 3000 or 10 000 ppm (equal to 0, 13.9, 138, 424, and 1393 mg/kg bw per day for males, 0, 16.4, 155, 497 and 1601 mg/kg bw per day for females).

All animals were observed for mortality and clinical signs during the study and body weights and food consumption were recorded. At termination of treatment all surviving animals were subjected to haematology, blood biochemistry, necropsy and organ weight analysis.

There was no mortality nor were there treatment-related changes in clinical signs in any dose group of either sex. Body weight, food consumption, and food conversion efficiency for all dose groups were comparable to controls.

Haematology and blood biochemistry revealed no treatment-related changes in any dose group of either sex. Some statistically significant changes in total leukocyte count (1000 ppm females), proportion of large unstained cells (3000 ppm, males), glutamate oxaloacetate transaminase (GOT) (1000 ppm males; 100 and 3000 ppm, females) and total bilirubin (100 and 3000 ppm, females) were not dose-related and were considered to be not treatment-related.

No treatment-related changes in gross findings at necropsy nor in organ weights were reported. Statistically significant effects on the relative liver weight of males at 100 ppm (−11% compared to control) and 3000 ppm (−7% compared to control) were considered not treatment-related and not adverse.

There was no evidence of flutianil-related toxicity in male or female mice fed a diet containing flutianil up to 10 000 ppm. Therefore the no-observed-adverse-effect level (NOAEL) was 10 000 ppm (equal to 1393 mg/kg bw per day for males, 1601 mg/kg bw per day for females), the highest dose tested (Harada, 2009a).

Study 2

In a 90-day oral toxicity study, flutianil technical (99.26%; Lot no. 04LF6) was administered in feed to groups of 10 CD1 ((SPF) ICR [Crlj:CD1(ICR)]) mice of both sexes at dose levels of 0, 1000, 3000, or 10 000 ppm (equal to 0, 138, 409 and 1387 mg/kg bw per day for males, 0, 159, 481 and 1555 mg/kg bw per day for females) for a period of 13 weeks.

All animals were observed for mortality and clinical signs during the study and their body weights and food consumption were recorded. At termination of treatment all surviving animals were subjected to haematology, blood biochemistry, necropsy, organ weight analysis and histopathology.

There were no treatment-related deaths and body weights, food consumption and food conversion efficiency for all dose groups were comparable to controls. One male mouse in the mid-dose (3000 ppm) group was found dead at the first week of treatment. The cause of this death was diagnosed by the study author as spontaneous in nature (dysuria with distended urinary bladder) and thus not treatment-related.

Haematology and blood biochemistry revealed no treatment-related changes in any dose group of either sex. A significant decrease (ca 30%) in creatinine concentration was observed in males in the 1000 and 10 000 ppm groups, but not at 3000 ppm. This change was not dose-related and was not accompanied by any histopathological finding or changes in blood urea nitrogen (BUN) and was therefore not considered to be treatment-related.

At necropsy, there were no treatment-related changes in gross findings or organ weights in any dose group of either sex. A statistically significant decrease in relative spleen weight (−23% compared to control) in males dosed at 3000 ppm was not dose- or treatment-related and no histopathological correlate was observed.

Atrophy of seminiferous tubules of the testis occurred in one male at the top dose, and in one male in the 3000 ppm dose group that was found dead during week 1, following dysuria. One such finding per group is within the historical control incidences in the same strain from the same laboratory Historical controls ranged from 0 to 1 animal/control group in each study for the 17 studies conducted between 2000 and 2010, and in the eight studies conducted over a five year period around the study under consideration (2002–2007).

Massive hepatocyte necrosis was present in one female in the 3000 ppm dose group; this individual was also found to have gross hepatic lesions at necropsy, but no comparable finding was observed in animals at 10 000 ppm. Hepatic microgranuloma occurred in two females in the top-dose group. Historical control data (HCD) from 2000 to 2010 (see above) in the same strain and from the same laboratory recorded microgranulomas in 0 to 2 female animals (in groups of 10 animals). Furthermore, in the eight studies conducted within the five year period around the study under consideration (2002–2007), incidences of microgranuloma in the liver ranged from 0 to 2 in females. Taking into account the documented spontaneous incidence of liver microgranuloma and the absence of statistical significance, this finding was not considered to be related to treatment with flutianil. No signs of inflammation were recorded in the animals which would support histopathological finding of microgranuloma in liver.

The NOAEL was 10 000 ppm (equal to 1387 mg/kg bw per day), the highest dose tested (Harada, 2009b, Kitazawa, 2014a).

Rat

Study 1

In a 28-day oral toxicity study flutianil (99.38%; Lot. no. 04AF1) was administered in the diet to six Wistar Hannover rats/group of each sex at dose levels of 0, 20, 200, 2000 or 20 000 ppm (equal to 0, 1.54, 16.1, 159 and 1555 mg/kg bw per day for males, 0, 1.82, 17.1, 172 and 1714 mg/kg bw per day for females).

All animals were observed for mortality and general clinical condition during the study and their body weights and food consumption recorded. Ophthalmology and urinalysis were conducted at week 4 of treatment. At the end of the treatment period all surviving animals were subjected to haematology, blood biochemistry, necropsy and organ weight analysis. Histopathological examination was performed on the liver, kidneys, and thyroids of both sexes and the hearts of females.

There were neither unscheduled deaths nor treatment-related changes in general clinical condition, body weight, food consumption, food efficiency, ophthalmology, urinalysis, haematology. nor notable gross findings at necropsy for either sex.

A statistically significant decrease in plasma total bilirubin was observed at 2000 and 20 000 ppm in males (83% of control value) In females the decrease was even more pronounced at 20 000 ppm (71% of control value) but without statistical significance. All findings were within the historical control values observed in studies conducted in the five years around the study under consideration. The decrease in total bilirubin was considered to be an effect of questionable significance and adversity.

In addition, a statistically significant decrease in plasma glucose (to 83% of control) was noted in males at 20 000 ppm. As there was no change in glucose concentration in subsequent 90-day or chronic toxicity studies with flutianil (although these included only lower doses), this effect was considered unrelated to treatment. There were no treatment-related effects on glucose in females.

The incidence of hyaline droplet deposition in the kidney in males at 200, 2000, and 20 000 ppm was 2/6, 2/6, and 5/6, respectively, which was considered dose-dependent and significantly increased at 20 000 ppm when compared to controls. As regards the severity of this finding, one male each in 200 and 2000 ppm had hyaline droplet deposition of lower (+) and higher (++) severity while at 20 000 ppm four males had droplets of higher severity (++) and only one at the lower level (+). Absolute and relative kidney weights increases (111% and 114% of control, respectively) were statistically significant in males at 20 000 ppm. No effects on kidney and associated clinical chemical parameters were observed in females. Subsequent immunohistochemical staining data (90-day study) showed the hyaline droplet formation to be related to the presence of α2μ-globulin, which is a finding of no human relevance.

In females, there was a significant reduction in relative heart weight at doses of 200 ppm (89% of control) and 2000 ppm (86% of control). Absolute heart weight was significantly reduced (to 87% of control) at 20 000 ppm. There were no associated clinical chemistry or histopathological findings relating to the heart, and heart weights were not reduced in a subsequent 90-day study or in the chronic toxicity and carcinogenicity study.

The NOAEL was 20 000 ppm (1555 mg/kg bw per day in males and 1714 mg/kg bw per day in females), the highest dose tested (Harada, 2009c, Kitazawa, 2014b).

Study 2

In a 90-day oral toxicity study flutianil (purity 99.38%; Lot no. 04AF1) was administered to 10 Wistar Hannover rats per sex at each of the following dose levels: 0, 20, 200, 2000 or 20 000 ppm (equal to 0, 1.22, 12.5, 122 and 1271 mg/kg bw per day for males, 0, 1.46, 14.3, 149 and 1500 mg/kg bw per day for females).

All animals were observed for mortality and clinical signs including detailed clinical and functional observations throughout the study. Body weight and food consumption were also recorded. At termination of treatment all surviving animals were subjected to ophthalmology, urinalysis, haematology, blood biochemistry, necropsy, organ weight analysis and histopathology.

There were no mortalities and no treatment-related clinical signs (including any from detailed clinical and functional observations) for any dose group of either sex during the study. Body weight and food consumption for each dose group were comparable to controls.

Ophthalmology, urinalysis and haematology showed no treatment-related changes in any dose group of either sex. Statistically significant prolongation of prothrombin time (PT; 122% of control value) and activated partial thromboplastin time (APTT; 109% of control value) was noted in males in the 20 000 ppm group (without any effect on platelet concentration), but in females a statistically significant shortening of PT (95%) was observed in the 20 000 ppm group. Not all increased values were within the range of the HCD, but changes in PT and APTT were considered inconsistent between sexes (prolongation versus shortening) and, given the small size of the change, of questionable adversity without observed changes in other haematological parameters.

As in the 28-days oral study, blood biochemistry revealed a significant decrease in plasma total bilirubin. In males dosed at 2000 and 20 000 ppm, the bilirubin level was 86% of controls, and the reduction was statistically significant at the top dose. In females, the statistically significant decrease in total bilirubin was 86% for the 2000 ppm group and 71% for the 20 000 ppm group when compared to controls. The changes in bilirubin were within the HCD for males, while changes in females at the top dose were lower than in any other documented study. In other dose groups there were no treatment-related changes in any parameter for either sex. There were no effects on liver marker enzymes in any group. Regarding the 28-days study findings, the decrease in total bilirubin as an isolated finding was considered an effect of questionable significance or adversity.

At necropsy, no treatment-related gross findings were noted in any dose group of either sex.

Organ weight analysis (Table 10) revealed a statistically significant increase in the relative liver weight of males and females in the 20 000 ppm group (109% and 113% of the control, respectively). In addition, a statistically significant increase in relative adrenal weight (117% of control) was noted in females from the 20 000 ppm group (Table 10). In the absence of any accompanying histopathological findings for the adrenals, this effect was considered non-adverse. In other dose groups there were no treatment-related changes in organ weights for either sex.

Centrilobular hepatocellular hypertrophy (grade: slight) was observed in 7/10 males in the 20 000 ppm group but was not observed in females. The slight increase in liver weight and the centrilobular hepatocellular hypertrophy in males were considered to be an adaptive response and therefore, non-adverse. In addition, with increasing doses males showed an increase in the severity of hyaline droplet deposition in the renal proximal tubular cells with the incidence of moderate deposition reaching statistical significance for the 20 000 ppm group (Table 10). Immunohistochemical staining demonstrated that the hyaline droplets in the proximal tubular cells were positive for α2μ-globulin.

Table 10. 90-day study in the rat: organ weight and relevant histopathology findings

		Male					Female				
Dose level (ppm)		**0**	**20**	**200**	**2000**	**20 000**	**0**	**20**	**200**	**2000**	**20 000**
						Organ weights					
Liver	Absolute ± SD (g) [% control]	9.29 ±1.18	8.85 ±0.80 [95]	9.27 ±0.85 [100]	9.35 ±0.78 [101]	9.98 ±0.94 [107]	5.12 ±0.43	5.27 ±0.54 [103]	5.26 ±0.38 [103]	5.31 ±0.43 [104]	5.47 ±0.44 [107]
	Relative to bw ± SD [% control]	2.35 ±0.10	2.29 ±0.11 [97]	2.42 ±0.17 [103]	2.46 ±0.22 [105]	2.56 ±0.16* [109]	2.32 ±0.13	2.43 ±0.16 [105]	2.42 ±0.10 [104]	2.42 ±0.15 [104]	2.61 ±0.21** [113]
Adrenals	Absolute ± SD (mg) [% control]	67.8 ±8.5	64.9 ±5.5 [96]	66.9 ±10.0 [99]	64.7 ±10.0 [95]	69.9 ±11.3 [103]	66.6 ±5.8	70.6 ±3.1 [106]	69.1 ±7.7 [104]	75.1 ±7.6 [113]	73.9 ±10.1 [111]
	Relative to bw ± SD [% control]	1.7 ±00.1	1.7 ±0.1 [100]	1.7 ±0.3 [100]	1.7 ±0.3 [100]	1.8 ±0.2 [106]	3.0 ±0.3	3.2 ±0.2 [107]	3.2 ±0.4 [107]	3.4 ±0.3 [113]	3.5 ±0.5* [117*]

	Male					Female				
Dose level (ppm)	**0**	**20**	**200**	**2000**	**20 000**	**0**	**20**	**200**	**2000**	**20 000**
	Histopathology [number of individuals examined]									
Liver: incidence of centrilobular hepatocellular hypertrophy (slight)	0 [10]	0 [10]	0 [10]	0 [10]	**7**** [10]	0 [10]	0 [10]	0 [10]	0 [10]	0 [10]
Kidney: incidence of hyaline droplet deposition in the proximal tubular cells	5+ 2++ [10]	6+ 2++ [10]	6+ 2++ [10]	4+ 6++ [10]	3+ **7++*** [10]	NR	NR	NR	NR	NR
Total incidence	7	8	8	10	10	NR	NR	NR	NR	NR

Grade: + slight; ++ moderate; NR: effect not relevant to females; bw: Body weight

* $p < 0.05$; ** $p < 0.01$; Dunnett's multiple comparison test

Taking into consideration the non-relevance to humans of the male rat-specific kidney effects, the NOAEL was 20 000 ppm (equal to 1271 mg/kg bw per day), the highest dose tested (Harada 2009d; Kitazawa, 2014b).

Dog

Study 1

In a 28-day oral toxicity study flutianil technical (purity 99.38%; Lot no. 04AF1) was administered to beagle dogs (two per sex per dose) in gelatine capsules at dose levels 0, 10, 300 or 1000 mg/kg bw per day. Deviation from the OECD guideline (two instead four dogs per group) was not considered critical in this instance since the study was used only as range-finding study for a subsequent 90-days study.

There were no compound-related effects on mortality, body weight, food consumption, organ weights, the results of ophthalmological examinations, haematology, clinical chemistry, or gross and histological pathology. Discharge of faeces containing test article-like material was observed in the top dose group and is assumed to be related to low oral absorption of flutianil, as was shown in rats.

The NOAEL in the 28-days dog study was 1000 mg/kg bw per day, the highest dose tested (Nagashima, 2006).

Study 2

In a 90-day subchronic oral toxicity study, flutianil (purity 99.38%; Lot no. 04AF1) was administered to four beagle dogs per sex per group by capsule at dose levels of 0, 30, 300 or 1000 mg/kg bw per day.

Faeces containing a test article-like substance were observed sporadically (8–14 times in males, 1–15 times in females) during the administration period in all males and females at the top dose. Test article in faeces was assumed to be due to low absorption of flutianil in dogs, as already demonstrated in rats.

There were no test article-related effects on body weight, body weight gain, food consumption, the results of ophthalmological examination or urinalysis.

As regards haematological examination, no statistically significant or notable haematological changes were observed in males. In females at the end of the study, statistically significant changes were seen in fibrinogen (decreased at all doses) and APTT (shortened at the highest dose; Table 11). As decreased fibrinogen would be expected in combination with increased rather than decreased coagulation time, shortened APTT and decreased fibrinogen were not considered correlated, and therefore considered not to be treatment-related.

In males, no effects on blood chemistry were observed. Statistically significant decreases in alkaline phosphatase (ALP) only in the two intermediate dose groups at weeks 7 and 13 were considered neither treatment-related nor biologically relevant.

There were no dose-related or statistically significant changes in gross pathology or organ weight.

Minimal to mild atrophy of the seminiferous tubules was observed in control, 30, 300 and 1000 mg/kg bw per day groups at an incidence of 1/4, 3/4, 0/4 and 3/4 respectively. The severity of the lesion was marginally increased at 1000 mg/kg bw (minimal to mild) compared with the control or the 30 mg/kg bw group (only minimal). The HCD provided covered a period of 10 years, approximately five years before and five years after the study under consideration, but the way in which the data was presented made interpretation difficult. It was however obvious from the data that mild atrophy in the seminiferous tubules is a rare finding; in HCD it had been observed in only one animal (nine months old) in 29 studies. That there were two animals with this finding in the current study is therefore outside what would be expected from the background controls. For minimal atrophy in HCD, there were nine instances reported out of 106 animals from 29 studies. It was stated that the range was from 0–67% which means that the highest incidence of minimal atrophy of seminiferous tubules per study was two out of three animals. In the HCD, individual incidences for atrophy of seminiferous tubules per study and the number of studies where it had been recorded were not included.

The mild infiltration of prostate cells of one dog at the highest tested dose of 1000 mg/kg bw per day was also observed in only one dog (nine months old) from 29 background studies (see Table 11).

The treatment-related atrophy of reproductive system cells in pubertal dogs is difficult to differentiate from the presence of still-immature tissues, since their morphology is similar. Neither atrophy of seminiferous tubules in testes nor cell infiltration in prostate were observed as potentially treatment-related findings in one-year dog study. Additionally, as no true dose–response relationship was observed, both findings were considered incidental rather than treatment-related.

The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Hoshiya 2009a, Ishikawa, 2014).

Table 11. 90-day study in the dog: haematology in females and histopathological findings in males

Dose (mg/kg bw per day)	0	30	300	1000
			Female	
Fibrinogen	286 ± 15	195 ± 53*	195 ± 10*	213 ± 23*
APTT	14.2 ± 0.8	14.9 ± 1.0	13.7 ± 0.8	12.0 ± 1.3*
			Male (number of dogs/grade)	
Testes: atrophy, seminiferous tubular	1/+	3/+	0	1/+, 2/++
Prostate: cell infiltration	1/+	1/+	2/+	1/+, 1/++

APTT: Activated partial thromboplastin time * $p < 0.05$, Dunnett′s test

Histopathology findings (grade): + = minimal; ++ = mild

Study 3

In a one-year dog oral study, flutianil (purity 99.22%; Lot no. 05DF2) was administered via capsules to groups of beagle dogs (four males and four females per group) at dose levels of 0, 30, 300 or 1000 mg/kg bw per day for 52 weeks.

No unscheduled deaths occurred during the administration period.

Faeces containing test article-like substance were observed sporadically during the administration period in males and females in the 1000 mg/kg bw group. As in the 90-day dog study, this was considered to be due to low absorption of flutianil, something also observed in rats.

No test article-related effects were observed on body weight or body weight gain, food consumption nor the results of urinalysis, ophthalmological examination or gross pathological findings.

Slight, but statistically significant differences were seen in some haematological and blood chemistry parameters. The changes were not considered related to test substance administration since they were either detected before the commencement of the treatment (weeks −2 and −1), were not dose-related or were absent at later measurement points.

A small number of statistically significant changes of relatively small magnitude (Table 12) were noted in kidney weights (males) and lung weights (females), but they were not accompanied by any significant histopathological findings. In contrast to the 90-day dog study, there were no changes in the gonads which could be considered treatment-related. Interstitial cell infiltration of the prostate was observed in the control, 30, 300 and 1000 mg/kg bw per day groups at incidences of 1/4, 0/4, 1/4 and 1/4 respectively, and was therefore not considered treatment-related. As regards the atrophy of the seminiferous tubules only one such male was identified in the control group. Minimal interstitial cell infiltration in the prostate was recorded in one animal each from the control, 300 and 1000 ppm, groups.

Table 12. One-year study in the dog: statistically significant organ weight changes

		Male				Female			
Dose mg/kg bw/day		**0**	**30**	**300**	**1000**	**0**	**30**	**300**	**1000**
Kidney weight absolute, (g)	Right	20.6 ±2.8	25.0 ±3.4	22.4 ±1.6	26.1 ±3.0*	No effect			
relative to control					127%				
Kidney weight absolute, (g)	Left	21.2 ±2.1	25.1 ±2.3	22.3 ±2.5	25.0 ±2.4	No effect			
relative to control					118%				
Lung weight: absolute, (g)		No effect				58.4 ±5.4	61.0 ±4.4	64.1 ±5.8	68.3 ±5.0*
Lung weight: relative (%)		No effect				0.65 ±0.01	0.71 ±0.04	0.74 ±0.06*	0.79 ±0.04**

* $p < 0.05$, ** $p < 0.01$

The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Hoshiya, 2009b, Ishikawa, 2014).

(b) Dermal application

In a 28-day dermal toxicity study flutianil (purity 99.22%; Lot no. 05DF2) was applied in a peanut oil vehicle to the shaved skin of 10 Wistar (Han) rats per sex at dose levels of 0, 100, 500 or 1000 mg/kg bw. Treatment was applied for six hours per day, seven days of the week for a 29-day period. Doses were selected based upon the results of a preliminary study where no effects were observed in animals treated for five days with 1000 mg/kg bw per day.

Observations and records were made of mortality, clinical signs (including neurological observations and locomotor activity), food consumption, ophthalmic examinations, clinical pathology, organ weight measurements and anatomic pathology evaluations (macroscopic and microscopic). The same observations were made for rats that formed additional recovery groups; these were treated with either 0 or 1000 mg/kg bw per day flutianil and sacrificed 14 days after the end of the treatment period.

No mortality was observed in the study. No clinical signs that could be attributed to the systemic effects of flutianil were seen in any of the animals. Overall, the skin reactions in treated animals were mild and most signs resolved during the treatment-free period. The incidence of desquamation and scabbing was similar in treated animals and controls. Erythema was observed occasionally in a few control animals and a few animals treated with 100 or 500 mg/kg bw per day, but not in animals treated with 1000 mg/kg bw per day.

There were no compound-related effects on body weight, food consumption, ophthalmic signs, haematology, urinalysis, organ weights or gross investigation and histopathology. Regarding the functional observation battery (FOB) there were no treatment-related effects, indicating the absence of neurological changes.

The NOAEL in the 28-days dermal study was 1000 mg/kg bw per day, the highest dose tested (Lucock, 2008).

(c) Exposure by inhalation

In a subchronic inhalation toxicity study, flutianil (purity 99.19%; Lot no. 2A76) was administered by inhalation to male and female (nulliparous and non-pregnant) Crl:CD(SD) albino rats for four weeks. Four groups of 10 male and 10 female rats were exposed, nose only, for six hours per day, five days a week to target concentrations of 0 (air control), 0.01, 0.1, and 1 mg/L flutianil over a four-week period; a total of 21 exposures. The mass median aerodynamic diameter (MMAD) ranged from 1.9–2.1 µm at the low dose, 2.1–2.3 µm at the mid dose and 2.1–2.5 µm at the high dose.

There were no test substance-related deaths in this study. Exposure to flutianil did not result in test substance-related adverse changes in body weight, body weight gain, daily food consumption, or daily food efficiency. No test substance-related clinical signs of toxicity or ophthalmological abnormalities were observed during the course of the study.

There were no adverse, treatment-related changes in haematology or clinical chemistry parameters at any concentration tested.

None of the gross observations made at necropsy were considered test substance-related.

At 1 mg/L statistically significant effects on liver weight (males, females), lung (females) and thyroid (males) were observed (Table 13). These effects and their magnitude were considered adverse, since additionally histopathological correlates were recorded.

Table 13. 28-day inhalation study in rats: absolute and relative organ weights

Concentration (mg/L)		0	0.01	0.1	1
			Male		
Liver:	Absolute weight (g)	10.847	9.430#	11.049	11.438
	Liver weight/brain weight × 100 Compared to control	514.061	464.618	539.738	542.367 106%
	liver weight/body weight × 100 Compared to control	2.776	2.643	2.861	3.122# 112%
			Female		
	Absolute weight (g) Compared to control	6.413	6.388	6.462	7.485# 117%
	Liver weight/brain weight × 100 Compared to control	333.066	336.037	334.668	383.348# (115)
	Liver weight/body weight × 100 Compared to control	2.865	2.897	2.963	3.384* 118%
			Female		
Lung:	Absolute weight (g) Compared to control	1.227	1.212	1.183	1.358# 111%
	Lung weight/brain weight × 100 Compared to control	63.694	63.826	61.377	69.618 109%
	Lung weight/body weight × 100 Compared to control	0.549	0.551	0.543	0.613# 112%
			Male		
Thyroid:	Absolute weight (g) Compared to control (%)	0.019	0.021	0.020	0.023# 121%
	Thyroid weight/brain weight × 100 Compared to control	0.880	1.033	0.957	1.074# (122)
	Thyroid weight/body weight × 100 Compared to control	0.005	0.006#	0.005	0.006# 120%

# $p < 0.05$; Dunnett's two-sided; parametric

* $p < 0.05$; Dunnett's two-sided; non-parametric

In 1 mg/L groups, nasal mucous cell hypertrophy/hyperplasia, lung centriacinar inflammation, liver hepatocellular hypertrophy, thyroid follicular cell hypertrophy (males only), and increased renal tubular hyaline droplets (males only) were recorded (Table 14).

Table 14. 28-day inhalation study in rats: histological findings

Sex	Male				Female			
Concentration (mg/L)	**0**	**0.01**	**0.1**	**1**	**0**	**0.01**	**0.1**	**1**
Number of rats/group:	10	10	10	10	10	10	10	10
Nose								
Hypertrophy/hyperplasia, mucous cell	0	0	0	5	0	0	0	10
Lungs								
Inflammation, centriacinar	0	1	0	4	0	1	0	4
Liver								
Hypertrophy, hepatocellular	0	0	0	6	0	0	0	6
Thyroid								
Hypertrophy, follicular cell	0	0	1	3	0	NE	NE	0
Kidneys								
Hyaline droplets, tubular	0	1	1	10	0	NE	NE	0

NE: Not examined

The minimal nasal mucous cell hypertrophy/hyperplasia and centriacinar inflammation of the lungs (with increased lungs weight) were considered to be a local response to exposure of a slight airway irritant and not relevant to dietary exposure. Hepatocellular hypertrophy and increased liver weight (< 20%) were not considered adverse. Increased thyroid weight in males in 1 mg/L group, with histopathological correlates (follicular cell hypertrophy) were considered an adverse finding, since no mechanism (for example, increased hepatic thyroxine glucuronidation) was investigated.

A minimal increase in renal tubular hyaline droplets in all 1 mg/L males suggested that the test compound or its metabolite binds to α2μ-globulin and slightly increases its retention in the renal tubular epithelium. As in other short-term studies with flutianil in rats this was considered an effect specific to the male rat with no human relevance.

The no-observed-adverse-effect concentration (NOAEC) in the 28-days inhalation study with 21 exposures was 0.1 mg/L, based on increases in thyroid weight and associated hypertrophy observed at 1 mg/L (Ng, 2013).

2.3 Long-term studies of toxicity and carcinogenicity

The long-term toxicity and carcinogenic potential of flutianil has been evaluated in a 78-week carcinogenicity study in mice and a 104-week study in rats. The 104-week rat study included a 52-week long-term toxicity phase.

Mouse

In a carcinogenicity study, flutianil (purity 99.26%; Lot no. 04LF6) was administered to CD1 mice, (52 individuals per sex per dose) in the diet for 78 weeks at dose levels of 0, 1000, 3000 or 10 000 ppm (equal to 0, 106, 321 and 1084 mg/kg bw per day for males, 0, 105, 316 and 1063 mg/kg bw per day for females). All animals were observed daily for mortality and general clinical signs during the study and their body weights and food consumption periodically recorded. At termination of treatment, all surviving animals were euthanized and subjected to haematology, necropsy, organ weight analysis and histopathology. No clinical biochemistry or urinalysis was investigated. With respect to animals killed in extremis or found dead during the treatment period, all such animals were also subjected to necropsy and histopathology. In addition, differential leukocyte counts were performed on blood smears from those killed in extremis.

There were no treatment-related differences in the numbers of premature decedents or scheduled terminations, and survival rates were acceptable (60% in males, 69% in females).

An increased incidence of soiled fur in males was the only clinical sign which was dose-related and reached statistical significance at the top dose. No comparable finding was observed in females.

Body weight, food consumption, and food efficiency for each dose group were comparable to controls.

Haematological examinations revealed no treatment-related changes in any group of either sex.

As regards necropsy findings (premature decedents and scheduled terminations), males at 10 000 ppm (1084 mg/kg bw per day) showed a significant increase in the incidence of soiled fur in abdominal/external genital regions and softening/atrophy of the testes, whereas no macropathological or clinical effects were observed in females of the top dose (1063 mg/kg bw per day). Most of the animals for which softening and atrophy of the testes were recorded, showed these signs at scheduled sacrifice and were not premature decedents.

There were no treatment-related changes in any organ weight, irrespective of sex or dose.

Histopathological investigation revealed no treatment-related changes in non-neoplastic lesions in any dose group of females. As regards males, a non-statistically significant increase in testis seminiferous tubule atrophy and oligospermia was observed at 10 000 ppm (Table 15). In the case of both atrophy and oligospermia, most animals were recorded at scheduled sacrifice and were not premature decedents. All males with oligospermia also showed atrophy of the testes. No clear differences between doses could be seen with respect to bilateral and unilateral effects. Historical control data included in the study report contained only data for tumour incidences. Since macroscopic observations on testes (atrophy and softening) did not correlate with any change in testis weight and could not be confirmed in histopathology examinations (no statistically significant increase in incidence or severer grades at the highest dose), the macroscopic findings, it was concluded, were not treatment-related.

Table 15. Long-term study in mice: necropsy and non-neoplastic histological findings in males (premature decedent and scheduled termination animals)

Sex	**Male**			
Dose (ppm)	**0**	**1000**	**3000**	**10 000**
		Necropsy findings		
Number of mice per group	52	52	52	52
Number of premature decedents	16	19	15	21
Number of scheduled deaths	36	33	37	31
Testis				
Softening	3	4	3	11*
among premature decedents	1	0	1	3
at scheduled death	2	4	2	8*
Atrophy	1	3	2	8*
among premature decedents	0	0	0	1
at scheduled death	1	3	2	7*
		Non-neoplastic changes		
Number of mice per group	52	52	52	52
Testis				
Seminiferous tubules atrophy	13	11	17	18
among premature decedents	3	4	3	3
grade +	2 (1B; 1U)	0	0	0
grade ++	1 (1B)	2 (2U)	2 (1B; 1U)	2 (1B; 1U)
grade +++	0	2 (2U)	1 (1U)	1 (1B)

Sex	Male			
Dose (ppm)	**0**	**1000**	**3000**	**10 000**
at scheduled death	10	7	14	15
grade +	3 (1B; 2U)	2 (1B; 1U)	8 (4B; 4U)	4 (2B; 2U)
grade ++	5 (2B, 3U)	1 (1B)	3 (2B; 1U)	5 (3B; 2U)
grade +++	2 (1B; 1U)	4 (3B, 1U)	3 (2B, 1U)	6 (4B; 2U)
Epididymis				
Number of mice per group	52	20[a]	17[a]	52
Number of premature decedents	16	19	15	21
Number of scheduled deaths	36	1	2	31
Oligospermia	6	3	0	11
among premature decedents	0	2	0	1
grade +	0	2 (2U)	0	1 (1U)
grade ++	0	0	0	0
grade +++	0	0	0	0
at scheduled death	6	1	0	10
grade +	0	0	0	2 (2B)
grade ++	1 (1B)	0	0	0
grade +++	5 (1B; 4U)	1 (1B)	0	8 (4B; 4U)

B: Bilateral; U: Unilateral; Severity grades: + mild, ++ moderate, +++ severe

[a] Examination was limited to animals with gross lesions at terminal kill and unscheduled deaths and the data were not subjected to statistical analysis

* $p \leq 0.05$, Fisher′s exact probability test

There were no statistically significant increases in neoplastic lesions in males or females, irrespective of the time of death.

The NOAEL for toxicity was 10 000 ppm (equal to 1063 mg/kg bw per day), the highest dose tested.

At the doses tested, there was no treatment-related increase in tumour incidence when compared to controls. The NOAEL for carcinogenicity was 10 000 ppm (equal to 1063 mg/kg bw per day), the highest dose tested (Harada, 2009e, Kitazawa, 2014a).

Rat

In a combined chronic/carcinogenicity study, flutianil technical (purity 99.28%; Lot no. 04LF6) was administered to Wistar rats in the diet to males at 0, 60, 600, 2000 or 6000 ppm (equal to 0, 2.45, 35.2, 81.9, and 249 mg/kg bw per day) and to females at 0, 60, 2000, 6000 or 20 000 ppm (equal to 0, 3.15, 111, 334, and 1130 mg/kg bw per day) for a duration of 52 weeks for satellite group, or 104 weeks in the case of the main group. Since in the 90-day rat study, hyaline droplet deposition in the kidney was considered likely to accelerate the occurrence of chronic progressive nephropathy in male rats, the top dose in male rats was reduced to 6000 ppm in this lengthier study.

Fifty-one Wistar Hannover rats were used in the main study groups; in the satellite groups 12 or 21 (at the highest dose) animals were used per sex and dose. During the treatment period, mortality, clinical signs, body weight, and food consumption were monitored for all animals. Ophthalmological examinations were performed on all animals in the highest-dose and control groups of both sexes at 52 weeks and 104 weeks of treatment. In addition, 10 animals from each group were subjected to functional observations at 49 weeks of treatment and to urinalysis, haematology, and blood biochemistry around 13, 26, 52, 78, and 104 weeks of treatment. All surviving animals in the satellite group after 52 weeks of treatment, and those in the main group after 104 weeks of treatment, were euthanized and subjected to necropsy, organ weight analysis, and histopathology.

There were no effects due to treatment on mortality, clinical signs, functional observations, body weight, food consumption, ophthalmology, urinalysis or gross pathology.

As regards the haematology findings (Table 16), there was a significant decrease in prothrombin time (PT) for females in the carcinogenicity study at week 104 at doses of 2000, 6000 and 20 000 ppm. No such effect was observed in males. The decrease in PT seen with 20 000 ppm females was outside the range of HCD mean ± SD, but the changes in the treated females at two years brought the values closer to those of one-year-old animals and were not considered to be adverse. No effects on PT were observed in the long-term study after 52 weeks of treatment. No other dose- or treatment-related effect was observed on haematological parameters.

Table 16. Rat chronic toxicity/carcinogenicity study: haematological findings (mean ± SD)

	Male					Female				
Dose level (ppm)	**0**	**60**	**600**	**2000**	**6000**	**0**	**60**	**2000**	**6000**	**20 000**
Prothrombin time (s) week 52	17.2 ± 0.9	16.8 ± 0.9	16.8 ± 0.9	18.2 ± 1.9	17.8 ± 1.3	16.5 ± 0.3	16.7 ± 0.6	16.3 ± 0.5	16.3 ± 0.5	16.0 ± 0.7
Prothrombin time (s) week 104	18.3 ± 1.0	17.3 ± 1.0	18.3 ± 1.4	18.2 ± 1.3	18.0 ± 1.7	18.2 ± 0.9	17.5 ± 0.7	17.3 ± 0.3*	17.4 ± 0.4*	16.9 ± 0.8**
HCD (s) week 104								17.8 ± 0.7		

HCD: Historical control data: four studies (113 animals) from the same lab, all examinations from 2007, including the current study;
SD: Standard deviation; * $p < 0.05$, ** $p < 0.01$

Blood biochemistry (Table 17) revealed a significant decrease in plasma total bilirubin in females at 20 000 ppm after 14, 78 and 104 weeks of treatment (33–43% lower than in controls). A non-statistically significant decrease in high-dose females was also observed at weeks 26 and 52. No other dose- or treatment-related changes in other blood biochemistry parameters were observed in females or males.

Table 17. Rat chronic toxicity/carcinogenicity study: blood biochemistry findings in female rats

Dose level (ppm)	**0**	**60**	**2000**	**6000**	**20 000**
Total bilirubin (mg/dL)					
week 14	0.08 ± 0.02	0.07 ± 0.01	0.06 ± 0.01	0.07 ± 0.02	0.05 ± 0.01**
week 26	0.10 ± 0.03	0.10 ± 0.02	0.08 ± 0.01	0.09 ± 0.03	0.07 ± 0.02
week 52	0.12 ± 0.03	0.11 ± 0.02	0.11 ± 0.03	0.10 ± 0.02	0.09 ± 0.02
week 78	0.14 ± 0.03	0.14 ± 0.05	0.10 ± 0.03	0.10 ± 0.02	0.08 ± 0.03**
week 104	0.12 ± 0.03	0.11 ± 0.02	0.10 ± 0.02	0.09 ± 0.02	0.08 ± 0.02**

* $p < 0.05$, ** $p < 0.01$

There was a significant increase (17%) in relative liver weights in females at 20 000 ppm after 52 weeks of treatment; this was not observed in any other group. Increases in liver weight did not reach statistical significance in any group when evaluated after 104 weeks (Table 18). These changes in liver weight were considered not to be adverse.

There were no significant treatment-related changes in organ weights of male rats during the chronic 52 week study. Relative and absolute thyroid weights at 600, 2000 and 6000 ppm were more than 10% greater than the control values, but no dose–effect relationship was observed. No changes in thyroid weight were observed after 104 weeks treatment. Absolute and relative uterus weights were more than 30% greater than the control values (not statistically significant) in the top-dose females after 52 and 104 weeks of treatment. After 52 weeks this was due to a single female at the top dose; when this was excluded the increase in uterus weight at the top-dose, compared to controls, was 12% and 19%, on an absolute and relative basis, respectively. After 104 weeks treatment, uterus weight was increased by ca 20% in animals treated with 6000 ppm. In this case also the large increase in uterus weight was mostly

due to one female in each of the 6000 ppm and 20 000 ppm groups, although the impact of the one animal uterus weight on the mean was smaller than after 52 weeks. In males after 104 weeks of treatment an increase in absolute and relative kidney weight of greater than 20% was recorded.

Table 18. Rat chronic toxicity/carcinogenicity study: organ weights (mean ± SD)

	Male					Female				
Dose level (ppm)	**0**	**60**	**600**	**2000**	**6000**	**0**	**60**	**2000**	**6000**	**20 000**
					Liver					
Absolute weight (g), 52 weeks	11.03 ± 1.12	12.43 ± 1.84	11.21 ± 1.66	11.20 ± 2.05	10.85 ± 1.30	5.87 ± 0.57	5.95 ± 0.53	6.43 ± 1.31	6.26 ± 0.71	6.68 ± 0.89
Compared to control										114%
Relative weight (%), 52 weeks	2.14 ± 0.17	2.32 ± 0.22	2.17 ± 0.18	2.18 ± 0.12	2.18 ± 0.16	2.05 ± 0.15	2.11 ± 0.11	2.19 ± 0.17	2.19 ± 0.12	2.40 ± 0.23**
Compared to control										117%
Absolute weight (g), 104 weeks	11.06 ± 1.86	12.12 ± 2.50	10.23 ± 1.33	11.65 ± 2.24	12.15 ± 2.35	7.19 ± 0.59	7.54 ± 2.03	7.87 ± 1.29	8.40 ± 3.34	9.17 ± 3.93
Compared to control										128%
Relative weight (%), 104 weeks	2.15 ± 0.24	2.44 ± 0.51	2.21 ± 0.29	2.22 ± 0.39	2.47 ± 0.54	2.29 ± 0.30	2.30 ± 0.33	2.24 ± 0.22	2.45 ± 0.69	2.69 ± 0.81
Compared to control										117%
					Thyroid					
Absolute weight (g), 52 weeks	22.8 ± 5.4	24.1 ± 4.1	31.3 ± 17.6	32.2 ± 19.8	27.2 ± 11.2	20.7 ± 3.8	19.4 ± 3.7	23.1 ± 11.7	23.8 ± 9.6	22.1 ± 7.4
Compared to control										
Relative weight (%), 52 weeks	0.004 ± 0.001	0.005 ± 0.001	0.006 ± 0.003	0.006 ± 0.003	0.006 ± 0.003	0.007 ± 0.001	0.007 ± 0.001	0.008 ± 0.003	0.008 ± 0.003	0.008 ± 0.003
Compared to control	-	125%	150%	150%	150%	-	100%	114%	114%	114%
					Uterus					
Absolute weight (g), 52 weeks	-	-	-	-	-	828 ± 277	959 ± 321	794 ± 260	846 ± 488	1121 ± 720
Compared to control										135%
Relative weight (%), 52 weeks	-	-	-	-	-	0.29 ± 0.10	0.35 ± 0.14	0.29 ± 0.14	0.30 ± 0.18	0.41 ± 0.27
Compared to control										141%
Absolute weight (g), 104 weeks	-	-	-	-	-	815 ± 155	847 ± 310	734 ± 271	1015 ± 478	1209 ± 518
(% control)									125%	148%
Relative weight (%), 104 weeks	-	-	-	-	-	0.26 ± 0.05	0.26 ± 0.10	0.21 ± 0.07	0.31 ± 0.17	0.38 ± 0.19
Compared to control									119%	146%
					Kidney					
Absolute weight (g), 104 weeks	2905 ± 596	3102 ± 850	2648 ± 377	3011 ± 739	3488 ± 1246	2023 ± 156	1997 ± 393	2065 ± 307	2013 ± 290	2256 ± 606
Compared to control					120%					
Relative weight (%), 104 weeks	0.57 ± 0.13	0.62 ± 0.21	0.57 ± 0.09	0.58 ± 0.16	0.72 ± 0.31	0.65 ± 0.1	0.61 ± 0.08	0.59 ± 0.07	0.60 ± 0.04	0.67 ± 0.13
Compared to control	-				126%	-				

In male rats at 52 weeks a significant and treatment-related increase in hyaline droplet deposition in proximal tubular cell of the kidney was observed at 2000 and 6000 ppm (Table 19). Immunohistochemical staining demonstrated that the hyaline droplets in the proximal tubular cells were positive for α2μ-globulin. Other kidney lesions that occurred at greater frequency (not statistically significant) in treated male rats were urinary casts, tubular basophilic change, and calculus. Only the top-dose and control group kidneys were examined in female rats. Examination revealed increased tubular basophilic change and increased urinary casts in the 20 000 ppm dose group, but the incidence of these findings was not statistically significant.

There was a no statistically significant increase in liver lesions in the top-dose male rats, and an increase in bile duct hyperplasia in top dose females. Increased incidence in the number of lesions was seen in the top dose males in the thyroid and pituitary (but these findings were not evident at 104 weeks). In the top dose females, increased incidences of lesions were evident in the thyroid and pituitary, pancreas, ovary, and uterine horn and were also apparent at 104 weeks in the ovary and uterine horn. The incidences were within the appropriate historical control data of the same laboratory (2007–2013). Other organs examined showed no difference in the incidence of lesions in the treated groups compared to the control group.

Table 19. Rat chronic study: summary of non-neoplastic lesions at 52 weeks

	Male					Female				
Dose level (ppm)	**0**	**60**	**600**	**2000**	**6000**	**0**	**60**	**2000**	**6000**	**20 000**
					Liver					
Foci of cellular alteration (eosinophilic cell type)	1/12	NI	NI	NI	4/20	0/12	NI	NI	NI	0/21
Microgranuloma	0/12	NI	NI	NI	3/20	1/12	NI	NI	NI	0/21
Bile duct hyperplasia	0/12	NI	NI	NI	0/20	1/12	NI	NI	NI	7/21
					Kidney					
Urinary cast	4/12	3/12	3/11	2/12	13/20	4/12	NI	NI	NI	9/21
Tubular basophilic change	7/12	3/12	5/11	3/12	15/20	1/12	NI	NI	NI	5/21
Proximal tubular cell, hyaline droplet deposition	4/12	6/12	8/11	12/12**	18/20**	0/12	NI	NI	NI	0/21
					Pancreas					
Decreased zymogen granules	0/12	NI	NI	NI	0/20	0/12	NI	NI	NI	1/21
Acinar cell atrophy	1/12	NI	NI	NI	2/20	2/12	NI	NI	NI	6/21
					Thyroid					
Hydropic degeneration of follicular cells	0/12	1/1	1/1	2/2	2/20	0/12	NI	1/1	1/1	2/21
C-cell hyperplasia	0/12	0/1	0/1	0/2	2/20	0/12	NI	1/1	1/1	1/21
					Pituitary					
Cyst, intermediate lobe	0/12	NI	NI	NI	4/20	0/12	0/1	0/3	0/1	0/21
Anterior cell hyperplasia	1/12	NI	NI	NI	1/20	1/12	1/1	0/3	0/1	3/21
Aberrant craniopharyngeal structure	0/12	0/1	0/1	0/2	0/20	0/12	0/1	0/3	0/1	1/21
					Ovary					
Follicular cyst	-	-	-	-	-	0/12	NI	0/1	NI	1/21
Interstitial cell hyperplasia	-	-	-	-	-	0/12	NI	0/1	NI	2/21
					Uterus					
Uterine horn luminal dilatation	-	-	-	-	-	0/12	1/1	1/1	0/1	3/21

NI: Not investigated * $p < 0.05$, ** $p < 0.01$; Histopathology was evaluated by Fisher′s exact probability test (one-tailed)

At 104 weeks there were no statistically significant differences in the number of adverse non-neoplastic lesions in the treated groups compared to the control groups (Table 20). The only observed statistically significant difference from control males was the decreased incidence of oedema in the interstitium of the testes, but the adversity of this finding was questionable. Similarly to the chronic study, hyaline droplet deposition in the proximal tubular cells of the kidney was a persistent finding in treated males at all doses, and was observed also in control animals. Only the top dose and control group kidneys were examined in females, revealing a slight increase in urinary casts at the top dose (a finding also seen at

52 weeks), and an increased incidence of chronic nephropathy. Other kidney lesions in females showed comparable incidence to that in control animals.

There was an increase in extramedullary haematopoiesis in the spleen at the top dose in both sexes but no changes in red blood cell (RBC) parameters.

In males at the top dose there was a slight increase in liver foci of cellular alteration (both eosinphilic and basophilic; a similar increase was seen at 52 weeks), islet cell hyperplasia in the pancreas, and in cysts and plantar granulomas of the skin. In females there were an increased number of lesions in the ovary and uterine horn. The observed findings were within appropriate historical control data (2007–2013).

In both sexes, the numbers of non-neoplastic lesions in all other organs were generally similar in the treated groups and controls.

Table 20. Rat carcinogenicity study: summary of non-neoplastic and neoplastic lesions (all animals including those found dead or killed in extremis)

Parameter	**Males**					**Females**				
Dose level (ppm)	**0**	**60[a]**	**600**	**2000**	**6000**	**0**	**60**	**2000[a]**	**6000**	**20 000**
					Liver					
Foci of cellular alteration (eosinophilic cell type)	18/51	4/18[#]	0/6[#]	3/11[#]	23/51	10/51	1/21[#]	1/12[#]	1/17[#]	8/51
Foci of cellular alteration (basophilic cell type)	15/51	3/18[#]	0/6[#]	2/11[#]	23/51	17/51	0/21[#]	1/12[#]	1/17[#]	18/51
Foci of cellular alteration (vacuolated cell type)	1/51	2/18[#]	1/6[#]	0/11[#]	4/51	0/51	0/21[#]	0/12[#]	0/17[#]	0/51
Bile duct hyperplasia	21/51	1/18[#]	1/6[#]	0/11[#]	20/51	26/51	7/21[#]	2/12[#]	4/17[#]	28/51
Cholangioma	-	-	-	-	-	0/51	0/21[#]	0/12[#]	1/17[#]	1/51
					Lung					
Lung: alveolar histiocyte accumulation	3/51	6/15[#]	0/4[#]	0/6[#]	7/51	3/51	5/20[#]	5/12[#]	6/18[#]	5/51
Lung: alveolar epithelial cell hyperplasia	2/51	1/15[#]	0/4[#]	0/6[#]	4/51	1/51	0/20[#]	1/12[#]	0/18[#]	1/51
					Kidney					
Urinary cast	6/51	2/50	8/51	10/51	5/51	6/51	4/19[#]	1/9[#]	2/16[#]	8/51
Tubular basophilic change	12/51	9/50	12/51	11/51	13/51	17/51	9/19[#]	5/9[#]	3/16[#]	12/51
Proximal tubular cell, hyaline droplet deposition	19/51	26/50	20/51	26/51	23/51	0/51	0/19[#]	0/9[#]	0/16[#]	1/51
Calculus	5/51	11/50	4/51	9/51	9/51	10/51	5/19[#]	1/9[#]	1/16[#]	6/51
Pyelitis/ pyelonephritis	0/51	2/50	0/51	1/51	4/51	0/51	0/19[#]	0/9[#]	0/16[#]	1/51
Chronic nephropathy	34/51	28/50	26/51	32/51	37/51	14/51	4/19[#]	1/9[#]	5/16[#]	21/51

Parameter	Males					Females				
Dose level (ppm)	**0**	**60[a]**	**600**	**2000**	**6000**	**0**	**60**	**2000[a]**	**6000**	**20 000**
					Testis					
Testis: oedema of interstium	22/51	2/14[#]	2/10[#]	2/11[#]	8/51**	-	-	-	-	-
					Pancreas					
Pancreas: islet cell hyperplasia	0/51	0/10[c]	0/4[c]	0/6[c]	2[b]/51	0/51	1/17	0/7	0/13	0/51
Pancreas: acinar cell hypertrophy	1/51	0/10[c]	0/4[c]	0/6[c]	2[b]/51	0/51	0/17	0/7	0/13	2/51
Pancreas: islet cell adenomas	1/51	0/10[c]	0/4[c]	0/6[c]	4[b]/51	1/51	0/17	0/7	0/13	0/51
					Spleen					
Increased extramedullary hematopoisis	20/51	6/14[#]	1/5[#]	8/11[#]	26/51	15/51	7/19[#]	0/7[#]	7/13[#]	19/51
					Skin					
Epidermal cyst	2/41[#]	4/38	6/33[#]	4/37[#]	7/41[#]	0/20[#]	1/22[#]	1/21[#]	2/20[#]	2/27[#]
Plantar granuloma	36/41[#]	33/38[#]	27/33[#]	32/37[#]	39/41[#]	13/20[#]	14/22[#]	13/21[#]	8/20[#]	18/27[#]
					Uterus					
Uterine horn luminal dilatation	-	-	-	-	-	2/51	0/17[#]	1/15[#]	0/19[#]	2/51
Uterine horn luminal dilatation endometrial gland	-	-	-	-	-	6/51	2/17[#]	5/15[#]	5/19[#]	8/51
Ovary atrophy	-	-	-	-	-	2/51	3/21[#]	1/10[#]	1/16[#]	7/51

[a] One male (60 ppm group) and one female (2000 ppm group) were excluded from evaluation because of accidental death.

[b] All animals (terminal kill after 104 weeks and found dead/killed in extremis)

[c] Only animals found dead/killed in extremis in 60, 600 and 2000 ppm groups were investigated

* $p < 0.05$, ** $p < 0.01$; Histopathology was evaluated by Fisher′s exact probability test (one-tailed)

[#] Only tissues of animals showing macroscopic lesions examined; no statistical analysis conducted on these tissue findings

After 52 weeks of treatment only benign uterine horn endometrial stromal polyps showed slightly higher incidence in top-dose females (3/21 compared to 0/12 in control; see Table 19); the finding was slightly above the historical control data (at most two cases per study) for the same laboratory, 2006–2012. After 104 weeks of treatment it was observed that the incidence of uterine horn endometrial stromal polyps was higher in the controls (7/51) than at the top dose (4/51).

After long-term treatment (all animals, terminal kill and animals killed in extremis/found dead) there was no increase in the incidence of tumours in any organ, except a very slight increase in adenomas in the islet cells of the pancreas, which was observed in top-dose males (4/51 compared to 1/51 in controls; see Table 20)) and exceeded the historical control data (0–3/51 cases per study, median 1). Two males with islet cell hyperplasia in the high-dose group did not develop islet cell adenomas. All four males with islet cell adenomas at the high dose also exhibited pancreatic masses in the macroscopic investigation but no islet cell hyperplasia was observed. There was no increase in islet cell carcinomas in either female or male rats at any dose level.

Bile duct cholangioma, a benign lesion, occurred in 1/17 and 1/51 females in the 6000 and 20 000 ppm groups but not at all in the concurrent controls. These differences in incidence were not statistically significant; no bile duct cholangiomas were recorded in the four HCD studies included in the dossier. There were no corresponding histological alterations, such as bile duct injuries, inflammation,

oval cell proliferation, and/or cholangiofibrosis. There were no malignant bile duct tumours in any female rat. Bile duct hyperplasia at the end of the study was comparable in controls (60%) and the top-dose animals (58%). The occurrence of bile duct cholangioma in the mid- and top-dose groups (one animal each) appears to be incidental.

None of the differences in incidence of tumours in male or female rats between those of the top-dose and concurrent control groups was statistically significant according to a Fisher exact test, but the combined incidence of islet cell adenomas and hyperplasia in males was positive in a Cochran–Armitage trend test.

The kidney was the most sensitive organ in male rats. The NOAEL for male rats was 600 ppm, based on hyaline droplet deposition in proximal tubule cells. Since the kidney findings were considered likely to be due to α2μ-globulin nephropathy, which is a condition specific to male rats and not relevant to humans, this finding was not used for the human risk assessment.

The NOAEL for toxicity was 2000 ppm (81.9 mg/kg bw per day), based upon a slight increase in liver foci of cellular alteration (both eosinphilic and basophilic with a similar increase at 52 weeks) observed at 6000 ppm (equal to 249 mg/kg bw per day) in male rats.

The NOAEL for carcinogenicity was at 2000 ppm (81.9 mg/kg bw per day), based upon an equivocally increased incidence in pancreatic islet cell adenomas in males at the highest dose of 6000 ppm (equal to 249 mg/kg bw per day) (Harada, 2009f, Kitazawa 2014c).

2.4 Genotoxicity

Flutianil was assayed for mutagenesis in four histidine-requiring strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and the tester strain of *Escherichia coli* (WP2 *uvr*A) in a study compliant with current OECD TG 471 requirements (Ballantyne, 2005). Flutianil was also assayed for its ability to induce mutation at the *tk* locus (5-trifluorothymidine [TFT] resistance) in mouse lymphoma L5178Y $tk^{+/-}$ cells in a study compliant with the OECD TG 476 (adopted 21 July 1997). Cytogenetic effects were evaluated in an in vitro chromosomal aberration assay in human lymphocyte cultures (Kumaravel, 2005) compliant with the OECD TG 473 (adopted 21 July 1997). Moreover, flutianil was assayed in vivo for its potential to induce micronuclei in the polychromatic erythrocytes of bone marrow in intraperitoneally treated mice, up to a dose of 2000 mg/kg bw (Buskens, 2010) in a study compliant with the OECD TG 474 (Adopted 21 July 1997).

The results obtained (summarized in Table 21) did not show any genotoxic potential in any of the assays employed. For the in vivo micronucleus test, despite the absence of significant clinical signs and direct bone marrow toxicity, the use of the intraperitoneal route of administration was considered sufficient to ensure higher systemic bioavailability of the test item and consequent exposure of the target tissue bone marrow. In addition, significant exposure of blood and bone was demonstrated in the ADME studies with flutianil in rats. The combination of a valid bacterial reverse mutation assay, an in vitro mammalian gene mutation assay and an in vitro micronucleus test fulfils the basic requirements to cover the three genetic end-points (gene mutations, structural and numerical chromosome aberrations) for the genotoxicity evaluation. Overall the results of these studies indicated that there was no concern with respect to the genotoxicity of flutianil

Table 21. Summary of in vitro and in vivo genotoxicity studies performed with flutianil

End-point	Test object	Experimental conditions	Batch/ Purity	Results	Reference
		In vitro			
Bacterial gene mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2 uvrA	Range finder and plate incorporation test: 5, 15.81, 50, 158.1, 500, 1581 and 5000 µg/plate in acetone (± S9) Pre-incubation test: 20.48, 51.2, 128, 320, 800, 2000 and 5000 µg/plate in acetone (− S9) 10.24, 25.6, 64, 160, 400, 1000 and 2500 µg/plate in acetone (TA100, TA1535, WP2 uvrA) (+ S9) 4.096, 10.24, 25.6, 64, 160, 400 and 1000 µg/plate in acetone (TA98, TA1537) (+ S9)	04AF1/ 99.38%	Negative[a]	Ballantyne (2005)
Mammalian gene mutation	L5178Y $tk^{+/-}$ mouse lymphoma cells	Range finding test (± S9): 12.5, 25, 50, 100, 200 and 400 µg/mL in acetone Experiment 1 (± S9): 10, 20, 30, 40, 50, 60, 80, 100, 150 and 200 µg/mL in acetone Experiment 2 (± S9): 10, 20, 30, 40, 50, 60, 70, 80, 100 and 150 µg/mL in acetone	04AF1/ 99.38%	Negative[b]	Lloyd (2005)
Chromosome aberration	Human lymphocytes	Experiment 1; short treatment 96.64, 151.0 and 235.9 µg/mL in acetone (− S9) 188.7, 235.9 and 294.9 µg/mL in acetone (+ S9) Experiment 2; short treatment 174.3, 193.7, 239.1 and 450.0 µg/mL in acetone (+ S9) Experiment 2; continuous treatment 114.4, 127.1, 141.2 and 364.5 µg/mL in acetone (− S9)	04AF1/ 99.38%	Negative[c]	Kumaravel (2005)
		In vivo			
Micronucleus induction	Bone marrow erythrocytes	500, 1000 and 2000 mg/kg bw once via i.p. for 24 h and 2000 mg/kg bw for 48 h to five male NMRI mice	04AF1/ 99.38%	Negative[d]	Buskens (2010)

bw: Body weight; S9: The 9000 × *g* supernatant fraction from rat liver homogenate

[a] Study compliant with the current OECD TG 471

[b] Study compliant with the OECD TG 476 (adopted 21 July 1997)

[c] Study compliant with the OECD TG 473 (adopted 21 July 1997)

[d] Study compliant with the OECD TG 474 (adopted 21 July 1997)
No decrease in the ratio of polychromatic to normochromatic erythrocytes compared to the concurrent vehicle control group was observed. However, despite the absence of significant clinical signs and direct bone marrow toxicity, the use of the intraperitoneal route of administration is considered to be sufficient to ensure higher systemic bioavailability of the test item and consequent exposure of the target tissue bone marrow

OECD TG: Organisation for Economic Co-operation and Development test guideline

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Rat

Study 1

In a range-finding one-generation reproductive toxicity study, groups of eight Wistar Hannover [BrlHan:WIST@Jcl (GALAS)] rats of each sex received flutianil (purity 99.22%; Lot no. 05DF2) in the diet at concentrations of 0, 20, 200, 2000 or 20 000 ppm continuously for a period of three weeks prior to mating, and until weaning of their F1 offspring. Doses were equal (in the parental animals) to a mean intake of 0, 1.1, 11.3, 111.2, and 1146 mg/kg bw per day in males, 0, 2.2, 21.1, 227 and 2271 mg/kg bw per day in females. This study was conducted to select dose levels of flutianil technical prior to a definitive two-generation reproduction toxicity study in rats.

In the parental animals there were no effects on mortality, clinical signs of toxicity, body weight, body weight gain or feed consumption. There were no treatment-related changes in reproductive performance, such as incidence of females with normal estrous cycles, mating index, fertility index, gestation index, duration of gestation or number of implantation sites. Sex ratios of pups in treated groups was unaffected. It was not possible to evaluate sperm parameters or male reproduction since males were treated for only three weeks prior to mating.

As regards organ weights, a slight and not statistically significant increase was observed in absolute and relative uterus weight in parental females dosed at 2000 and 20 000 ppm; this was attributed primarily to two individual females in each group with larger uteri. There was a significant increase in relative liver weight in females at 2000 ppm (13% increase) and 20 000 ppm (16% increase). Absolute liver weights were increased in females at 20 000 ppm and 2000 ppm (18% and 12% higher respectively compared to control) but not with statistical significance. Neither histopathology nor blood chemistry were investigated to further clarify the effects on liver weight. Only mild effects on liver weight (< 10%) were observed in males in all dose groups. Thyroid was enlarged in one female in each of the 2000 and 20 000 ppm groups. In these two dose groups absolute and relative thyroid weights were increased by more than 20%. In males no dose response was observed for the effects of treatment on thyroid weight.

No treatment-related clinical signs or mortality were observed in offspring. There were no effects on body weight or gross pathology. In high-dose females absolute uterus weight was 30% higher than in controls and relative weight 27% so.

Based upon the effects on thyroid weight, without histopathological or blood chemistry investigations, NOAEL in parental females was 200 ppm (equal to 21.1 mg/kg bw per day). Parental males were not affected by treatment with flutianil. Since no effects were observed on reproductive performance, the reproductive NOAEL was 20 000 ppm (equal to 1146 mg/kg bw per day in males, 2271 mg/kg bw per day in females) the highest dose tested. Based upon the effects on uterus weight, without histopathological or blood chemistry investigations, the NOAEL for offspring females was 2000 ppm (equal to 227 mg/kg bw per day). Offspring males were not affected by treatment with flutianil (Shimizu, 2009a).

Study 2

In a two-generation reproduction study, flutianil (purity 99.22%; Lot no. 05DF2) was administered to 24 Wistar Hannover rats per sex and per group in the diet at concentrations of 0, 200, 2000 or 20 000 ppm for a period of 10 weeks prior to mating, and then until weaning of their offspring. Calculated intakes of flutianil for each group are summarized in Table 22.

No mortality nor treatment-related clinical signs were recorded during the study.

Changes in body weight (+ 7%), body weight gains (+10%) and food consumption (+15%) were increased with statistical significance in F0 generation males at 20 000 ppm during the study, but the same parameters were unaffected in the F1 generation. Mean body weights of F0 and F1 females in all treated groups were comparable to those in the control group throughout the study period.

Table 22. Test substance intake (mg/kg bw per day) two-generation rat reproductive toxicity study

Sex	Doses Period of treatment	Generation	200 ppm	2000 ppm	20 000 ppm
Males	Premating period (10 weeks)	F0	13.9	142	1468
		F1	15.2	155	1581
	Breeding period (7 weeks)	F0	9.3	93	984
		F1	9.3	91	963
Females	Premating period (10 weeks)	F0	16.6	171	1754
		F1	17.1	176	1765
	Gestation + lactation period	F0	23.5	244	2414
		F1	22.9	231	2238

In F1 parental animals in all treated groups, mean age and body weight at completion of preputial separation or vaginal opening were comparable to those of control group. No abnormalities in estrous cyclicity were found in any group of F0 or F1 females. Mean estrous cycle lengths were between 4.0 and 4.1 days in all groups including the control in both F0 and F1 generations. All animals mated and the number of days until mating was comparable between control and treated groups in both generations. Fertility index was comparable between the control and dosed animals in the F0 generation; in the F1 generation it was slightly lower (87.5%) in the 20 000 ppm group, since only 21 of 24 animals were pregnant. No effects were observed on gestation index, duration of gestation or sex ratio. Number of implantation sites at 20 000 ppm in the F1 generation was slightly lower (11) compared to controls and remaining treated groups (12.6, 12.2, 12.6). Although the magnitude of this decrease is considered marginal it is outside the historical control range. The implantation sites in the HCD provided for F1 generation animals at conducting laboratory in the five-year period 2004–2008 (seven studies) ranged between 11.1 and 13.0 days (mean 11.9 days). The number of corpora lutea was not determined.

The mean number of F2 pups delivered by the 20 000 ppm group (10.0) was significantly lower than that in the control group (11.8). The difference was partially attributable to the fact that one animal delivered only two pups, but was also consistent with the slightly lower number of implantation sites in animals at this dose. There was no indication of increased fetal resorptions and/or deaths.

There were no dose- or treatment-related changes in sperm motility, sperm morphology, epididymal sperm count or sperm head count.

The only gross pathological finding with a tendency to be higher in treated males was kidney pelvic dilation in the F0 generation (F0: 0/24, 0/24, 0/23, 2/23; F1: 2/22, 3/24, 4/24, 3/21).

To eliminate the effect of the F0 males at the top dose having a higher body weight, relative organ weights only were considered for the males of the F0 generation. F0 and F1 males in the 20 000 ppm group displayed statistically significant increases in the relative weights (ca 15%) of their liver and adrenals (ca 10%). F0 and F1 females displayed statistically significant increases in the absolute (10% and 18% respectively) and relative (15% and 11% respectively) liver weights. A statistically significant increase in thyroid weights (absolute and relative) was only seen in F1 females in the 20 000 ppm group (ca 18%). A statistically significant increase in absolute kidney weight was seen in F0 males (12%) at 20 000 ppm, but not in the F1 generation, and no significant changes were observed in relative kidney weight (ca +5%) in males at this dose.

In the 2000 and 20 000 ppm parental groups the incidence of hyaline droplet deposition in the proximal tubular cells of F0 and F1 males was higher than in the control group and grading analysis of the kidney lesions revealed a significant increase in the severity of this alteration. Grade 2 proximal tubular cell hyaline droplet deposition was increased to a statistically significant extent in males of the F0 generation at 2000 and 20 000 ppm, and in males of F1 generation at 20 000 ppm. This finding was not considered relevant to humans. F0 and F1 males in the 20 000 ppm group exhibited an increase in centrilobular hepatocellular hypertrophy. There were no treatment-related histopathological findings in females.

Mean body weights of F1 male and female pups in the 2000 ppm group, and males in the 20 000 ppm group, significantly exceeded those of the control groups on day 21 of lactation. No significant changes in body weight were found in any of the other dose groups, including all male and female pups in the F2 generation. There were no treatment related changes in mean absolute and relative organ weights of F1 and F2 weanlings in any of the treated groups. The increase in relative and absolute uterus weight in F1 and F2 weanlings was very slight (at most +11% at 20 000 ppm) and was not statistically significant. Some significant differences were observed in F1 female weanlings, however, these differences were considered incidental as there was no clear dose–response relationship.

The parental NOAEL was 2000 ppm (equal to 171 mg/kg bw per day in females), based upon increased thyroid weight in females at 20 000 ppm.

The NOAEL for reproductive performance was 2000 ppm (equal to 91 mg/kg bw per day in males, 171 mg/kg bw per day in females) based upon a slightly reduced number of implantation sites and the number of pups delivered by the F1 parental generation at 20 000 ppm.

The NOAEL for offspring was 20 000 ppm (equal to 963 mg/kg bw per day) the highest dose tested (Shimizu 2009b).

(b) Developmental toxicity

Rat

In a range-finding developmental toxicity study, flutianil (purity 99.38%; Lot no. 04AF1) formulated in 0.5% carboxymethyl cellulose (CMC) was administered orally via gavage to 32 presumed-pregnant Wistar Hannover rats randomly assigned to four dosage groups (eight rats per group) once daily, on gestation days (GDs) 6 to 19 at dosages of 0, 100, 333 or 1000 mg/kg bw per day.

Clinical observations, body weights and food consumption were recorded. All rats were sacrificed on GD 20 and examined for the number and distribution of corpora lutea, implantation sites and uterine contents. A gross necropsy of the thoracic, abdominal and pelvic viscera was performed. The gravid uterus was excised and weighed. Fetuses were weighed and examined for gross external alterations and sexed.

All rats survived to scheduled sacrifice. There were no clinical observations or histopathological findings that were considered treatment-related. The number of pregnant females for the 0, 100, 333 and 1000 mg/kg bw per day groups were 8, 6, 7 and 7, respectively. Body weights, body weight gain, absolute and relative feed consumption changes were generally comparable across all dosage groups. There was no test material-related effect on the number of corpora lutea, implantations, resorptions or live fetuses/litters, gravid uterine weights or placental morphology. There were no notable effects on the thoracic, abdominal or pelvic viscera.

Totals of 78, 61, 77 and 78 live fetuses from the 0, 100, 333 and 1000 mg/kg bw per day dosage groups, respectively, were examined externally for fetal alterations. No gross external alterations were observed. There was no test substance-related effect on fetal body weights, sex ratios or percentage of male fetuses/litter.

The NOAEL for both maternal and embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (York, 2006a).

In a developmental toxicity study, flutianil (purity 99.38%; Lot no. 04AF1) in aqueous 0.5% CMC was administered to 25 female Wistar Hannover rats per group. The test substance was administered by gavage once daily to rats on GDs 6 to 19 of presumed gestation at doses of 0 (vehicle), 100, 333 or 1000 mg/kg bw per day. The rats were observed for viability at least twice daily and for general appearance weekly. Rats were also examined for clinical observations, abortions, premature deliveries and deaths before and 60 ± 10 minutes after dosae administration and at sacrifice. Body weights were recorded weekly during the acclimation period, on GD 0 and daily during the dosage period, and at sacrifice. Food consumption values were recorded on GDs 0, 6, 9, 12, 15, 19 and 20.

The rats were sacrificed on GD 20 and given a caesarean section. A gross necropsy of the thoracic, abdominal and pelvic viscera was performed. Placental weights were recorded and fetuses

were weighed and examined for sex and gross external alterations. Approximately a half of the fetuses in each litter were examined for soft tissue alterations and retained for possible future skeletal evaluation. The remaining fetuses were examined for skeletal alterations.

All rats survived until scheduled sacrifice. The number of pregnant females for the 0, 100, 333 and 1000 mg/kg bw per day groups were 21 (84%), 22 (88%), 23 (92%) and 22 (88%), respectively. Doses of flutianil technical as high as 1000 mg/kg bw per day did not affect clinical or necropsy observations, body weight or body weight gain, absolute or relative feed consumption or any parameter evaluated at caesarean section. Although not statistically significant, there was a slight increase in early resorptions at the high dose (23 compared with 10 in controls). There was no evident alteration in the sex ratio. The litter averages for corpora lutea, implantations, litter sizes, live fetuses, early and late resorptions, fetal body weights, placental weight, percentage resorbed conceptuses and percent live male fetuses were all comparable among the four dosage groups and did not significantly differ. Flutianil technical did not cause any effect on gross, soft tissue or fetal development. There was a marginal (not statistically significant) elevation in the overall proportions of litters with delayed sternal ossification at the mid- and high-dose levels (13.6% of litters affected at 333 mg/kg bw per day and 22.7% of litters (five fetuses in five litters) affected at 1000 mg/kg bw. The incidence in the control and 100 mg/kg bw per day groups were one fetus in one litter. Based on a marginal increase at 300 and 1000 mg/kg bw per day, delayed sternal ossification was not considered to be treatment-related.

The NOAEL for maternal and fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (York, 2006b).

Rabbit

In a dose range-finding developmental toxicity study, flutianil (purity 99.22%; Lot no. 05DF2) formulated in 0.5% CMC was administered orally via gavage to three groups of six time-mated female New Zealand White (NZW) rabbits once daily from GD 6 to GD 28. Dose levels were 0, 100, 300 or 1000 mg/kg bw per day, administered using a dose volume of 5 mL/kg. All animals were observed twice daily for mortality and moribundity. Clinical observations, body weights and food consumption were recorded at appropriate intervals. On GD 29 a laparohysterectomy was performed on each surviving female. The uteri, placentae and ovaries were examined, and the number of fetuses, early and late resorptions, total implantations and corpora lutea were recorded. Gravid uterine weights were recorded, also net body weights, and net body weight changes were calculated. The fetuses were weighed and examined for external malformations and developmental variations.

No treatment-related mortalities were observed. One female each in the 100 and 300 mg/kg bw groups was euthanized in extremis on GDs 8 and 17 due to confirmed or suspected limb fracture. All other females were euthanized and examined on GD 29. There were no test material-related internal findings at necropsy.

Maternal body weight gains were variable in all groups (including the control), and no apparent test substance-related trends were observed. Mean food consumption throughout gestation, net body weights, net body weight changes and gravid uterine weights in all test material-treated groups were also similar to the control group.

Post-implantation loss in the 1000 mg/kg bw per day group was 12.4% per litter, higher than in the control group (5.0% per litter). This had been explained as being due to two females which had post-implantation losses of three fetuses each. However, no difference in the number of total live fetuses per dam was seen between the top-dose group (9.2 ± 1.60) and the controls (9.0 ± 1.90). Litter parameters in the 300 mg/kg bw per day dose group were comparable to the controls. In the 100 mg/kg bw per day group the number of viable fetuses was lower than in the control group. This was explained by higher pre-implantation losses which occurred in two females that had pre-implantation losses of four and seven fetuses respectively. Post-implantation loss at 100 mg/kg bw per day was also higher (25.6% per litter) than in the control group (5% per litter).

Mean fetal weight in the 1000 mg/kg bw per day group was similar to the control group. A statistically significant increase in mean fetal weight was noted in 100 mg/kg bw per day group, and is likely to be the result of the smaller litter size.

No treatment-related external fetal malformations or developmental variations were observed in any fetus.

Since no effects on dams were observed, the maternal NOAEL was 1000 mg/kg bw per day. Although slightly higher post-implantation losses were noted in the 1000 mg/kg bw per day group, the mean number of viable fetuses in this group was similar to the control group. Therefore, the fetal NOAEL was also 1000 mg/kg bw per day, the highest dose tested (Knapp, 2007a).

In a developmental toxicity study in rabbits, flutianil (purity 99.22%; Lot no. 05DF2) in aqueous 0.5% CMC was administered to groups of 25 time-mated female NZW rabbits by gavage at dose levels of 0, 100, 300 or 1000 mg/kg bw from GDs 6 to 28. Body weight was recorded on day 0, day 4, and daily from days 6–29. All animals were observed twice daily for mortality and moribundity. Clinical observations, body weights and food consumption were recorded at appropriate intervals. On GD 29 a laparohysterectomy was performed on each surviving female. The uteri, placentae and ovaries were examined, and the numbers of fetuses, early and late resorptions, total implantations and corpora lutea were recorded. Gravid uterine weights were recorded along with net body weights and net body weight changes were calculated. Placental weights were also recorded. The fetuses were weighed, sexed and examined for external, visceral and skeletal malformations and developmental variations.

One female each in the 100, 300 and 1000 mg/kg bw per day groups was found dead during GDs 23–28. The deaths in the 300 and 1000 mg/kg bw per day groups were attributed to intubation error based on the macroscopic findings (dark red or red fluid contents in the thoracic cavity and dark red discoloration of the lungs). One additional female at 100 mg/kg bw per day was euthanized in extremis on GD 15 due to rales, pale ears/eyes and red material on the base of the tail; no relationship to the test article was evident. Also at 100 mg/kg bw per day one additional female aborted on GD 28, following 10 days of low food consumption and decreased defecation.

There were no test article-related clinical findings at the daily examinations and no clinical findings approximately one hour following dose administration.

Mean maternal body weights, body weight gains, net body weights, net body weight gains and gravid uterine weights in the 100, 300 and 1000 mg/kg bw per day groups were similar to those in the control group. No test article-related effects on mean food consumption were observed in these groups.

Intrauterine growth and survival were unaffected by test article administration at dose levels of 100, 300 and 1000 mg/kg bw per day. There were no statistically significant differences between the total number of malformations or number of variations in the treated groups compared to the control group.

Hydrocephaly (three fetuses in one litter) was the only observed malformation in the 1000 mg/kg bw per day group for which further exploration was necessary. No early or late resorptions or dead fetuses were recorded for this dam. The nine live fetuses were however amongst the fetuses with lowest body weights in the study. In the 39 HCD studies on time-mated NZW rabbits from the same contract research organisation (but adifferent testing site) included in the study report, maximum incidence for hydrocephaly was two fetuses in one litter (range: 0–2 fetuses per study), observed in only one study; 32 studies were without any hydrocephaly finding. In total, hydrocephaly was recorded in only seven out of 798 litters and eight out of 6881 fetuses in this HCD set and was considered a very rare finding. Additionally, data from 83 HCD sets on artificially inseminated NZW rabbits (from the same testing site as this study) were also included in the study report. In 12 studies within the five years period (2002 to 2007) around the time of this flutianil study, no single case of hydrocephaly was recorded. In only one study from 1998 (nine years before the flutianil study was conducted), three fetuses in one litter were recorded with hydrocephaly. This confirms the condition as very rare finding in NZW rabbits in this testing laboratory, independ of whether they were time-mated or artificially inseminated. Additional to the HCD included in the study report, further HCD from the same laboratory from 2005–2007, 2007–2009, 2010–2012 and 2011–2013 (all January dates) were submitted. The same HCD were provided to ECHA RAC (European Chemicals Agency, Committee for Risk Assessment) during the public consultation on the EU classification and labelling procedure for flutianil in 2015 (ECHA, 2015). In HCD from January 2005–January 2007 hydrocephalus was found in eight out of 922 examined litters (0.87% of all examined control litters), and in 12 out of 7621 examined fetuses (0.16% of all examined control fetuses) with a single finding of four fetuses with hydrocephalus in one litter. In HCD from

January 2007–January 2009 moderate or marked hydrocephalus was found only in two litters out of 936 examined litters (0.21%), and in six fetuses out of 7708 examined fetuses (0.078%), with single finding of five fetuses with hydrocephalus in one litter. In HCD from January 2010–January 2013 (in total 86 studies) hydrocephalus was observed in three litters with a maximum of one fetus per litter.

Since litter is the relevant unit and at 1000 mg/kg bw per day only one litter was affected, 3 fetuses with hydrocephaly in one dam were considered not to be a consequence of the treatment with flutianil.

The maternal and the fetal NOAEL was 1000 mg/kg bw per day, the highest dose tested (Knapp, 2007b).

2.6 Special studies

(a) Neurotoxicity

No studies of neurotoxicity were submitted for flutianil.

No evidence of neurotoxicity was observed in the available subchronic and chronic toxicity studies with mice, rats, or dogs. Furthermore, detailed clinical observations and FOBs, in accordance with guidelines, were carried out for all male and female rats in the 90-day subchronic feeding study and the chronic feeding study. An FOB was also performed on rats in the 28-day dermal toxicity test. There were no treatment-related alterations in the clinical signs or the parameters measured in the FOBs.

(b) Immunotoxicity

In the immunotoxicity study (US EPA OCSPP 870.7800) flutianil (purity 99.19%; Lot no. 2A76) was administered to male Han Wistar (RccHan™;WIST) rats (10 per dosage group) in the diet at concentrations of 3250, 6500 or 13 000 ppm (equal to 304, 616 and 1251 mg/kg bw per day) for four weeks. The objective of this study was to make a functional assessment of the humoral T-lymphocyte-dependent antibody response (TDAR), using a modification of the Jerne plaque-forming cell (PFC) assay. Eight male rats (positive control) received cyclophosphamide at 50 mg/kg bw, administered by intraperitoneal injection on day 27. On day 25 (four days prior to necropsy), all animals received a single intravenous dose (1 mL by bolus injection) of sheep red blood cells (sRBCs; 2×10^8 cells/mL) in 0.9% saline.

During the study, clinical condition, body weight, food consumption, water consumption, organ weight (spleen and thymus), macropathology and PFC investigations were undertaken.

There were no unscheduled deaths, no treatment-related clinical signs, no adverse effect on body weight gain, food and water consumption nor on organ weights. There were no notable treatment-related macroscopic findings.

In the plaque-forming assay, there were no statistically significant differences from controls in the number of cells per spleen, PFC per 10^6 viable cells, or PFC per spleen in any of the groups which received flutianil. Treatment with a single 50 mg/kg bw dose of the immunosuppressant cyclophosphamide on day 27 resulted in a statistically significant decrease, and almost complete inhibition in male rats of the antibody response to a challenge with sRBCs.

It was concluded that dietary administration of flutianil to male rats at 3250, 6500 or 13000 ppm for four weeks caused no treatment-related effects. There was also no effect on immune function, as assessed by the measurement of antigen-specific T-cell-dependent antibody formation.

The NOAEL for functional immunotoxicity of flutianil was therefore 13 000 ppm (1251 mg/kg bw per day), the highest dose tested (Moore, 2012).

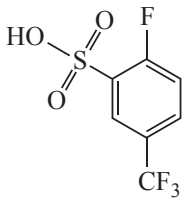
(c) Studies on metabolites

Studies were conducted on soil, water and plant metabolites OC 56635 and OC 53276, as well as on OC 63421, the sodium salt of OC 56635. None of these metabolites had been identified in rat metabolism studies.

For metabolites OC 53279, OC 56574, Bz5 and Bz6, identified in plant and/or animal commodities, QSAR assessments for genotoxicity were provided. None of these four metabolites had been identified in rat metabolism studies.

Metabolite OC 56635 (2-fluoro-5-(trifluoromethyl)benzenesulfonic acid)

Figure 7. Structure of metabolite OC 56635



Metabolite OC 56635 is a substituted benzenesulfonic acid and therefore expected to be acidic. In Guthrie (1978) the pK_a of sulfonic acid is stated to be −2.8; in OECD QSAR Toolbox 4.4.1 it was calculated to be −3.49. In order to avoid animal suffering from a compound presumed corrosive, additional studies were conducted with its sodium salt, OC 63421. The applicant argued that the free acid would ionise rapidly and completely at typical ambient pHs, and would never be found as the free acid in the environment. As a result exposure to the free acid, as such, would never occur.

A summary of the data is presented below.

Table 23. Toxicity studies conducted with OC 56635

Species	Strain	Sex	Route	Purity	LD_{50} (mg/kg bw)	Reference
Acute rat study (up-and-down procedure)						
Rat	Wistar	Female	Oral	99.6%	2000* > LD_{50} > 300	Kim, 2008

*In the oral preliminary study for micronucleus assay in mice, all animals treated with 1000 and 2000 mg/kg bw died within 24 hours. If no substantial difference between rats and mice in the absorption or toxic dynamics of OC 56635 is assumed, the LD_{50} in rats might be expected to fall between 300 and 1000 mg/kg bw, clearly more acutely toxic than flutianil.

In an acute oral toxicity study, groups of female Han Wistar rats were given a single oral dose of OC 56635 (purity 99.6%) in water according to an up-and-down procedure. In a preliminary range-finding assessment 2000 mg/kg bw were administered by gavage to a single female animal. Five days later a single female animal was administered 300 mg/kg bw. In the main study four female animals were administered 300 mg/kg bw. All animals were monitored for mortality, clinical signs and body weight changes during the 14 days after administration and were subjected to gross necropsy on day 14.

The animal treated with 2000 mg/kg bw died on day 1 of the treatment period. Noted before death were abnormal gait, decrease in locomotor activity, decreased respiration, eyelid closure, lacrimation, soiled perineal region and piloerection. For all animals treated with 300 mg/kg bw, no changes were observed in body weight gain nor any clinical signs noted. No treatment-related macroscopic abnormalities were seen at necropsy in animals administered 300 or 2000 mg/kg bw.

The oral LD_{50} of OC 56635 was considered to be higher than 300 mg/kg bw and below 2000 mg/kg bw (Kim, 2008).

Metabolite OC 56635 was assayed for mutation in four histidine-requiring strains (TA98, TA100, TA1535, TA1537) of *Salmonella typhimurium* and tester strain *Escherichia coli* WP2 *uvr*A pKM101 in a study compliant with the current OECD TG 471 guidance (Lee, 2008a). OC 56635 was also assayed for its ability to induce mutation at the *tk* locus (5-trifluorothymidine resistance) in mouse lymphoma L5178Y $tk^{+/-}$ cells (Zheng, 2008) in a study compliant with the OECD TG 476, (adopted 21 July 1997).

Metabolite OC 56635 was also assayed in vivo for its potential to induce micronuclei in polychromatic erythrocytes from the bone marrow of intraperitoneally-treated mice, up to dose of 300 mg/kg bw (Lee, 2008b), essentially compliant with the OECD TG 474 (1997 version). The results obtained did not show genotoxic potential in any of the assays employed. Additionally, despite the absence of direct bone marrow toxicity in the in vivo micronucleus test, the use of the intraperitoneal route of administration and the presence of relevant clinical signs (for example inanimation) indicate an adequate systemic exposure to the test compound.

Based on the results of these studies the Meeting concluded that there is no concern with respect to genotoxicity of metabolite OC 56635.

Table 24. Summary of in vitro and in vivo genotoxicity studies conducted with OC 56635

Study	Test system/ species	Purity	Experimental conditions	Result	Reference
			In vitro		
Bacterial gene mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2 *uvr*A pKM101	99.6%	Range-finding (cytotoxicity): 0, 8.19, 20.5, 51.2, 128, 320, 800, 2000 and 5000 µg/plate in water (± S9) Main study (1st and 2nd test) (gene mutation): 0, 313, 625, 1250, 2500 and 5000 µg/plate in water (± S9)	Negative[a]	Lee, 2008a
Mammalian gene mutation	L5178Y/$tk^{+/-}$ mouse lymphoma cells	99.6%	Range-finding (cytotoxicity): 0, 10.9, 21.9, 43.8, 87.5, 175, 350, 700, 1400 and 2800 µg/mL in water (± S9; 3 h and 24 h) Main study (gene mutation): 0, 21.9, 43.8, 87.5, 175, 350, 700, 1400 and 2800 µg/mL in water (± S9, 3 h and 24 h)	Negative[b]	Zheng, 2008
			In vivo		
Micronucleus induction	Bone marrow erythrocytes of male ICR mice	99.6%	Range-finding (not GLP): 0, 30, 100, 300, 1000 and 2000 mg/kg bw Bone marrow collection time trial (not GLP): 300 mg/kg bw Main experiment (GLP): 0, 75, 150 and 300 mg/kg bw once via intraperitoneal injection	Negative[c]	Lee, 2008b

bw: body weight; S9: 9000 × *g* supernatant fraction from rat liver homogenate;: Good laboratory practice; h: hours.

a Study compliant with the current OECD TG 471

b Study compliant with OECD TG 476, (adopted 21 July1997)

c The study essentially complies with the OECD TG 474 (adopted 21 July 1997) with the exception that at the 48-hour sampling time (high dose only) the induction of micronuclei was evaluated in three animals only in a trial for bone marrow collection, not according to GLP. No decrease was observed in the ratio of polychromatic to normochromatic erythrocytes compared to the concurrent vehicle control group. However, despite the absence of direct bone marrow toxicity, the use of the intraperitoneal route of administration and the presence of relevant clinical signs (for example inanimation) indicate an adequate systemic exposure to the test compound.

OECD TG: Organisation for Economic Co-operation and Development test guideline

Quantitative structure–activity relationship (QSAR) assessments (Derek Nexus 6.1.0 and OECD QSAR Toolbox, version 4.2) were provided (Gledhill, 2020).The Ames test prediction from In Derek Nexus was negative. With the OECD QSAR Toolbox no alert for in vitro (Ames) or in vivo (Micronucleus) mutagenicity by ISS was recorded. An alert for DNA binding (by OASIS) was given (triggered by the CF3 function). The same alert was given for flutianil. The alert is considered of low relevance since experimental data showed that OC 56636 is negative. The OECD QSAR Toolbox allocated OC 56635 to Cramer class III.

Metabolite OC 63421 (sodium 2-fluoro-5-(trifluoromethyl)benzenesulfonate)

Figure 8. Structure of metabolite OC 63421

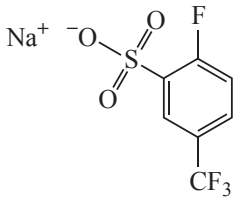


Table 25. Toxicity studies conducted with OC 63421

Species	Strain	Sex	Route	Purity	Result	Reference
Acute rat study						
Rat	Wistar	Female	Oral	99.7%	LD_{50} > 2000 mg/kg bw	Prinsen, 2009
Repeated dose (28-day) oral toxicity						
Rat	Wistar	Males & females	Oral	99.7%	NOAEL: 1380 mg/kg bw/day	Lina, 2009

In an acute rat toxicity study with OC 63421 no mortality nor clinical signs were observed. The oral LD_{50} of OC 63421 in female Wistar Hannover rats was > 2000 mg/kg bw (Prinsen, 2009).

In a 28-day oral toxicity study OC 63421 (Lot no E0810008; purity 99.7%) was administered in feed to five Wistar rats per group per sexes at dose levels of 0, 5000, 16 000 or 50 000 ppm (equal to 0, 400, 1380, and 4740 mg/kg bw per day for males, 0, 430, 1400, and 4860 mg/kg bw per day for females).

All animals were observed for mortality and clinical signs during the study and body weights and food consumption recorded. At termination of treatment all surviving animals were subjected to haematology, blood biochemistry, gross pathology, organ weight analysis and histopathology.

There was no mortality nor treatment-related changes in clinical signs in any dose group of either sex. No significant or biologically relevant changes in body weight were recorded. The body weight gain over the study duration was unaffected in males, but 19% lower in females at the high dose, though this had no statistical significance. Food consumption in high-dose males was lower than in controls overt the first seven days of the study but total food consumption over the course of the study was 20% higher than in controls. In top-dose females the overall food consumption was 8% higher. Food conversion efficiency was reduced at the high dose in both sexes (15% in males and 21% in females).

As regards haematological parameters, the RBC count was 8% lower than controls (statistically significant) in males of the high-dose group. Mean corpuscular volume (MCV) was increased to a statistically significant extent in the mid- and high-dose males, but the increase was only 4% compared to controls in both cases. Also, reticulocyte count was higher than controls in high-dose males (statistically significant at +29%). No effects on haematology were observed in females.

Blood biochemistry revealed no treatment-related changes in any dose group of either sex. No treatment-related changes in gross findings at necropsy, organ weights or histopathology were recorded for treated-animals that were different to controls.

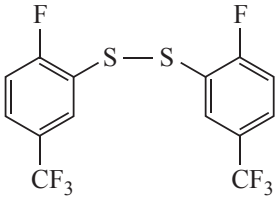
Based upon increased food consumption and reduced food conversion efficiency in both sexes, as well as slight effects on some haematological parameters, the NOAEL for OC 63421 (the salt of OC 56635) was 16 000 ppm, equal to 1380 mg/kg bw per day (Lina, 2009).

Metabolite OC 56634, (bis[2-fluoro-5-(trifluoromethyl) phenyl]disulfide)

Assessments using QSAR (Derek Nexus 6.1.0 and OECD QSAR Toolbox, version 4.4) were run (Gledhill, 2020). In Derek Nexus the Ames prediction was negative. Using OECD QSAR Toolbox no alert was recorded for in vitro (Ames) or in vivo (micronucleus) mutagenicity by ISS. An alert was produced for DNA binding (by OASIS). This same alert was given for flutianil. The alert was considered of low relevance since experimental data showed that flutianil is not genotoxic. OECD QSAR Toolbox allocated OC 56634 to Cramer class III. Structural similarity to flutianil (Dice coefficient) was estimated to be 50%.

Metabolite OC 53276, ((2Z)-{[2-fluoro-5-(trifluoromethyl)phenyl]sulfinyl}[3-(2-methoxyphenyl)-1,3-thiazolidin-2-ylidene]ethanenitrile)

Figure 9. Structure of metabolite OC 53276



Metabolite OC 53276 was assayed for mutatagenic potential in four histidine-requiring strains (TA98, TA100, TA1535, TA1537) of *Salmonella typhimurium* and tester strain *Escherichia coli* WP2 *uvr*A in a study compliant with the current OECD TG 471 (Lee, 2010a). It was also assayed for the ability to induce mutation at the *tk* locus (5-trifluorothymidine [TFT] resistance) in mouse lymphoma L5178Y $tk^{+/-}$ cells (Ahn, 2010) in a study compliant with the OECD TG 476, (adopted 21 July 1997). Moreover, OC 53276 was assayed in vivo for its potential to induce micronuclei in the polychromatic erythrocytes from the bone marrow of intraperitoneally treated mice, up to dose of 2000 mg/kg bw (Lee, 2010b) in a study compliant with the OECD TG 474 (adopted 21 July 1997). The results obtained did not show any genotoxic potential in any of the assays employed. In addition, in the in vivo micronucleus test, despite the absence of clinical signs and direct bone marrow toxicity, the use of the intraperitoneal route of administration was considered sufficient to ensure higher systemic bioavailability of the test item and consequent exposure of the target bone marrow.

Based on the results of these studies it was concluded that there is no concern with respect to the genotoxicity of metabolite OC 53276.

Table 26. Summary of the in vitro and in vivo genotoxicity studies conducted with OC 53276

Study	Species	Purity (%)	Experimental conditions	Result	Reference
			In vitro		
Bacterial gene mutation	*Salmonella typhimurium* TA98, TA100, TA1535,TA1537 and *Escherchia coli* WP2 *uvr*A pKM101	99.16	Range-finding study(cytotoxicity): 0, 8.19, 20.5, 51.2, 128, 320, 800, 2000 and 5000 µg/plate in water (± S9) Main study (1st and 2nd test) (gene mutation): 0, 313, 625, 1250, 2500 and 5000 µg/plate in water (± S9)	Negative[a,b]	Lee, 2010a
Mammalian gene mutation	L5178Y/*tk* $^{+/-}$ mouse lymphoma cells	99.16	Range-finding study (cytotoxicity): 2.90, 7.24, 18.1, 45.3, 113, 283, 707, 1768 and 4420 µg/mL in DMSO (± S9; 3h and 24 h) Main study (gene mutation): a) 28.1, 56.3, 113, 225, 450, 900, 1800 and 3600 µg/mL in DMSO; (+ S9; 3 h) b) 0.94, 1.88, 3.75, 7.5, 15, 30, 60, and 120 µg/mL. (– S9; 3 h) c) 0.94, 1.88, 3.75, 7.5, 15, 30, 60 and 120 µg/mL. (– S9; 24 h)	Negative[a]	Ahn, 2010

Study	Species	Purity (%)	Experimental conditions	Result	Reference
			In vivo		
Micronucleus induction	Bone marrow erythrocytes of male ICR mice	99.16	Range-finding (not GLP): 0, 30, 100, 300, 1000 and 2000 mg/kg bw Bone marrow collection time trial (not GLP): 2000 mg/kg bw Main experiment (GLP): 0, 500, 1000 and 2000 mg/kg bw once via intraperitoneal injection	Negative[c]	Lee, 2010b

[a] Study compliant with OECD TG 476 (adopted 21 July1997)

[b] Only 2-aminoanthracene was used as positive control with S9 mix. In the study report and also upon request, no confirmation of the manufacturer on effectiveness of S9 (Lot 09103008) was provided due to the expiry period of retention of documents. However, the dataset available for this metabolite includes also a mammalian gene mutation assay in L5178Y/$tk^{+/-}$ mouse lymphoma cells, showing a clear negative result. On this basis, the gene mutation end-point appears to be adequately investigated

[c] Study compliant with OECD TG 474 (adopted 21 July 1997). No decrease in the ratio of polychromatic to normochromatic erythrocytes compared to the concurrent vehicle control group was observed. However, the use of the intraperitoneal route of administration is considered to be sufficient to ensure higher systemic bioavailability of the test item and consequent exposure of the target bone marrow tisue

bw: Body weight; S9: 9000 × *g* supernatant fraction from rat liver homogenate;

OECD TG: Organisation for Economic Co-operation and Development test guideline

Assessments using QSAR (Derek Nexus 6.1.0 and the OECD QSAR Toolbox, version 4.2) were provided (Gledhill, 2020). In Derek Nexus the Ames prediction was negative. Using the OECD QSAR Toolbox no alert was raised for the in vitro (Ames) by ISS. An alert was produced for DNA binding (by OASIS) and in vivo (micronucleus) mutagenicity by ISS. The same alerts were given for flutianil. These alerts were considered of low relevance since experimental data showed that OC 53276 was not genotoxic. The OECD QSAR Toolbox allocated OC 53276 to Cramer class III. Structural similarity to flutianil (Dice coefficient) was estimated to be 94.74%.

Metabolite OC 53279, (2Z)-{[2-fluoro-5-(trifluoromethyl)phenyl]sulfanyl}[4-hydroxy-3-(2-methoxyphenyl)-1,3-thiazolidin-2-ylidene]acetonitrile

Assessments using QSAR (Derek Nexus 6.1.0 and the OECD QSAR Toolbox, version 4.2) were provided (Gledhill, 2020). In Derek Nexus the Ames prediction was negative. Using the OECD QSAR Toolbox no alert was raised for in vitro (Ames) by ISS. An alert was given for DNA binding (by OASIS) and in vivo (micronucleus) mutagenicity by ISS. The same alerts were given for flutianil. These alerts were considered of low relevance since experimental data showed that flutianil is not genotoxic. The OECD QSAR Toolbox allocated OC 53279 to Cramer class III. Structural similarity to flutianil (Dice coefficient) was estimated to be 87.72%.

Metabolite OC 56574, (2Z)-{[2-fluoro-5-(trifluoromethyl)phenyl]sulfanyl}[3-(2-methoxyphenyl)-1-oxo-1,3-thiazolidin-2-ylidene]acetonitrile

Assessments using QSAR (Derek Nexus 6.1.0 and the OECD QSAR Toolbox, version 4.2) were provided (Gledhill, 2020). In Derek Nexus the Ames prediction was negative. Using the OECD QSAR Toolbox no alert was raised for in vitro (Ames) by ISS. An alert was given for DNA binding (by OASIS) and in vivo (micronucleus) mutagenicity by ISS. The same alerts were given for flutianil. These alerts were considered of low relevance since experimental data showed that flutianil is not genotoxic. The OECD QSAR Toolbox allocated OC 53574 to Cramer class III. Structural similarity to flutianil (Dice coefficient) was estimated to be 94.74%.

Metabolite Bz5, S-[2-(methanesulfinyl)-4-(trifluoromethyl)phenyl]cysteine

Assessments using QSAR (Derek Nexus 6.1.0 and the OECD QSAR Toolbox, version 4.2) were provided (Gledhill, 2020). In Derek Nexus the Ames prediction was negative. Using the OECD QSAR Toolbox no alert was saised for *in vitro* (Ames) by ISS. An alert was given for DNA binding (by OASIS) and in vivo (micronucleus) mutagenicity by ISS. The same alerts were given for flutianil. These alerts were considered of low relevance since experimental data showed that flutianil is not genotoxic. The OECD QSAR Toolbox allocated Bz5 to Cramer class III. Structural similarity to flutianil (Dice coefficient) was estimated to be 41.67%.

Metabolite Bz6, S-[2-fluoro-5-(trifluoromethyl)phenyl] hydrogen carbonothioate

Assessments using QSAR (Derek Nexus 6.1.0 and the OECD QSAR Toolbox, version 4.2) were provided (Gledhill, 2020). In Derek Nexus the Ames prediction was negative. Using the OECD QSAR Toolbox no alert was raised for in vitro (Ames) or in vivo (micronucleus) mutagenicity by ISS. An alert was given for DNA binding (by OASIS). The same alert was given for flutianil. The alert was considered of low relevance since experimental data showed that flutianil is not genotoxic. The OECD QSAR Toolbox allocated Bz6 to Cramer class III. Structural similarity to flutianil (Dice coefficient) was estimated to be 55.81%.

(d) Impurities

Flutianil technical material has very high purity (>99%) with only a few impurities and theses specified at a very low level (0.1%) (US EPA label amendment[1]). Batches used in toxicological studies support the specification of the technical material.

3. Observations in humans

No information was provided on the health of workers involved in the manufacture or use of flutianil. No information on accidental or intentional poisoning in humans was available.

4. Microbial aspects

Target site

The precise mode of action of flutianil is unknown; it has a novel MOA against powdery mildew. It is classified under the Fungicide Resistance Action Committee (FRAC) code U13.

4.1 Mechanism and type of antimicrobial action

No available information.

4.2 Microbiome of the human gastrointestinal tract

No available information.

4.3 Antimicrobial spectrum of activity

No available information.

4.4 Antimicrobial resistance mechanisms and genetics

No available information.

1 https://www3.epa.gov/pesticides/chem_search/ppls/011581-00005-20200130.pdf

Comments

Biochemical aspects

The absorption, distribution and excretion of flutianil was investigated in rats following the administration of flutianil uniformally labelled in either the methoxyphenyl ring or the fluorotolyl ring, as a single oral low dose (10 mg/kg bw), a single oral high dose (1000 mg/kg bw) or 14 unlabelled daily oral low doses followed by a radioactive low dose (Needham, 2006; Hardwick, 2009a, b; Wicksted, 2012). Based on the sum of the radioactivity in urine, bile, cage wash plus debris and the residual carcass, approximately 20% of the oral dose was absorbed at 10 mg/kg bw [^{14}C] flutianil. There was no significant sex difference following administration of either radiolabel. Urinary excretion of radioactivity from methoxyphenyl-labelled flutianil was slightly lower (ca 10%) following repeat dosing (Hardwick, 2009c) than from a single dose (ca 20%) (Hardwick, 2009a), but this might have been due to variation across studies. At 1000 mg/kg bw, based on urinary excretion data (bile not measured), absorption was approximately 1% (Hardwick, 2009a).

At the low dose (10 mg/kg bw), maximum plasma concentrations were achieved within 13 hours following administration of fluorotolyl-labelled flutianil and within three hours following administration of methoxyphenyl-labelled flutianil. At the high dose level, concentrations were generally below the limit of detection. In contrast to plasma, maximum whole blood concentrations were achieved within eight hours following administration of fluorotolyl-labelled flutianil and within five hours following administration of methoxyphenyl-labelled flutianil at the low dose level. Peak blood levels of radioactivity were approximately four-fold higher than peak plasma levels. At the high dose, maximum blood concentrations were achieved within four hours following administration of fluorotolyl-labelled flutianil but could not be determined following administration of methoxyphenyl-labelled flutianil. There were no significant sex differences in plasma or blood kinetics of radioactivity following administration of either radiolabelled form (Hardwick, 2009a).

Flutianil was widely distributed throughout the tissues following oral administration and was rapidly eliminated at 10 mg/kg bw. Generally, radioactivity seemed to be associated with the organs of metabolism and excretion, and fatty tissues. Liver, after fat, was the tissue containing the highest level of radioactivity at all time points. Tissue residues were not proportional to dose, and with the exception of residues in the gastrointestinal tract, an increase in dose levels from 10 to 1000 mg/kg bw resulted in an increase in tissue residues of much less than 100-fold. Thus, absorption was saturated at the high dose level. At comparable times after dosing (24 and 48 hours) tissue concentrations of radioactivity were generally greater in rats that received fluorotolyl-labelled flutianil than in rats given methoxyphenyl-labelled flutianil. At the low dose, clearance of radioactivity from the tissues was relatively rapid although slightly greater following administration of methoxyphenyl-labelled flutianil. At the high dose many tissues and blood exhibited a relatively slow clearance, with significant levels remaining after 48 hours.

Repeated administration was less extensively investigated than single doses, but there was no evidence it resulted in a major effect on the absorption, distribution, or excretion of flutianil (Hardwick, 2009c).

Excretion was extensive; over 90% in 48 hours. Faecal elimination was the major route of excretion and accounted for 70–95% of AD, varying with label position and dose level (Hardwick, 2009a).

Samples of urine, faeces and bile from the studies described above were analysed for metabolites. In urine, no parent flutianil was detected in any group dosed, irrespective of cannulation status or label position. The major peak (called either Met 6 or Met 11, which are structural isomers) from the urine of rats dosed with fluorotolyl-labelled flutianil was identified as a mercapturate conjugate of a hydroxylated methylsulphoxy trifluoromethyl ring structure, and this accounted for up to 5.5% of AD following a single dose, and up to 11% of AD following repeated low dose administration. Several other minor metabolites were identified which resulted from cleavage of the parent compound between the methoxyphenyl and fluorotolyl ring structures. The major component in the faeces was the parent compound (94–98% of the faecal elimination) and two minor, tentative metabolites, OC 53429 and OC 53982, resulting from sequential defluorination of the parent compound, without cleavage of the two ring structures. The majority of metabolites remained unidentified. The metabolic profile of flutianil was independent of sex, single or repeated administration and of dose level (Hardwick, 2009c; Wicksted, 2012).

Toxicological data

The acute oral median lethal dose (LD_{50}) of flutianil was > 5000 mg/kg bw (Lowe, 2015a) and the dermal LD_{50} was > 5000 mg/kg bw (Lowe, 2015b). The inhalation median lethal concentration (LC_{50}) of flutianil was > 5.17 mg/L (Dreher, 2007). Flutianil was not irritating to skin but transiently irritating to eyes in rabbits (Suzuki, 2006a, b) and was not skin sensitizing in the Guinea pig maximization test (Suzuki, 2006c).

In repeated-dose toxicity studies on mice and rats, the main effects were adaptive changes in the liver. In male rats, hyaline droplets in the proximal tubular cells increased by treatment were positive for α2μ-globulin and were therefore concluded not to be relevant to humans. In dog studies, no treatment-related effects were observed.

In a 90-day dietary toxicity study in mice, flutianil was administered at dietary concentrations of 0, 1000, 3000 or 10 000 ppm (equal to 0, 138, 409 and 1387 mg/kg bw per day for males, 0, 159, 481 and 1555 mg/kg bw per day for females). The NOAEL was 10 000 ppm (equal to 1387 mg/kg bw per day), the highest dose tested (Harada, 2009b; Kitazawa, 2014a).

In a 90-day dietary toxicity study in rats, flutianil was administered at dietary concentrations of 0, 20, 200, 2000 or 20 000 ppm (equal to 0, 1.22, 12.5, 122 and 1271 mg/kg bw per day for males, 0, 1.46, 14.3, 149 and 1500 mg/kg bw per day for females). The NOAEL was 20 000 ppm (equal to 1271 mg/kg bw per day), the highest dose tested (Harada, 2009d; Kitazawa, 2014b).

In a 90-day oral toxicity study flutianil was administered to beagle dogs by capsule at dose levels of 0, 30, 300 or 1000 mg/kg bw per day. The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Hoshiya, 2009b; Ishikawa, 2014).

In a one-year oral toxicity study flutianil was administered to dogs via capsule at dose levels of 0, 30, 300 or 1000 mg/kg bw per day. The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Hoshiya, 2009a; Ishikawa, 2014).

In a 78-week carcinogenicity study in mice flutianil was administered in the diet at concentrations of 0, 1000, 3000 or 10 000 ppm (equal to 0, 106, 321 and 1084 mg/kg bw per day for males, 0, 105, 316 and 1063 mg/kg bw per day for females). The NOAEL for toxicity was 10 000 ppm (equal to 1063 mg/kg bw per day), the highest dose tested. The NOAEL for carcinogenicity was 10 000 ppm (equal to 1063 mg/kg bw per day), the highest dose tested (Harada, 2009e; Kitazawa, 2014a).

In a 104-week combined chronic toxicity and carcinogenicity study in rats, flutianil was administered in the diet to males at concentrations of 0, 60, 600, 2000 or 6000 ppm (equal to 0, 2.45, 35.2, 81.9, and 249 mg/kg bw per day), and at 0, 60, 2000, 6000 and 20 000 ppm (equal to 0, 3.15, 111, 334, and 1130 mg/kg bw per day) to females. The NOAEL for toxicity was 2000 ppm (equal to 81.9 mg/kg bw per day), based upon a slight increase in liver foci of cellular alteration in males (a similar increase was seen at 52 weeks in both eosinophilic and basophilic foci) at 6000 ppm (equal to 249 mg/kg bw per day). The NOAEL for carcinogenicity was 2000 ppm (equal to 81.9 mg/kg bw per day), based upon an equivocally increased incidence of pancreatic islet cell adenomas at 6000 ppm in males (equal to 249 mg/kg bw per day) (Harada, 2009f; Kitazawa, 2014c).

The Meeting concluded that flutianil was carcinogenic in male rats but not in mice or female rats.

Flutianil was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found.

The Meeting concluded that flutianil is unlikely to be genotoxic.

In view of the lack of genotoxicity, the absence of carcinogenicity in mice and female rats and the fact that only benign pancreatic tumours were observed at the highest dose tested in male rats, the Meeting concluded that flutianil is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation reproductive toxicity study in rats, flutianil was administered at dietary concentrations of 0, 20, 200, 2000 or 20 000 ppm (equal to 0, 9.3, 91 and 963 mg/kg bw per day in males, 0, 16.6, 171 and 1754 mg/kg bw per day in females). The NOAEL for parental effects was 2000 ppm (equal to 171 mg/kg bw per day), based upon increased absolute and relative thyroid weight in females at 20 000 ppm. The reproductive NOAEL was 2000 ppm (equal to 91 mg/kg bw per day),

based upon a slightly reduced number of implantation sites and the number of pups delivered by the F1 parental generation at 20 000 ppm. The offspring NOAEL was 20 000 ppm (equal to 963 mg/kg bw per day), the highest dose tested (Shimizu, 2009b).

In a developmental toxicity study in rats, flutianil was administered by gavage at dose levels of 0, 100, 333 or 1000 mg/kg bw per day. The maternal and fetal NOAEL was 1000 mg/kg bw per day, the highest dose tested (York, 2006b).

In a developmental toxicity study in rabbits, flutianil was administered by gavage at dose levels of 0, 100, 300 or 1000 mg/kg bw per day. The maternal and fetal NOAEL was 1000 mg/kg bw per day, the highest dose tested (Knapp, 2007b).

The Meeting concluded that flutianil is not teratogenic.

No evidence of neurotoxicity was reported in routine toxicological studies with flutianil.

The Meeting concluded that flutianil is unlikely to be neurotoxic.

In an immunotoxicity study (functional assessment of the humoral T-lymphocyte-dependent antigenic immune response, TDAR) in male rats, flutianil was administered at dietary concentrations of 0, 3250, 6500 or 13 000 ppm (equal to 0, 304, 616 and 1251 mg/kg bw per day) for 28 days. The NOAEL for systemic toxicity was 13 000 ppm (equal to 1251 mg/kg bw per day), the highest dose tested. No specific indications of immunotoxicity were noted, therefore the NOAEL for immunotoxicity was 13 000 ppm (equal to 1251 mg/kg bw per day), the highest dose tested (Moore, 2012).

The Meeting concluded that flutianil is not immunotoxic.

Toxicological data on metabolites and/or degradates

Metabolites OC 56635, OC 53276, OC 53279, OC 56574, OC 56634, Bz5 and Bz6 were identified in residues of plant and animal origin, none of them were identified, or identified at relevant amounts, in rat metabolism studies.

For metabolites OC 53279, OC 56574, OC 56634, Bz5 and Bz6, identified in plant and animal commodities, QSAR assessments for negative genotoxicity predictions were provided. Genotoxicity studies (in vitro and in vivo) were conducted with metabolites OC 56635 and OC 53276 and were negative.

An acute toxicity study in the rat with OC 63421, the sodium salt of OC 56635, revealed an LD_{50} of > 5000 mg/kg bw (Prinsen, 2009): an equivalent study with OC 56635 identified an LD_{50} of between 300 and 2000 mg/kg bw (Kim, 2008). A 28-day rat toxicity study with OC 63421, the sodium salt of OC 56635, revealed a NOAEL of 16 000 ppm (equal to 1380 mg/kg bw per day), based upon increased food consumption and reduced food conversion efficiency in both sexes, as well as slight effects on some haematological parameters observed at 50 000 ppm (equal to 4740 mg/kg bw per day) (Lina, 2009).

As regards the metabolite OC 63421 (sodium salt of OC 56635), the Meeting concluded that the acceptable daily intake (ADI) of its parent flutianil is sufficiently protective, since the NOAEL in the 28-day rat study with OC 63421 was > 1000 mg/kg bw per day. This also applies to the acid form of OC 63421, the metabolite OC 56635.

For metabolites OC 53276, OC 53279 and OC 56574, the Meeting concluded that the ADI for flutianil is considered sufficiently protective, based upon their close structural similarity to flutianil and absence of functional groups likely to confer higher biological activity.

Based on the available data, the Meeting concluded that the metabolites OC 56634, Bz5 and Bz6 could be evaluated using the threshold of toxicological concern (TTC) approach based on Cramer class III, with a threshold of 1.5 µg/kg bw per day.

Microbiological data

Flutianil has a novel mode of action against powdery mildew, which is classified under the FRAC code U 13. No information is available on mechanism and type of any antimicrobial action on the microbiome of the human gastrointestinal tract, antimicrobial spectrum of activity or antimicrobial resistance mechanisms and genetics.

Human data

No information was provided on the health of workers involved in the manufacture or use of flutianil. No information on accidental or intentional poisoning in humans was available.

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

Toxicological evaluation

The Meeting established an ADI of 0–0.8 mg/kg bw for flutianil, based on the NOAEL of 82 mg/kg bw per day in the two-year chronic toxicity and carcinogenicity study in rats, and using a safety factor of 100. The margin between the upper bound ADI and the LOAEL for an equivocal increase in pancreatic tumours in male rats was 300.

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

A toxicological monograph was prepared.

Levels relevant to risk assessment of flutianil

Species	Study	Effect	NOAEL	LOAEL
Mouse	90-day study of toxicity [a]	Toxicity	10 000 ppm, equal to 1387 mg/kg bw per day [c]	-
	Two-year study of toxicity and carcinogenicity [a]	Toxicity	10 000 ppm, equal to 1063 mg/kg bw per day [c]	-
		Carcinogenicity	10 000 ppm, equal to 1063 mg/kg bw per day [c]	-
Rat	90-day study of toxicity [a]	Toxicity	20 000 ppm, equal to 1271 mg/kg bw per day [c]	-
	Two-year study of toxicity and carcinogenicity [a]	Toxicity	2000 ppm, equal to 81.9 mg/kg bw per day	6000 ppm, equal to 249 mg/kg bw per day
		Carcinogenicity	2000 ppm, equal to 81.9 mg/kg bw per day	6000 ppm, equal to 249 mg/kg bw per day
	Two-generation study of reproductive toxicity [a]	Reproductive toxicity	2000 ppm, equal to 91 mg/kg bw per day	20 000 ppm, equal to 963 mg/kg bw per day
		Parental toxicity	2000 ppm, equal to 171 mg/kg bw per day	20 000 ppm, equal to 963 mg/kg bw per day
		Offspring toxicity	20 000 ppm, equal to 963 mg/kg bw per day [c]	-
	Developmental toxicity study [b]	Maternal toxicity	1000 mg/kg bw per day [c]	-
		Embryo and fetal toxicity	1000 mg/kg bw per day [c]	-
Rabbit	Developmental toxicity study [b]	Maternal toxicity	1000 mg/kg bw per day [c]	-
		Embryo and fetal toxicity	1000 mg/kg bw per day [c]	-
Dog	28-day, thirteen-week and one-year studies of toxicity [d, e]	Toxicity	1000 mg/kg bw per day [c]	-

Species	Study	Effect	NOAEL	LOAEL
Metabolite OC 63421 (sodium 2-fluoro-5-(trifluoromethyl)benzenesulfonate)				
Rat	Four-week study of toxicity [a]	Toxicity	16 000 ppm, equal to 1380 mg/kg bw per day	50 000 ppm, equal to 4740 mg/kg bw per day

[a] Dietary administration

[b] Gavage administration

[c] Highest dose tested

[d] Two or more studies combined

[e] Capsule administration

Acceptable daily intake (ADI) applies to flutianil, OC 56635/OC 63421, OC 53276, OC 53279 and OC 56574, expressed as flutianil

0–0.8 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 flutianil

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Moderately rapid (T_{max} 3–8 h). Poorly absorbed < 20% (based on bile and urine at 10 mg/kg bw)
Dermal absorption (*in vitro*)	8% for a 50 g/L formulation; 23% for a 0.02 g/L dilution
Distribution	Widely distributed
Potential for accumulation	Low at 10 mg/kg bw
Rate and extent of excretion	Extensively excreted, > 90% in 48 h at 10 mg/kg bw, in bile, urine and predominantly faeces (80%)
Metabolism in animals	Limited (10% of the administered dose) Cleavage at sulfur bridge, oxidation and conjugation
Toxicologically significant compounds in animals and plants	Flutianil and OC 56635, OC 63421, OC 53276, OC 53279 and OC 56574
Acute toxicity	
Rat, LD_{50}, oral	> 5000 mg/kg bw
Rat, LD_{50}, dermal	> 5000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.17 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Transiently irritating
Guinea pig, dermal sensitization	Not sensitizing (Magnussen & Kligman)
Mouse, dermal sensitization	No data
Short-term studies of toxicity	
Target/critical effect	Thyroid hypertrophy, inhalation route
Lowest relevant oral NOAEL	1000 mg/kg bw per day, highest dose tested (dog)
Lowest relevant dermal NOAEL	1000 mg/kg bw per day, highest dose tested (rat)
Lowest relevant inhalation NOAEC	0.1 mg/L (rat)

Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Liver foci and pancreatic adenoma (male rat)
Lowest relevant NOAEL	81.9 mg/kg bw per day (rat)
Carcinogenicity	Carcinogenic in male rats [a]
Genotoxicity	Unlikely to be genotoxic
Reproductive toxicity	
Target/critical effect	Increased thyroid weight in females; reduced implantation rates and pup numbers
Lowest relevant parental NOAEL	171 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	963 mg/kg bw per day, highest dose tested (rat)
Lowest relevant reproductive NOAEL	91 mg/kg bw per day (rat)
Developmental toxicity	
Target/critical effect	None
Lowest relevant maternal NOAEL	1000 mg/kg bw per day, highest dose tested (rat/rabbit)
Lowest relevant embryo/fetal NOAEL	1000 mg/kg bw per day, highest dose tested (rat/rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	No study
Subchronic neurotoxicity NOAEL	No study
Developmental neurotoxicity NOAEL	No study
Immunotoxicity	1251 mg/kg bw per day, highest dose tested (rat)
Studies on toxicologically relevant metabolites	
OC 56635	Oral LD_{50} 300–2000 mg/kg bw (rat) Unlikely to be genotoxic (negative *in vitro* and *in vivo*)
OC 63421	Oral LD_{50} > 2000 mg/kg bw (rat) Unlikely to be genotoxic (salt of OC 56635) 28-day NOAEL 1380 mg/kg bw per day, reduced body weight and haematology changes
OC 56634	Unlikely to be genotoxic (QSAR)
OC 53276	Unlikely to be genotoxic (negative in vitro and in vivo)
OC 53279	Unlikely to be genotoxic (QSAR)
OC 56574	Unlikely to be genotoxic (QSAR)
Bz5	Unlikely to be genotoxic (QSAR)
Bz6	Unlikely to be genotoxic (QSAR)
Microbiological data	No data submitted
Human data	No data submitted.

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

Summary

	Value	Study	Safety factor
ADI	0–0.8 mg/kg bw [a]	Two-year study of toxicity and carcinogenicity (rat)	100
ARfD	Unnecessary		

[a] Applies to flutianil, OC 56635 / OC 63421, OC 53276, OC 53279 and OC 56574, expressed as flutianil

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ISOPROTHIOLANE (addendum)

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Explanation

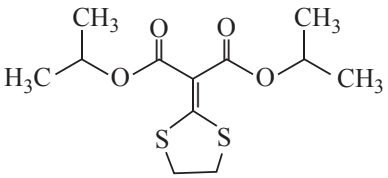
Isoprothiolane is the ISO-approved name for diisopropyl 1,3-dithiolan-2-ylidenemalonate (IUPAC), with the Chemical Abstract Service number 50512-35-1. Isoprothiolane is a systemic fungicide with protective and curative action which is used on rice crops. In bananas it is used to control black sigatoka (*Mycosphaerella fijiensis*). Isoprothiolane inhibits the penetration and elongation of infecting hyphae by inhibiting the formation of infecting peg or cellulase secretion.

Isoprothiolane was previously evaluated by the FAO/WHO Joint Meeting on Pesticide Residues (JMPR) in 2017, when an acceptable daily intake (ADI) of 0–0.1 mg/kg body weight (bw) was established, based on a no-observed-adverse-effect level (NOAEL) of 10.9 mg/kg bw per day identified in a two-year study of toxicity and carcinogenicity in rats, using a safety factor of 100. This ADI was also applicable to the isoprothiolane metabolites M-2, M-3 and M-5. The Meeting in 2017 concluded that it was unnecessary to establish an acute reference dose (ARfD).

Isoprothiolane is being evaluated by the current Meeting in support of the FAO panel review of isoprothiolane for additional maximum residue limits (MRLs) for bananas. The new information on isoprothiolane included: 28-day and 90-day oral studies of toxicity in mice, a developmental toxicity study in rabbits, in vitro mammalian cell gene mutation assay, a bacterial reverse mutation assay and in vivo micronucleus assay on isoprothiolane. Also, with respect to its metabolites: acute toxicity studies and genotoxicity studies on metabolites 4-hydroxy-isoprothiolane and isoprothiolane-monosulfoxide, and QSAR predictions for 4-hydroxy-isoprothiolane and isoprothiolane-monosulfoxide.

All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with the 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.

Figure 1. Chemical structure of isoprothiolane



Evaluation for acceptable intake

1. Biochemical aspects

No new data available.

2. Toxicological studies

2.1 Short-term studies of toxicity

(a) Oral administration

Mouse

Study 1

In a 28-day repeated-dose study, isoprothiolane (Lot No. H-003; purity 99.4%) was administered to four groups of Crl:CD-1 (ICR) mice (six mice/sex per group; four weeks of age) at dietary concentrations of 0, 500, 1500 or 5000 ppm (equal to 0, 92.2, 266 and 870 mg/kg bw per day for males, 0, 107, 347 and 1042 mg/kg bw per day for females). All animals were observed for viability / mortality, and clinical signs were recorded twice daily during the treatment. Food consumption and body weights were recorded on days 1, 4, 8, 15, 22 and 29 during treatment. Urine samples were collected from nonfasted animals, in the final week. Blood samples were taken at termination of the study for haematology and clinical chemistry. All animals were subjected to detailed postmortem gross examination. Organ weights were recorded for liver, brain, heart, spleen, kidneys, thymus, adrenals, epididymides, testes, ovaries and uterus. No histopathological examination was conducted.

No mortality or clinical signs were noted during the study. Body weight change was unaffected by treatment and food consumption was not significantly affected. There were no significant differences in any of the urinary findings. In the haematological examination, mean corpuscular haemoglobin concentration (MCHC) was significantly decreased at 5000 ppm in males (32.57 g/dL compared with 34.10 g/dL in controls; $p < 0.05$), and haemoglobin (Hb) concentration (8.1% decrease compared to control value) and haematocrit (Ht; a 6.2% decrease compared to control value) were significantly decreased at 5000 ppm in females (Table 1). For these findings, the relationship to treatment was unclear because the changes were slight and only three animals were examined. Other differences, in both sexes, were not significant or were considered unrelated to treatment as there was no dose–response relationship. Blood biochemical examination showed albumin : globulin ratio (A:G) and a particular protein fraction of albumin at 5000 ppm were significantly reduced in males, however this was considered unrelated to administrationof the test article as no significant difference was found in the total amount of albumin. Also in males at 1500 and 5000 ppm a protein fraction of α1-globulin (α1-G) was significantly increased, however this was considered unrelated to administration as no significant difference was found in other protein fractions. Other differences, in both sexes, were not significant or were considered unrelated to treatment as there was no dose–response relationship. With organ weight measurement, absolute and relative liver weight was significantly increased in males and females at 5000 ppm and, in females only, the absolute and relative heart weight were significantly reduced at 5000 ppm. These liver and heart weight findings were considered to be related to treatment. Other differences were either not

significant or considered unrelated to treatment as there was no dose–response relationship. At necropsy no abnormal findings were observed in either sex. In summary, effects were observed in organ weights in both males and females at 5000 ppm, but no effects of treatment were noted at 1500 or 500 ppm.

Table 1. Key findings of the 28-day dietary toxicity study in mice (Namiki, 2019a)

	Male				Female			
Diet concentration (ppm)	**0**	**500**	**1500**	**5000**	**0**	**500**	**1500**	**5000**
Intake (mg/kg bw per day)	**0**	**92.2**	**266**	**870**	**0**	**107**	**347**	**1042**
Haematology, n = 3								
HCT (%)	43.63	42.47	42.90	41.80	43.90	43.43	43.07	41.17*
HGB (g/dL)	14.87	14.30	14.10	13.60	14.77	14.33	14.20	13.57**
MCHC	34.10	33.67	32.87	32.57*	16.50	16.47	16.47	15.77
Organ weights, n = 6								
Liver, absolute (g)	2.0	2.1	2.2	3.3**	1.4	1.7	1.6	2.1**
Liver, relative (%)	5.9	6.1	6.6	9.2**	5.4	6.0	6.1	8.1**
Heart, absolute (mg)	168.7	168.7	166.8	169.3	145.0	136.3	142.0	118.7**
Liver, relative (mg/100 g)	498.5	480.9	490.5	478.8	547.1	494.1	546.1	463.8**

* $p < 0.05$, ** $p < 0.01$ Dunnett's multiple comparison test; Source: Namiki, 2019a

The NOAEL (without histopathological evaluation) for isoprothiolane was 1500 ppm (equal to 266 mg/kg bw per day) based on decreased absolute and relative heart weight in females seen at the lowest-observed-adverse-effect level (LOAEL) of 5000 ppm (equal to 870 mg/kg bw per day) (Namiki, 2019a).

Study 2

In a 90-day study of toxicity, isoprothiolane technical (Lot no. H-003; purity 99.4%) was administered to Crl:CD1 (ICR) mice (10 mice/sex per group; four weeks of age) in diets containing the test article at 0 (control), 200, 1000 or 5000 ppm (equal to 0, 32.3, 168 and 793 mg/kg bw per day in males, 0, 40.1, 197, and 958 mg/kg bw per day in females). All animals were observed for viability / mortality and clinical signs were recorded twice daily during the treatment. Food consumption and body weights were recorded on days 1, 4, and 8 and once a week thereafter. Ophthalmology examinations were performed on all animals before the start of administration, and on all animals in the control and high-dose groups during week 13. Urine samples were collected from nonfasted animals in the final week 13. Blood samples were taken from five mice/sex per dose at termination of the study for haematology and clinical chemistry. Functional observation battery (FOB) and motor activity assessments were performed on all surviving animals in week 13. All animals were subjected to detailed postmortem gross examination. Organ weights were recorded for liver, brain, heart, spleen, kidneys, thymus, adrenals, epididymides, testes, ovaries and uterus. A histopathological examination was performed on selected organs and tissues from the control and high-dose groups only.

No changes were noted in clinical observations, functional observations, body weight, body weight gain, food consumption, urinalysis, ophthalmology or necropsy in any test article-treated group. In the 5000 ppm group, effects were noted on haematology, biochemistry, organ weight, and histopathological examination as follows, and are highlighted in Table 2. Red blood cells (RBC), Hb and/or Ht were significantly low, and mean corpuscular volume (MCV) and/or reticulocyte count were significantly high in both sexes. Activated partial thromboplastin time (APTT) was significantly prolonged in both sexes. With respect to biochemistry, alanine aminotransferase (ALT), total globulin, and/or total protein were significantly high, or tended that way, in both sexes. Considering organ weights, absolute and relative liver weight was significantly increased in both sexes. In histopathological examination of the liver the incidence of periportal hypertrophy of hepatocytes was increased, and single cell necrosis and focal necrosis were also observed. In the 200 and 1000 ppm groups, no changes that could be related to test article administration were noted in any parameter.

Table 2. Key findings of the 90-day dietary toxicity study in mice (Namiki, 2019b)

	Male				Female			
Diet concentration (ppm)	**0**	**200**	**1000**	**5000**	**0**	**200**	**1000**	**5000**
Intake (mg/kg bw per day)	**0**	**32.2**	**168**	**793**	**0**	**40.1**	**197**	**958**
Body weight, $n = 10$[a]								
Day 1	27.96	27.96	27.88	28.03	22.59	22.53	22.46	22.66
Day 91	38.70	40.19	39.73	38.70	29.45	30.74	31.17	30 19
Food consumption (g/mouse/day), $n = 10$[a]								
Day 1	6.24	6.04	6.03	4.93**	5.39	5.11	5.12	4.47**
Day 91	5.61	5.52	5.56	5.45	5.29	5.61	5.68	5.42
Haematology, $n = 5$								
Haematocrit (%)	43.66	43.28	43.32	40.88	45.12	43.30	43.98	40.12**
Haemoglobin (g/dL)	14.44	14.36	14.22	13.38	15.00	14.64	14.38	13.02**
RBC (10^4/µl)	954.5	935.0	889.4	847.2**	927.6	881.4	877.6	820.8**
MCV (fL)	45.74	46.36	48.72*	48.28*	48.64	49.14	50.14	49.00
MCHC (g/dL)	33.08	33.18	32.82	32.74	33.22	33.82	32.70	32.44
Reticulocytes (%)	4.304	5.168	5.322**	5.482**	4.288	4.840	5.356	6.276*
APTT (s)	22.48	19.88	23.02	29.66**	23.40	24.18	24.82	30.74**
Blood chemistry, $n = 5$								
ALT (IU/L)	28.8	32.4	31.8	52.4	25.2	27.4	31.8	46.8*
Total globulin (g/dL)	77.0	111.4	129.8	172.8**	85.2	65.8	94.8	120.0
Total protein (g/dL)	4.28	4.64	4.60	4.78*	4.46	4.44	4.48	4.40
Albumin (%)	56.76	57.16	59.42	57.18	62.64	60.98	62.73	58.00*
α1-G (%)	9.94	10.60	10.14	11.82	8.10	8.44	7.85	9.58
Organ weights, $n = 10$[a]								
Liver wt, absolute (g)	2.082	2.267	2.393	3.226**	1.639	1.696	1.974*	2.639**
Liver wt, relative (%)	5.394	5.649	6.024	8.329**	5.557	5.493	6.328**	8.741**
Heart wt, absolute (mg)	194.4	188.4	187.5	176.3	146.9	157.9	160.3	151.5
Heart wt, relative (mg/100g)	502.4	468.7	472.2	455.4*	498.8	513.6	514.1	503.2
Histopathology, $n = 10$[a]								
Liver:								
periportal hypertrophy hepatocytes	0	0	0	10	0	0	0	10
Necrosis, single cell	0	0	0	4	0	0	0	1
Necrosis, focal	0	0	0	1	0	0	0	1

[a] 1000 ppm females were $n = 9$; Source: Namiki, 2019b

MCV: Mean corpuscular volume; MCHC: Mean corpuscular haemoglobin concentration;

APTT: Activated partial thromboplastin time; ALT: Alanine aminotransferase; α1-G: α1-Globulin

* $p < 0.05$, ** $p < 0.01$ Dunnett's multiple comparison test;

The NOAEL was 1000 ppm (equal to 168 mg/kg bw per day) based on changes in haematology, clinical chemistry, liver weight changes and histopathological findings in the liver seen at the LOAEL of 5000 ppm (equal to 793 mg/kg bw per day) (Namiki, 2019b).

2.2 Genotoxicity

Recent geneotoxicity studies on isoprothiolane are summarized below in Table 3.

Table 3. Genotoxicity of isoprothiolane

Test system	Test object	Concentration range	Purity	Result	Reference
		In vitro			
Bacterial reverse mutation assay	*Salmonella typhimurium* strains TA 98, TA 100, TA 1535 and TA 1537 *Escherichia coli* strain WP2*uvrA*	*Without metabolic activation:* 3.86, 11.6, 34.8, 104 and 313 µg/plate (for TA 100, TA 1535 and TA 1537) 15.4, 46.3, 139, 417 and 1250 µg/plate (for TA 98) 61.7, 185, 556, 1667 and 5000 µg/plate (for WP2 *uvrA*) *With metabolic activation:* 3.86, 11.6, 34.8, 104 and 313 µg/plate (for TA 1535) 15.4, 46.3, 139, 417 and 1250 µg/plate (for TA 98, TA 100, TA 1537 and WP2 *uvrA*)	99.4%	Negative	Tsukushi, 2019a
Gene mutation in mouse lymphoma	L5178Y mouse lymphoma cells,	*Without metabolic activation:* 15 to 130 µg/mL *With metabolic activation:* 2 to 20 µg/mL	99.4%	Negative	Munechika, 2018
		In vivo			
Mouse bone marrow micronucleus	Slc:ICR mice	Oral gavage 0, 500, 1000 and 2000 mg/kg bw	99.4%	Negative	Tsukushi, 2019b

2.3 Reproductive and developmental toxicity

(a) Developmental toxicity

Rabbit

In a prenatal developmental toxicity dose range-finding study, isoprothiolane technical (Lot no. H-003; purity 99.4%) was administered in vehicle consisting of 1.0% w/v sodium carboxymethyl cellulose (CMC) solution (low viscosity) including 0.2% v/v Tween 80. The material was given via gavage once daily to groups of six mated female New Zealand White rabbits on gestation days (GDs) 7 through 28, at dose levels of 0, 15, 80, 300 or 600 mg/kg bw per day; the dosing volume was 5 mL/kg bw). Dams were observed for mortality, clinical signs, body weights and food consumption. On GD 29, dams were sacrificed, the uterine contents examined and fetuses investigated for viability, size, and external anomalies.

In the 600 mg/kg bw per day group, two females were euthanized in extremis on GDs 19 and 23, one female was found dead, and one female aborted during GDs 19–28. The two females that were euthanized following severe body weight losses (14.1% and 17.6% loss compared to their GD 7 body weights) also showed markedly reduced food consumption (generally 0–5 g/day beginning as early as GD 8), with corresponding incidences of decreased defaecation noted at the daily examinations beginning on GD 10 and continuing until each was euthanized. At necropsy, no gross findings were noted for either female. Clinical signs were observed in two surviving females such as decreased defaecation, sporadic occurrences of brown material on the anogenital area and/or hindlimbs (throughout the treatment period) and red material on the cage floor or in the cage pan. All females in the control, 15, 80 and 300 mg/kg bw per day groups survived to scheduled necropsy on GD 29. Body weight losses or reduced mean body weight gains, lower food consumption, and corresponding incidences of decreased

defaecation were also noted in the 300 mg/kg bw per day group. In the 600 mg/kg bw per day group, mean maternal body weight losses during GDs 7–10 and 10–13, and a lower mean body weight gain during GDs 13–20 were noted, both compared to the control group. The difference on GDs 8–9 was statistically significant. In the 300 mg/kg bw per day group, a mean body weight loss during GDs 10–13 and a lower mean body weight gain during GDs 20–29 were noted, both compared to the control group; the difference on GDs 28–29 was statistically significant. In the 600 mg/kg bw per day group, lower mean food consumption was noted during GDs 7–28 compared to the control group. In 300 mg/kg bw per day group, lower mean food consumption (not statistically significant) was observed throughout the treatment period. At necropsy no remarkable gross findings were noted in any of the treatment groups.

Lower mean fetal weights were noted for the two surviving females in the 600 mg/kg bw per day group, and for females in the 300 mg/kg bw per day group. There were no observable effects at 15 or 80 mg/kg bw per day.

Based on these results, dosage levels of 30, 100, and 300 mg/kg bw per day were selected for a definitive prenatal developmental toxicity study of isoprothiolane technical administered by oral gavage to New Zealand White rabbits (Millard, 2018).

In the main prenatal developmental toxicity study, isoprothiolane technical (Lot no. H-003; purity 99.4%) was presented in a vehicle consisting of a 1.0% w/v sodium CMC solution (low viscosity) with 0.2% v/v Tween 80. This was administered via gavage once daily to mated female New Zealand White rabbits (22 per group) on GDs 7 through 28 at doses of 0, 30, 100 or 300 mg/kg bw per day. The administration volume was 5 mL/kg bw per day. Dams were observed for mortality, clinical signs, body weights and food consumption. Animals were weighed individually on GD 0 (by supplier) and GDs 4–29 (daily) and food consumption was measured daily from GD 4 to GD 29. On GD 29, dams were killed and their uterine contents examined for implantations, resorptions and viable fetuses. All fetuses were euthanised, weighed and examined for external, skeletal and visceral anomalies.

All females survived to scheduled necropsy. Test substance-related occurrences of decreased defaecation, which corresponded to reduced mean food consumption, were noted sporadically at the daily examinations for six of the 22 females in the 300 mg/kg bw per day group. No test substance-related clinical observations were noted at daily examinations for rabbits in the 30 and 100 mg/kg bw per day groups. No test substance-related clinical observations were reported at any dosage level from an examination made approximately six hours after dose administration.

Table 4. Findings of the teratogenicity study in rabbits (Millard, 2019)

		Dose level (ppm)			
		0	**30**	**100**	**300**
Dams with viable fetuses		22	22	21	21
Body weight gain[a] (g):	GD 7–10	64	76	68	–21**
	GD 10–13	75	75	72	35*
	GD 7–29	413	355	367	178**
Body weight: day on GD 29 (kg)		3.74	3.73	3.70	3.54*
Food consumption:	GD 7–10	171	173	176	135**
(g/rabbit per day)	GD 10–13	151	156	156	115**
	GD 7–29	143	143	144	110**
Fetal weight (g)		42.8	40.9	42.7	39.1**
External malformations (%/litter)		0	0	0.6	0.4
Soft tissue malformations (%/litter)		1.9	2.4	3.0	1.9
Skeletal malformations (%/litter)		0.5	0.5	0.0	1.2
Total %/litter with malformations		2.3	2.9	3.6	3.5

GD: Gestation day; * $p < 0.05$, ** $p < 0.01$ Source: Millard, 2019

[a] Mean differences calculated from individual differences. Nongravid weight(s) not included in calculation of mean

Test substance-related mean body weight losses, lower mean body weight gains, and correspondingly reduced mean maternal food consumption were noted in the 300 mg/kg bw per day group throughout the treatment period, which resulted in a slightly lower (5.1%) mean absolute body weight on GD 29 and a greater net body weight loss compared to the control group (Table 4). Mean net body weight and gravid uterine weight in the 300 mg/kg bw per day group were comparable to the control group. Mean body weights, body weight gains, food consumption and gravid uterine weights in the 30 and 100 mg/kg bw per day groups were unaffected by test substance administration.

No test substance-related macroscopic findings were noted at any dosage level.

Test substance-related lower (male 6.8%, female 8.7% and combined 8.6%) mean fetal body weights compared to the control group were noted in the 300 mg/kg bw per day group. Intrauterine growth at 30 and 100 mg/kg bw per day and intrauterine survival at 30, 100, and 300 mg/kg bw per day were unaffected by test substance administration.

There were no test substance-related fetal malformations or developmental variations noted at any dosage level.

The NOAEL for maternal toxicity was 100 mg/kg bw per day based on lower food consumption and body weight gains, body weight losses, and decreased defaecation observed at the LOAEL of 300 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 300 mg/kg bw per day, the highest dose tested. Isoprothiolane had no effects that would suggest teratogenicity at any dose level tested (Millard, 2019).

2.4 Special studies

(a) In silico studies on metabolites

Toxicity studies were submitted for isoprothiolane metabolites M-3 (4-hydroxy-isoprothiolane; diisopropyl-4-hydroxy-1,3-dithiolan-2-ylidenemalonate) and M-1 (isoprothiolane-monosulfoxide; diisopropyl-1-oxo-1,3-dithiolan-2-ylidenemalonate). Metabolite M-3 is found in rat, mouse, goat fat, rice grain, grape, apple and root crops. Metabolite M-1 is found in mouse (including its glucuronide), rice grain, grape, apple and root crops.

The toxicity of two isoprothiolane metabolites, 4-hydroxy-isoprothiolane and isoprothiolane-monosulfoxide, was compared to that of the parent substance using multiple QSAR and read-across methods. Firstly, the Organisation for Economic Co-operation and Development (OECD) Toolbox v.4.0 was utilised to identify potential areas of concern for the metabolites that are present as a result of administration of the parent to test subjects. An alert for estrogen receptor binding was identified in the case of M-3. However, due to the hydroxy substituent not being attached to an aromatic ring and the alkyl chain being branched, the potential estrogen receptor binding of the M-3 was considered to be of no concern. No additional areas of concern were identified for the M-1 when compared to isoprothiolane. Secondly, additional QSAR methods (Derek Nexus, ToxTree and CAESAR) were used to predict and compare the toxicity of isoprothiolane and the two metabolites. All three substances were considered to be out of the applicability domain of CAESAR, so the output from this model was not considered further. Derek Nexus and ToxTree gave identical predictions for all substances across all the end-points investigated.

For general toxicity, no additional areas of concern were identified for either metabolite. Therefore, the metabolites are not expected to have greater potency than toxicological studies have shown for isoprothiolane. Regarding genotoxicity, ToxTree and Derek Nexus gave negative predictions for all substances. However, because the predictions for CAESAR were considered to be “out of domain”, the overall genotoxicity of the metabolites could not be concluded. Due to the absence of hydroxy and sulfoxide functional groups in the parent molecule, read across could not be performed between isoprothiolane and its metabolites. Therefore, further genotoxicity testing of the metabolites was deemed to be required (Pellizzaro & Holmes, 2017).

(b) Acute oral toxicity of metabolites M-3 and M-1

Table 5. Results of the acute toxicity study with isoprothiolane metabolites M-3 and M-1

Route	Species	Strain	Sex	Purity	LD_{50} (mg/kg bw)	Reference
		Metabolite M-3 (4-hydroxy-isoprothiolane)				
Oral	Rat	Sprague Dawley (Crl:CD)	Female	99.5%	300–2000	Munechika, 2019a
		Metabolite M-1 (isoprothiolane-monosulfoxide)				
Oral	Rat	Sprague Dawley (Crl:CD)	Female	100%	300–2000	Munechika, 2019b

The acute oral toxicity of 4-hydroxy-isoprothiolane (Lot no. 9AC0103Z; purity 99.5%), suspended in an aqueous solution of 0.5% w/v methyl cellulose 400, was investigated in female Sprague Dawley rats (three animals per group) using an "up-and-down" method. The test substance was administered by gavage (10 mL/kg bw) to each animal at a dosage of 300 or 2000 mg/kg bw. Mortality and clinical signs were recorded during the subsequent 14 days. All animals were weighed on the day of test substance administration (day 0), days 1, 7 and the day of necropsy.

All animals died between day 1 and day 5 in the 2000 mg/kg bw group. In the 300 mg/kg group, no animals died, there were no abnormal clinical signs, no effect on body weight and no abnormal findings at necropsy. The acute oral LD_{50} for 4-hydroxy-isoprothiolane (M-3) was greater than 300 mg/kg bw and less than 2000 mg/kg bw for female rats (Munechika, 2019a).

The acute oral toxicity of monosulfoxide-isoprothiolane (Lot no. 7AC0302N; purity 100%), suspended in an aqueous solution of 0.5% w/v methyl cellulose 400, was investigated in female Sprague Dawley rats (three animals per group) using an "up-and-down" method. The test substance was administered by gavage (10 mL/kg bw) to each animal at a dosage of 300 or 2000 mg/kg bw. Mortality and clinical signs were recorded during the subsequent 14 days. All animals were weighed on the day of test substance administration (day 0), days 1, 7 and the day of necropsy.

Two animals died on day 1 in the 2000 mg/kg bw group; reddish nasal discharge was observed at 0.5 hours in the surviving animal. In the 300 mg/kg group no animals died, there were no abnormal clinical signs, no effect on body weight and no abnormal findings at necropsy. The acute oral LD_{50} for monosulfoxide-isoprothiolane (M-1) was greater than 300 mg/kg bw and less than 2000 mg/kg bw for female rats (Munechika, 2019b).

(c) Genotoxicity of metabolites M-3 and M-1

Table 6. Results of genotoxicity studies with isoprothiolane metabolites M-3 and M-1

Test system	Test object	Concentration range	Purity	Result	Reference
		Metabolite M-3 (4-hydroxy-isoprothiolane)			
Bacterial reverse mutation assay	*Salmonella typhimurium* strains TA 98, TA 100, TA1535 and TA1537 *Escherichia coli* strain WP2*uvrAp*	*With and without metabolic activation:* 0, 17, 52, 164, 512, 1600 and 5000 µg/plate (2 assays)	99.7%	Negative	Gijsbrechts, 2018a
In vitro human lymphocyte assay	Human lymphocytes	*With and without metabolic activation:* 0, 100, 500 and 600 µg/mL	99.7%	Negative	Verbaan, 2018
		Metabolite M-1 (isoprothiolane-monosulfoxide)			
Bacterial reverse mutation assay	*Salmonella typhimurium* strains TA 98, TA 100, TA1535 and TA1537 *Escherichia coli* strain WP2*uvrAp*	*With and without metabolic activation:* 0, 52, 164, 512, 1600 and 5000 µg/plate	100%	Negative	Gijsbrechts, 2018b
In vitro human lymphocyte assay	Human lymphocytes	*Without metabolic activation:* 0, 10, 50 and 90 µg/mL *With metabolic activation:* 0, 10, 90 and 110 µg/mL	100%	Negative	Buskens, 2018

3. Observations in humans

No new data available.

Comments

Biochemical aspects

No new data available.

Toxicological data

In a 28-day study of toxicity in mice, isoprothiolane was administered at dietary concentrations of 0, 500, 1500 or 5000 ppm (equal to 0, 92.2, 266 or 870 mg/kg bw per day for males, 0, 107, 347 and 1042 mg/kg bw per day for females). The NOAEL was 1500 ppm (equal to 266 mg/kg bw per day) based on decreased absolute and relative heart weight in females seen at the LOAEL of 5000 ppm (equal to 870 mg/kg bw per day) in the absence of histopathological examination (Namiki, 2019a).

In a 90-day study of toxicity in mice, isoprothiolane was administered at dietary concentrations of 0, 200, 1000 or 5000 ppm (equal to 0, 32.3, 168 and 793 mg/kg bw per day for males, 0, 40.1, 197 and 958 mg/kg bw per day for females). The NOAEL was 1000 ppm (equal to 168 mg/kg bw per day) based on changes in haematology, clinical chemistry, liver weight increases and histopathological findings in the liver seen at the LOAEL of 5000 ppm (equal to 793 mg/kg bw per day) (Namiki, 2019b).

Isoprothiolane was negative for genotoxicity in an additional bacterial reverse mutation assay (Tsukushi, 2019a), gene mutation in mouse lymphoma assay (Munechika, 2018) and an additional in vivo mouse micronucleus assay (Tsukushi, 2019b).

The Meeting confirmed the conclusion of the 2017 Meeting that isoprothiolane is unlikely to be genotoxic in vivo

In an additional developmental toxicity study in rabbits, isoprothiolane was administered by gavage at dose levels of 0, 30, 100 or 300 mg/kg bw per day. The NOAEL for maternal toxicity was 100 mg/kg bw per day based on lower food consumption and body weight gains, body weight losses, and decreased defaecation observed at the LOAEL of 300 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 300 mg/kg bw per day, the highest dose tested. Isoprothiolane was not teratogenic in rabbits in this study (Millard, 2019).

The Meeting confirmed the conclusion of the 2017 Meeting that isoprothiolane is not teratogenic.

Toxicological data on metabolites and/or degradates

M-3 (4-hydroxy-isoptothiolane)

The acute oral median lethal dose (LD_{50}) for metabolite M-3 was greater than 300 mg/kg bw and less than 2000 mg/kg bw for female rats (Munechika, 2019a). Metabolite M-3 was tested for genotoxicity in bacterial reverse mutation and in vitro human lymphocyte assays in the presence and absence of metabolic activation. It gave a negative response in both assays (Gijsbrechts, 2018a; Verbaan, 2018).

The Meeting confirmed the conclusion of the 2107 Meeting that it was unlikely that the metabolite M-3 or its conjugates would be of greater toxicity than the parent, isoprothiolane.

M-1 (isoptothiolane-monosulfoxide)

The acute oral LD_{50} for metabolite M-1 was greater than 300 mg/kg bw and less than 2000 mg/kg bw for female rats (Munechika, 2019b). Metabolite M-1 was tested for genotoxicity in bacterial reverse mutation and in vitro human lymphocyte assays in the presence and absence metabolic activation. It gave a negative response in both assays (Gijsbrechts, 2018b; Buskens, 2018).

The JMPR 2017 Meeting concluded that:

> "...these metabolites [M-3, M-5, M-2] are not of greater toxicological concern than the parent and considered that they would be covered by the acceptable daily intake (ADI) established for isoprothiolane."

The new studies on M-3 support the previous conclusion from the JMPR 2017 meeting (FAO, 2017).

Metabolite M-1 was not detected in the rat metabolism study. No repeat-dose studies were available for metabolite M-1. It was negative for mutagenicity, therefore dietary exposure to M-1 should be compared to the threshold of toxicological concern (TTC) value for Cramer class III, that is 1.5 µg/kg bw per day.

Microbiological data

No data for antimicrobial activity or impact on the human gut microbiome were available.

Human data

No new data available.

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

Toxicological evaluation (addendum)

The Meeting re-affirmed an ADI of 0–0.1 mg/kg bw on the basis of a NOAEL of 300 ppm (equal to 10.9 mg/kg bw per day) from a two-year study of toxicity and carcinogenicity in rats, based on an increase in blood urea nitrogen (BUN) in females, and an increase in the relative weight of liver and kidneys in both sexes at 3000 ppm (equal to 115 mg/kg bw per day). A safety factor of 100 was applied. Although the NOAEL of 50 ppm (equal to 3.4 mg/kg bw per day), in the 90-day oral toxicity study in rats was lower, the LOAEL in that study was based on marginal effects seen at 300 ppm (equal to 20.9 mg/kg bw per day). The Meeting therefore concluded that the NOAEL of the two-year combined toxicity/carcinogenicity study was the more appropriate value on which to establish the ADI.

The Meeting re-affirmed that it was unnecessary to establish an ARfD for isoprothiolane in view of its low acute oral toxicity and absence of developmental toxicity or any other toxicological effects likely to be elicited by a single dose. The Meeting concluded that the lower food consumption and body weight losses seen in the early phase of dosing in the developmental toxicity study in rabbits were not an appropriate basis for an ARfD because the decrease in body weight was small in relation to the rabbits' overall body weights.

The reaffirmed ADI of 0–0.1 mg/kg bw can be applied to metabolites M-2, M-3 and M-5.

Addendum to levels relevant to risk assessment of isoprothiolane

Species	Study	Effect	NOAEL	LOAEL
Mouse	90-day study of toxicity[a]	Toxicity	1000 ppm, equal to 168 mg/kg bw per day	5000 ppm, equal to 793 mg/kg bw per day[c]
Rabbit	Developmental toxicity study[b]	Maternal toxicity	100 mg/kg bw per day	300 mg/kg bw per day[c]
		Embryo/fetal toxicity	100 mg/kg bw per day	300 mg/kg bw per day[c]

[a] Dietary administration [b] Gavage administration [c] Highest dose tested

Acceptable daily intake (ADI) applies to isoprothiolane, M-2, M-3 and M-5 expressed as isoprothiolane

0–0.01 mg/kg bw

Acute reference dose (ARfD) applies to isoprothiolane, M-2, M-3 and M-5 expressed as isoprothiolane

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.

Addendum to critical end-points for setting guidance values for exposure to isoprothiolane

Short-term studies of toxicity	
Target/critical effect	Liver, red blood cells, haemoglobin, haematocrit, alanine aminotransferase and total protein
Lowest relevant oral NOAEL	168 mg/kg bw per day (mouse)
Genotoxicity	Unlikely to be genotoxic
Developmental toxicity	
Target/critical effect	Decrease in body weight gain and food consumption
Lowest relevant maternal NOAEL	100 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	100 mg/kg bw per day (rabbit)
Studies on toxicologically relevant metabolites	
M-3 (4-hydroxy-isoprothiolane)	Acute oral LD_{50}: 300–2000 mg/kg bw (rat) Not genotoxic (Ames and human lymphocytes in vitro)
M-3 (4-hydroxy-isoprothiolane)	Acute oral LD_{50}: 300–2000 mg/kg bw (rat) Not genotoxic (Ames and human lymphocytes in vitro)

Summary

	Value	Study	Safety factor
ADI	0–0.01 mg/kg bw[a]	Two-year study of toxicity and carcinogenicity (rat)	100
ARfD	Not necessary		

[a] Applies to parent, metabolites M-2, M-3 and M-5

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Mefentrifluconazole

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Explanation

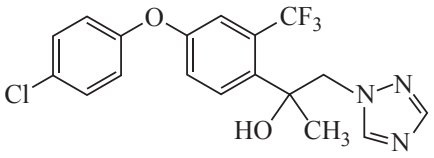
Mefentrifluconazole is the International Standards Organization (ISO)-approved common name for 2*RS*(-2-[4-(4-chlorophenoxy)-α,α,α-trifluoro-*o*-tolyl]-1-(1H-1,2,4-triazol-1-yl)propan-2-ol (IUPAC), Chemical Abstracts Service number 1417782-03-6. Chemically, mefentrifluconazole is a triazole compound. It is a racemic mixture of two (*R*- and *S*-) enantiomers. The substance is a novel fungicide that is used to control fungal diseases in various crops including cereals, oilseeds, fruits or vegetables. Its fungicidal mode of action (MOA) is by blocking ergosterol biosynthesis due to inhibition of the P450 sterol demethylase (CYP51) resulting in growth inhibition and cell membrane disruption.

Mefentrifluconazole 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 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

Figure 1. Chemical structure of mefentrifluconazole



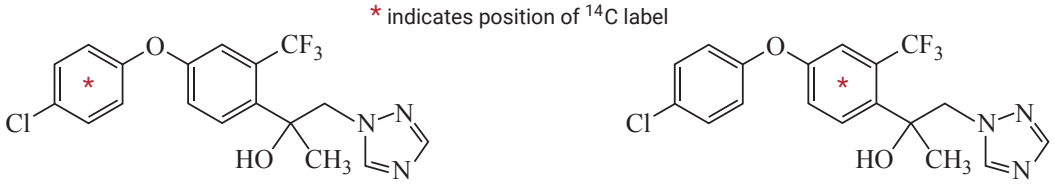
1.1 Absorption, distribution and excretion

(a) Oral route – rat

The toxicokinetics and metabolism in the rat of mefentrifluconazole were examined in a number of separate studies in which the test substance was radiolabelled at different sites in the molecule. The studies also differed with regard to their experimental design including the doses applied. They had in common that male and female Wistar rats [Crl:WI (Han)] were employed, and that the vehicle was always 0.5% carboxymethyl cellulose (CMC). The dosing volume was 10 mL/kg body weight (bw). All the analytical work in this study was performed by liquid scintillation counting (LSC).

In the first study, the test substance was either labelled in the chlorophenyl ring or in the trifluoromethyl ring (see Fig. 2) to investigate plasma kinetics, absorption, distribution, elimination, and tissue residues. Most of the individual experiments (including repeated dose administration) were performed with the chlorophenyl-labelled compound, but mass balance and biliary excretion were also examined using the trifluoromethyl label.

Figure 2. Radiolabelling of mefentrifluconazole in the chlorophenyl ring and in the trifluoromethyl ring



[*chlorophenyl*-^{14}C]mefentriflconazole
Mefentrifluconazole radiolabelled in the chlorophenyl ring

[*trifluoromethyl*-^{14}C]mefentriflconazole
Mefentrifluconazole radiolabelled in the trifluoromethyl ring

Plasma kinetics of chloromethyl-labelled mefentrifluconazole was examined in groups of three male and three female animals after a single oral gavage administration of either 5 or 180 mg/kg bw. Blood samples of 300–500 µL were taken, under anaesthesia, at 0.5, 1, 2, 4, 8, and 24 hours, and thereafter once daily until termination at 168 h after dosing. Based on the kinetic data reported below, and taking into account also the outcome of toxicological studies with repeated administration, it was decided to use the same dose levels also for subsequent mass balance, biliary excretion and tissue distribution experiments.

In the single dose, mass balance experiments, groups of four rats per sex were treated once with doses of 5 or 180 mg/kg bw chlorophenyl label or only of 180 mg/kg bw trifluoromethyl label. Urine and

faeces were collected in the intervals 0–6 h, 6–12 h, 12–24 h and subsequently in 24-hour intervals until termination after 168 h. For mass balance estimates, the cage wash was also checked for radioactivity. At termination, radioactivity was measured in the organs and tissues listed below for the tissue distribution experiments. In two of the groups, excreta had already been sampled over a 24-hour interval prior to dosing, and in some treatment groups exhalation was examined in two additional male rats for 48 hours. In a further experiment, four males and four females received pretreatment with 180 mg/kg bw per day of unlabelled mefentrifluconazole for 14 days prior to administration of a single final chlorophenyl-labelled dose. The same pattern of excreta collection was followed as in the single-dose trials.

The contribution of biliary excretion to total elimination was investigated in bile duct-cannulated rats. After recovery from surgery, at least four male and female rats per group were administered the test article at either 5 or 180 mg/kg bw (chlorophenyl label) or just at 180 mg/kg bw (trifluoromethyl label). Bile, urine, and faeces were collected in three hour intervals for the first 24 hours, in six hour intervals until 72 hours after dosing and thereafter in 12 hour intervals until termination at 168 hours. Analysis for the remaining radioactivity in the body at termination was confined to stomach and gut (both with contents) and residual carcass.

Tissue distribution was investigated with the chlorophenyl label only after single administration of either 5 or 180 mg/kg bw of test article. At different time points, three males and three females were killed and samples from a wide range of organs and tissues taken and analyzed for radioactivity. Table 1 provides an overview on sampling times for the various groups.

Table 1. Time points (hours after dosing with chlorophenyl-labelled mefentrifluconazole) at which three rats per sex and group were killed for determination of radioactivity in tissues

Dose	5 mg/kg bw	180 mg/kg bw
Male rats	1, 7, 30, 34 hours	2, 22, 38, 53 hours
Female rats	0.5, 3, 12, 24 hours	0.5, 4, 17, 24 hours

The selection of these time points was based on the results obtained from plasma kinetics. The following organs / tissues were checked for remaining radioactivity:

adipose tissue	gut and gut contents	pancreas
adrenal glands	heart	skin
blood cells and plasma	kidney	spleen
bone (left hind leg)	liver	stomach and stomach contents
bone marrow	lung	testes
brain	muscle (left hind leg)	uterus
carcass (residual)	ovaries	thyroid gland

The key toxicokinetic parameters measured are summarized in Table 2. Concentration maxima and areas under the concentration–time curve (AUC values) indicate an internal exposure that was clearly correlated to the dosing regimen. After a single low oral dose of 5 mg/kg bw, as well as after single dosing of 180 mg/kg bw, the internal dose (indicated as $AUC_{0\rightarrow\infty}$) was approximately twice as high for males compared to females. Absorption was moderately rapid and appeared a bit faster in females as also suggested by excretion data shown below.

Table 2. Toxicokinetic parameters measured in rat plasma after single oral administration of ^{14}C-chlorophenyl ring-labelled mefentrifluconazole

	Oral dose (mg/kg bw)	C_{max} (µg equiv./g)	T_{max} [h]	Initial $t_{½}$ [h]	Terminal $t_{½}$ (h)	$AUC_{0\rightarrow168}$ (µg equiv.× h/g)	$AUC\,0\rightarrow\infty$ (µg equiv.× h/g)
Males	1 × 5	2.04	1.2	7.68	85.7	34.9	39.6
	1 × 180	62.5	5.5	12.9	87.7	1650	1810
Females	1 × 5	1.67	0.5	2.56	62.1	15.7	15.3
	1 × 180	49.9	0.7	3.99	78.3	845	807

$t_{½}$: Half-life

The mass balance experiments demonstrated almost complete elimination occurring within the first three days following dosing (see Table 3) and with faeces as the major route. In contrast, urine was less important for excretion. Excretion by exhalation was negligible and little remaining radioactivity was detected in organs and tissues after seven days, with the highest relative levels of residue (exceeding those in blood or plasma) found in liver, gut contents, and carcass. This pattern of distribution and elimination was similar for male and female rats and was not fundamentally altered by dose level or by repeated administration, even though it was noted that total recovery of radioactivity, in both sexes, was lowest in the group receiving multiple doses.

Following single dose administration of trifluoromethyl-labelled test substance at a dose level of 180 mg/kg bw, urinary excretion accounted for ca 10% in both males and females. As with the chlorophenyl label, faeces was the predominant elimination route (76% in males and 85% in females over 168 hours) with the major part of excretion occurring within 72 hours. Organ and tissue residues were low. To conclude, these data suggested a high degree of similarity in the distribution and elimination behaviour of mefentrifluconazole whichever of the two different ring structures carry the radiolabel. The total recovery of radioactivity in male rats was lower with the trifluoromethyl label (86.5% of administered dose) compared to that with the chlorophenyl label (93.8%) but this is considered to reflect normal experimental variation rather than any true difference.

Table 3. Excretion balance and organ/tissue residues at termination (% of administered radioactivity) in the experiments with [chlorophenyl-^{14}C]mefentrifluconazole

Dose frequency	**1×**		**1×**		**(14 × unlabelled) + 1 × labelled**	
Dose	**5 mg/kg bw**		**180 mg/kg bw**		**180 mg/kg bw**	
Sex	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Urine 0–6 h	1.63	2.01	0.407	0.305	0.580	2.40
Urine 6–12 h	1.78	2.58	0.779	0.622	0.911	1.38
Urine 12–24 h	2.89	2.58	1.82	2.03	1.61	2.45
Urine 24–48 h	1.74	2.44	1.85	2.25	1.04	2.36
Urine 48–72 h	0.550	1.64	0.808	1.24	0.617	1.10
Urine 72–96 h	0.167	0.476	0.364	0.949	0.129	0.444
Urine 96–120 h	0.076	0.199	0.101	0.383	0.069	0.221
Urine 120–144 h	0.073	0.166	0.051	0.150	0.058	0.115
Urine 144–168 h	0.053	0.142	0.036	0.131	0.030	0.083
Subtotal urine	**8.95**	**12.2**	**6.22**	**8.06**	**5.05**	**10.6**
Cage wash (CW)	0.32	0.67	0.43	0.92	0.28	0.63
Faeces 0–6 h	0.002	1.16	0.050	0.004	0.699	2.12
Faeces 6–12 h	1.09	2.66	0.576	0.246	11.2	1.56
Faeces 12–24 h	26.2	41.4	31.0	32.0	40.5	34.1
Faeces 24–48 h	49.4	32.4	34.2	26.9	28.2	41.0
Faeces 48–72 h	9.90	7.92	16.0	16.8	4.72	7.32
Faeces 72–96 h	2.04	2.07	4.01	7.63	0.934	1.94
Faeces 96–120 h	0.600	0.820	0.953	1.20	0.277	0.529
Faeces 120–144 h	0.176	0.193	0.294	0.480	0.098	0.173
Faeces 144–168 h	0.097	0.105	0.126	0.312	0.071	0.127
Subtotal faeces	**88.9**	**87.7**	**86.9**	**85.5**	**86.3**	**80.4**
Subtotal tissues/CW	**0.741**	**0.928**	**0.702**	**1.20**	**0.472**	**0.774**
Subtotal volatiles	**0.128**	**NT**	**0.057**	**NT**	**NT**	**NT**
Total	**98.7**	**100.9**	**93.8**	**94.8**	**91.8**	**91.7**

NT: Not tested; CW: Cage wash

The experiments in bile duct-cannulated rats clearly revealed that oral absorption was much higher than suggested by the rather low amount that was excreted via urine or remained in the organs and tissues. Bile was the predominant elimination route, irrespective of dose, sex, or radiolabel. The only possible exception was the high-dose male group receiving [*chlorophenyl*-^{14}C]mefentrifluconazole in which urinary excretion was remarkably high, compared to that observed in the mass balance experiment. In sum, at the low dose, an oral absorption of ca 80% may be estimated, whereas absorption of the high dose was in the range of 60–70% suggesting some degree of saturation. Elimination via the bile was very fast, the vast majority occurring within the first 24 hours following dosing, a considerable proportion of this being almost immediate excretion within three hours of dosing. The relevant data are summarized in Table 4. As in the mass balance experiments, very little radioactivity (less than 1% in all groups) was found in the body (carcass and organs) at termination.

Table 4. Excretion in bile duct-cannulated rats (% of administered radioactivity) following a single dose of mefentrifluconazole

Radiolabel	**[*chlorophenyl*-^{14}C] label**				**[*trifluoromethyl*-^{14}C] label**	
Dose	**5 mg/kg bw**		**180 mg/kg bw**		**180 mg/kg bw**	
Sex	**Male**	**Female**	**Male**[a]	**Female**[a]	**Male**	**Female**
Urine, total, 0–168 h	10.1	18.4	34.4	10.2	11.3	10.7
Faeces, total, 0–168 h	6.8	6.4	11.4	16.7	30.7	26.7
Bile, total, 0–168 h	67.0	61.4	35.8	53.6	58.6	59.6
Bile, 0–3 h	33.3	32.7	8.4	21.7	18.5	16.6
Bile, 0–24 h	66.0	60.7	33.7	53.0	57.1	57.3

[a] Animals in these groups showed a remarkable variation that was attributed to frequently interrupted bile flow due to blocked cannulae or other cannulation problems. The figures in the table reflect mean values of the majority of animals in the respective group (that is three or four animals).

The distribution of radioactivity to the various organs and tissues over the course of time is described in Tables 5 and 6. In male and female animals concentrations generally declined in parallel to the radioactive residues in plasma. Highest values, exceeding those in plasma or blood, were determined at the end of the observation period, observed in the gut, stomach and gut contents, in the liver and sometimes also in the kidneys. With regard to the subsequent assessment of genotoxicity, it should be emphasized that radioactivity was detected in the bone marrow.

Table 5. Mean tissue concentration of radioactivity (in µg equiv./g tissue) after single oral administration of [chlorophenyl-^{14}C]mefentrifluconazole at a dose level of 5 mg/kg bw

Sex	**Males**				**Females**			
Time after dosing (h)	**1**	**7**	**20**	**34**[a]	**0.5**	**3**	**12**	**24**
Plasma	2.00	0.969	0.574	0.241	1.40	0.577	0.239	0.155
Blood	1.21	0.549	0.317	0.134	0.948	0.336	0.147	0.096
Heart	0.607	0.185	0.154	0.050	1.04	0.214	0.078	0.031
Lung	0.632	0.293	0.145	0.059	1.07	0.275	0.099	0.054
Spleen	0.329	0.105	0.054	0.026	0.630	0.138	0.040	0.021
Uterus	NA	NA	NA	NA	0.596	0.271	0.113	0.068
Pancreas	0.615	0.185	0.080	0.030	0.986	0.293	0.080	0.045
Adipose tissue	0.267	0.221	0.118	0.042	0.373	0.417	0.165	0.059
Muscle	0.169	0.068	0.033	0.015	0.307	0.086	0.024	0.016
Testes / ovaries	0.232	0.193	0.118	0.041	1.04	0.287	0.106	0.052
Adrenals	1.31	0.298	0.120	0.041	3.32	0.535	0.155	0.057
Thyroid	0.349	0.154	0.065	0.031	0.674	0.109	0.040	0.024

Sex	Males				Females			
Time after dosing (h)	1	7	20	34[a]	0.5	3	12	24
Bone marrow	0.310	0.097	0.047	0.022	0.383	0.060	0.020	0.012
Stomach	22.4	1.83	0.347	0.041	15.6	4.46	0.313	0.132
Liver	12.7	5.60	2.47	1.03	9.72	4.16	2.11	1.29
Brain	0.256	0.042	0.012	0.005	0.777	0.090	0.014	0.006
Kidney	1.52	1.10	0.433	0.189	1.93	0.683	0.399	0.234
Carcass	0.192	0.118	0.056	0.025	0.284	0.139	0.053	0.044
Skin	0.124	0.116	0.064	0.028	0.153	0.104	0.036	0.021
Gut	4.80	4.61	1.84	0.626	7.39	4.71	2.45	1.83
Bone	0.102	0.051	0.028	0.014	0.118	0.049	0.020	0.011
Stomach content	413.9	18.8	0.707	0.128	164.8	49.6	3.03	3.23
Gut content	26.1	99.6	53.3	15.2	26.6	68.0	83.5	46.0

[a] One animal excluded from mean (dosing not adequate)

Table 6. Mean tissue concentration of radioactivity (in µg equiv./g tissue) after single oral administration of [chlorophenyl-^{14}C]mefentrifluconazole at a dose level of 180 mg/kg bw

Sex	Males				Females			
Time after dosing (h)	2	22	38	53	0.5	4	17	24
Plasma	58.8	22.1	11.2	11.2	57.0	23.1	7.71	5.50
Blood	42.7	12.9	5.88	6.94	35.1	12.1	4.54	3.20
Heart	42.0	6.39	2.64	2.78	47.7	18.3	3.05	1.69
Lung	41.2	5.49	2.40	2.73	44.0	18.1	2.98	1.95
Spleen	23.9	2.63	1.02	1.12	27.9	11.2	1.24	0.840
Uterus	NA	NA	NA	NA	23.0	13.2	3.36	2.32
Pancreas	61.6	5.60	2.12	1.81	64.0	29.5	3.04	1.70
Adipose tissue	19.0	6.24	0.570	0.702	20.5	66.6	6.09	3.66
Muscle	64.9	16.6	2.64	4.35	17.8	8.82	0.795	0.573
Testes / Ovaries	23.3	5.19	2.15	1.90	46.3	21.6	3.97	2.24
Adrenals	99.0	9.32	2.41	2.68	136.0	52.1	6.59	3.59
Thyroid	40.9	9.03	3.16	2.92	50.0	16.8	20.0	2.86
Bone marrow	26.3	4.15	1.31	1.60	29.6	10.7	3.15	0.818
Stomach	322.8	5.72	9.08	4.86	683.3	385.7	18.1	20.4
Liver	207.2	93.1	16.1	21.0	212.4	84.8	39.4	30.1
Brain	29.7	0.976	0.229	0.235	39.6	13.5	0.638	0.277
Kidney	51.7	12.1	5.96	6.31	62.8	25.4	6.78	4.99
Carcass	12.0	2.50	0.873	1.41	8.87	4.79	1.71	1.21
Skin	15.0	2.62	0.882	1.27	5.58	3.18	1.12	0.836
Gut	178.2	83.7	17.8	24.6	119.8	123.4	115.6	78.7
Bone	8.70	1.39	0.565	0.784	7.93	3.33	1.41	0.864
Stomach content	1940.8	33.6	9.24	14.8	10657.4	1678.3	61.1	30.7
Gut content	2020.1	1243.1	346.5	330.4	1535.2	1744.6	1340.1	1134.5

To conclude, radiolabelled mefentrifluconazole was to a large extent absorbed, and rapidly so; it was widely distributed and nearly completely eliminated, mainly via bile and faeces. Applied dose, site of radiolabelling in the molecule and pre-treatment with unlabelled test substance had no major impact on its toxicokinetic parameters, apart from a slightly lower oral absorption at the high-dose level.

Longer half-lives and a greater AUC were seen in male rats compared with females, and this might indicate some saturation of excretion, at least in males. Highest residue levels were found in gut, liver, stomach, and, in particular, in gut and stomach contents. No evidence of accumulation was observed. (Wenker, 2015).

In a second study, mefentrifluconazole had been radiolabelled in the triazole moiety on either the 3- or the 5-position (see Fig. 3). As in the first study, plasma kinetics, mass balance, biliary excretion and tissue residues were examined in a number of separate experiments. The study design was similar but a slightly expanded by including additional (and also higher) oral doses, and introducing intravenous (i.v.) application into the plasma kinetics part of the experimental plan. In addition, a larger number of animals were used and the study itself was carried out in a different laboratory.

The plasma kinetics of triazole ring-labelled mefentrifluconazole was examined in groups of four male and four female rats after single oral gavage administration of 5, 40, 120, or 360 mg/kg bw of labelled test article. Blood samples of 100–200 µL were taken under anaesthesia from the retro-orbital sinus directly after administration and then at 1, 2, 4, 8, and 24 hours, thereafter once daily until termination at 168 hours after dosing. In addition, a further group of six male and six female rats received an i.v. dose (dosing volume 1 mL/kg bw) of 0.4 mg/kg bw. From these animals, blood was taken directly after administration, then at 0.5, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144 and 168 hours after dosing.

Figure 3. Radiolabelling sites in the triazole moiety of mefentrifluconazole

* indicates position of ^{14}C label

In the single dose, mass balance experiments, groups of four rats per sex received doses of 5 or 180 mg/kg bw. Urine was collected in the intervals 0–6 h, 6–12 h, 12–24 h and subsequently in 24-hour intervals until termination after 168 hours. For balance estimates, the cage wash was also checked for radioactivity. Faeces were collected in 24-hour intervals until termination. In two males from the low-dose group exhalation was monitored for 48 hours. At termination, radioactivity was measured in the same organs and tissues as in the first study (Wenker, 2015, see above). In the repeat dose experiment four males and four females received pretreatment of unlabelled mefentrifluconazole at 180 mg/kg bw per day for 14 days prior to administration of a single final dose that had been radiolabelled in the triazole moiety. The same pattern of excreta collection as in the single-dose trials was followed.

Biliary excretion was investigated in a total of six male and 10 female bile duct-cannulated rats, at the low-dose level, once again, of 5 mg/kg bw. A high dose of 180 mg/kg bw was applied, after recovery from surgery, to 11 male and six female rats. Bile was sampled in 3-hour intervals until termination at 72 h after dosing. Urine and faeces were collected in 24-hour intervals. Bile, excreta, cage wash, stomach and gut (both with contents) and residual carcass were examined for radioactivity.

Tissue distribution was investigated at four different time points after a single dose of either 5 or 180 mg/kg bw, each administered to three male and three female rats. On the basis of preceding examination of plasma kinetics these time points were selected to cover maximum plasma concentration, one-half, one-quarter, and one-eight of the maximum (see Tables 9 and 10). The same organs and tissues as in the previous study by Wenker (2015) were taken and analyzed for radioactivity.

Based on the plasmakinetic parameters, a rapid and nearly complete absorption can be assumed when the results after oral low dose (5 mg/kg bw) and intravenous (0.4 mg/kg bw) administration are compared (Table 7). With regard to the higher oral doses, a more or less linear correlation with internal exposure became apparent when the AUC is considered. However, with regard to C_{max} no further increase was observed when doses of 120 and 360 mg/kg bw were compared. This finding might suggest a decrease in the absorption rate after administration of high doses, confirming what is known from investigations of many other pesticides. The finding agrees with results of the mass balance experiments reported below.

Table 7. Toxicokinetic parameters measured in rat plasma after single oral or i.v. administration of [triazole-^{14}C]mefentrifluconazole

Dose (mg/kg bw)	Route	C_{max} (µg equiv./g)*		T_{max} (h)*		Half-life (h)		AUC (µg equiv. × h/g)	
		M	F	M	F	M	F	M	F
0.4	i.v.	1.35	1.17	-	-	12.46	10.03	3.6	1.3
5	Oral	3.04	2.07	1	1	43.83	34.11	38	17
40	Oral	23.08	13.77	1	1	20.43	41.94	296	119
120	Oral	53.73	34.37	1	1	17.39	58.38	886	467
(2nd peak)			20.41		8				
360	Oral	57.08	20.32	1	1	30.05	38.72	2629	1148
(2nd peak)		55.96	29.19	24	8				

M: Male F: Female

* For some dose groups and individual animals, two maxima were observed and attributed by the study authors to potential enterohepatic circulation.

Mass balance experiments with mefentrifluconazole labelled in the triazole moiety revealed a higher urinary excretion than with the labels in the chlorophenyl or trifluoromethyl positions, in particular in males of the single low-dose group, but faeces were still the major route of elimination. Excretion via exhalation was negligible and in nearly all groups organ/tissue residues accounted for less than 0.5% of the applied radioactivity, with the highest values measured in skin, liver, and carcass (Table 8). The majority of excretion had alrady occurred within 48 to 72 hours of dosing, and repeated administration did not alter the elimination pattern.

Table 8. Excretion in rats (% of administered radioactivity) following administration of [triazole-^{14}C]mefentrifluconazole (figures are rounded)

Administration	Single dose				Repeated dose (pretreatment with unlabelled test material)	
Dose	5 mg/kg bw		180 mg/kg bw		180 mg/kg bw	
Sex	Male	Female	Male	Female	Male	Female
Urine, total, 0–168 h	41	15	19	11	23	17
Faeces, total, 0–168 h	59	87	81	90	74	83
Cage wash	0.6	0.2	0.1	0.6	0.5	0.3
Tissues	1.2	0.4	0.2	0.2	0.3	0.3

As with the chlorophenyl or trifluoromethyl labels, biliary excretion contributed a lot to subsequent elimination via the faeces. In the groups receiving the low dose of 5 mg/kg bw, excretion via bile was found to account for 71% and 74% of the administered radioactivity in males and females, respectively, with the major part already excreted within 24 hours of dosing. In contrast, in the same dose groups total excretion of radioactivity via the urine after 72 hours was only 11% in males and 10% in females. At a dose level of 180 mg/kg bw mean excretion via bile was 42% and 46% of the administered radioactivity in males and females, respectively. Mean total excretion of radioactivity via urine after 72 hours was 7% for males and 11% for females. When these rates are summed up, oral absorption rates of more than 80% for the low dose and 50–60% for the high dose may be assumed. The first estimate is of similar magnitude to that found with chlorophenyl or trifluoromethyl labels, whereas at the high dose absorption was around 10% lower with the triazole label. Total body residues in this experiment (mostly in carcass or gut contents) were in the order of 1% of the applied radioactivity in all groups.

Radioactive organ and tissue residues over the course of time are shown in Tables 9 and 10 for the low and high doses, respectively. The distribution agreed well with the information obtained in the first study with a chlorpphenyl label (Wenker, 2015). Apart from stomach and gut and their contents, highest residues were found in the liver. For all organs and tissues a decline with time was seen.

Table 9. Mean tissue concentration of radioactivity (in µg equiv./g tissue) after single oral administration of [triazole-^{14}C]mefentrifluconazole at a dose level of 5 mg/kg bw

	Mean tissue concentration [µg equiv./g]							
	Males				Females			
	C_{max}	$C_{max}/2$	$C_{max}/4$	$C_{max}/8$	C_{max}	$C_{max}/2$	$C_{max}/4$	$C_{max}/8$
Time after dosing	**1 h**	**4 h**	**18 h**	**28 h**	**1 h**	**2 h**	**4 h**	**24 h**
Blood cells	0.88	0.55	0.33	0.20	0.30	0.12	0.14	0.04
Plasma	2.99	1.20	0.42	0.29	1.53	0.48	0.42	0.09
Lung	1.47	0.85	0.41	0.29	1.01	0.44	0.37	0.11
Heart	1.29	0.67	0.36	0.25	0.87	0.34	0.27	0.07
Spleen	0.76	0.61	0.40	0.26	0.55	0.36	0.32	0.08
Kidney	1.98	1.78	0.54	0.49	1.66	0.89	0.84	0.26
Adrenal glands	4.68	1.63	0.64	0.50	3.72	1.47	0.86	0.18
Testes/ovaries	0.57	0.70	0.40	0.26	1.06	2.01	0.82	0.11
Uterus	-	-	-	-	0.64	1.50	0.76	0.14
Muscle	0.58	0.61	0.38	0.26	0.41	0.22	0.20	0.07
Brain	0.72	0.53	0.33	0.22	0.59	0.26	0.19	0.06
Adipose tissue	0.62	0.36	0.11	0.05	0.54	0.70	0.96	0.17
Bone	0.32	0.29	0.16	0.12	0.16	0.09	0.08	0.02
Bone marrow	1.07	0.79	0.48	0.34	0.97	0.39	0.34	0.10
Thyroid	2.71	1.42	0.84	0.68	2.53	0.84	0.64	0.14
Pancreas	1.36	0.74	0.53	0.24	1.33	1.37	1.09	0.09
Stomach content	131.04	78.59	1.16	2.41	94.05	71.23	41.77	0.28
Stomach	14.23	10.87	0.96	0.67	19.73	15.47	6.23	0.30
Gut content	43.59	49.46	13.02	8.05	34.33	32.87	63.74	11.88
Gut	6.42	7.42	2.97	1.00	15.48	23.05	12.18	2.08
Liver	16.35	6.74	1.64	1.07	9.13	4.24	4.14	0.68
Skin	0.69	0.57	0.39	0.27	0.48	0.31	0.23	0.07
Carcass	0.67	0.55	0.36	0.24	0.52	0.55	0.37	0.10

C_{max}: Maximum concentration

Table 10. Mean tissue concentration of radioactivity (in µg equiv./g tissue) after single oral administration of [triazole-^{14}C]mefentrifluconazole at a dose level of 180 mg/kg bw

	Mean tissue concentration [µg equiv./g]							
	Males				Females			
	C_{max}	$C_{max}/2$	$C_{max}/4$	$C_{max}/8$	C_{max}	$C_{max}/2$	$C_{max}/4$	$C_{max}/8$
Time after administration	**1 h**	**24 h**	**36 h**	**48 h**	**1 h**	**8 h**	**24 h**	**34 h**
Blood cells	21.60	13.61	9.16	8.98	17.10	8.40	4.04	2.57
Plasma	70.96	18.03	11.20	10.43	52.86	17.32	6.93	2.85
Lung	47.87	15.63	10.10	11.21	70.49	20.16	6.24	3.15
Heart	45.94	14.37	9.17	18.55	48.22	18.09	5.13	2.30
Spleen	31.44	15.03	9.98	5.26	34.78	13.35	5.59	2.49
Kidney	65.36	22.06	13.37	13.54	69.81	26.98	8.90	4.13
Adrenal glands	123.94	26.44	12.71	13.15	144.30	52.10	11.04	4.70
Testes/ovaries	22.00	14.45	9.46	9.61	52.19	25.67	7.89	2.34
Uterus	-	-	-	-	31.22	16.63	7.88	2.79

	Mean tissue concentration [µg equiv./g]							
	Males				Females			
	C_{max}	$C_{max}/2$	$C_{max}/4$	$C_{max}/8$	C_{max}	$C_{max}/2$	$C_{max}/4$	$C_{max}/8$
Time after administration	**1 h**	**24 h**	**36 h**	**48 h**	**1 h**	**8 h**	**24 h**	**34 h**
Muscle	22.69	13.67	8.88	8.85	23.06	9.62	4.35	2.15
Brain	37.38	12.61	8.27	8.70	47.77	15.09	3.59	1.86
Adipose tissue	22.82	3.44	1.46	3.88	40.50	44.88	2.83	1.18
Bone	6.99	5.46	2.33	3.23	8.09	2.82	1.92	0.63
Bone marrow	35.12	18.65	10.73	11.25	34.22	13.20	5.45	3.15
Thyroid	70.82	36.53	16.11	17.07	74.52	29.11	25.12	5.76
Pancreas	59.52	14.30	9.18	9.60	76.61	25.21	6.91	2.58
Stomach content	6558.92	52.53	54.38	38.23	4056.53	725.01	22.33	16.68
Stomach	671.23	33.77	19.33	22.86	1373.12	95.21	17.38	6.01
Gut content	1349.46	909.28	572.58	185.46	1273.02	2395.29	978.73	550.58
Gut	201.79	104.52	59.29	24.84	399.59	178.25	139.13	38.93
Liver	267.46	41.53	29.38	17.11	221.46	91.19	29.87	13.83
Skin	21.49	14.28	11.65	12.61	34.50	14.38	4.32	2.56
Carcass	24.87	15.86	12.08	18.24	28.59	14.99	6.45	3.64

C_{max}: Maximum concentration

It may be concluded that mefentrifluconazole, also when radiolabelled in the triazole ring, is rapidly absorbed from the gastrointestinal tract (GIT), is widely distributed and eliminated fast, mainly via bile and faeces. The highest residues are found in gut and stomach and their contents, and in the liver. No evidence of accumulation was observed (Fabian & Landsiedel, 2016).

For the in vivo part of the study, all experiments were carried out in parallel with radiolabelling in the chlorophenyl ring and in the triazole moiety of mefentrifluconazole (see Figs 2 and 3). An overview on the study design is provided in Table 11 suggesting that the investigations were complimentary to those in the two other studies reported above.

Table 11. Treatment groups in the in-life phase of a metabolism study with chlorophenyl-labelled and triazole-labelled mefentriflucozole (single oral administration)

Group code	Dose (mg/kg bw)	Animals per label	Termination	Objectives
VM	5	4 males	One hour post dosing; (at or near the presumed maximum plasma concentration)	Sampling of liver, kidney, muscle, fat, blood/plasma, and carcass; Determination of metabolite pattern
VF	5	4 females		
WM	180	4 males		
WF	180	4 females		
DXM	180	10 males	Seven days post dosing	Excretion balance; Metabolites in urine and faeces
DXF	180	10 females		

The results of metabolite analysis are summarized in the following subsection. Sampling of urine and faeces in the groups DXM and DXF took place in the following intervals: 0–6 h, 6–12 h, 12–24 h and thereafter in one-day intervals until termination at 168 h for females or 170 h for males; all times post dose. With the triazole label, excretion was nearly complete after 72 h (>90%) with more than the half of applied radioactivity already excreted within 48 h. With the chlorophenyl label, elimination was more gradual, reaching 83% of total radioactivity at termination in males and 78% in females. As seen in the previously reported studies, faeces were the major route of elimination accounting for 69–83% of the dose whereas urinary excretion was rather limited at 4–14% of administered dose (Birk, Bogen & Doebbe, 2016).

(b) Oral route – mouse

The plasma kinetics of mefentrifluconazole were also investigated in the mouse. Groups of four male and four female C57BL/6JRj mice were dosed once, orally by gavage at target dose levels of 10, 50 and 75 mg/kg bw. The test item was radiolabelled in the triazole moiety. Blood samples were taken before dosing and at 0.5, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144 and 168 hours after test substance administration. Blood was separated into plasma and blood cells and radioactivity determined in each sample.

In the male group receiving the high dose, three out of four animals died within the 48 hours following substance application. According to the study authors, these deaths were not attributable to mefentrifluconazole but to inappropriate sampling procedures since incidental bleeding and blood loss had been reported after the scheduled samplings. This may have resulted in dehydration and anaemia. Even though this explanation is plausible, it is surprising that only high-dose males died whereas the difficulties with blood sampling also occurred with a few other animals from the low- and mid-dose groups. In an additional experiment, four replacement males were given the same high dose of 75 mg/kg bw. With these animals, blood was sampled after three hours but not after two or four hours; the other sampling times were the same as in the main study groups. All the replacement mice survived, as did all the other animals in the study.

The maximum plasma concentrations (C_{max}) showed a clear dose dependency with a sublinear C_{max} – dose relationship. At the high dose in male animals, C_{max} values of comparable magnitude were obtained at sampling time points of 0.5, 3 and 8 h post dosing; for female animals C_{max} values were 21.48 and 26.62 µg equiv./g at sampling time points of 0.5 and 8 h post dosing. At the mid-dose, C_{max} values were observed as 19.78 and 19.18 µg equiv./g, as well as 17.24 µg equiv./g for males and females; after dosing these peaks occurred after 1 h in males, and after 8 h in females, and again for males. In the low dose in males, C_{max} values were 5.66 µg equiv./g and occurred 8 h post dosing, whereas in females C_{max} values of 3.98 and 5.31 µg equiv./g were reached 1 h and 4 h post dosing. The observation that more than one C_{max} value was present in the high-dose groups of males and females, in the mid-dose group of males as well as in the low-dose group of females might indicate a potential for enterohepatic (re)circulation of the test substance and/or its metabolites.

Terminal half-lives were 80.4 h and 54.2 h in low-dose males and females, respectively. For mid-dose males and females, half-lives of 65.2 h and 40.1 h were calculated, whereas the respective values for high-dose animals were 31.8 h and 34.6 h.

At least for the low dose levels of 5 mg/kg bw (rat) or 10 mg/kg bw (mouse), the toxicokinetic parameters in both species were of similar magnitude (see Table 2 above for comparison).

The calculated AUC values in male and female mice were 151, 694 and 958 µg equiv. × hour/g as well as 127 478 and 1012 µg equiv. × hour/g for target doses of 10, 50 and 75 mg/kg bw, respectively. If the AUC : dose ratios at the higher dose levels are compared to those calculated for the low-dose experiments, the internal doses were clearly correlated to the administered oral doses, but some saturation of absorption became apparent at the maximum dose level of 75 mg/kg bw. Nonetheless, it may be concluded from this study that relatively high concentrations of mefentrifluconazole will be present in the blood following oral administration of high doses to mice. This might be useful in the interpretation of in vivo genotoxicity studies, in particular micronucleus assays, in which exposure of the target tissue is often of concern (Fabian & Landsiedel, 2014).

It must be emphasized that plasma concentrations of mefentrifluconazole were also determined as part of routine short-term toxicity studies in rats, mice and dogs. The results are reported below in the relevant subsections. It can be concluded that, whilst findings from the toxicity studies do not, at least, contradict the observations from the specific absorption, distribution, metabolism and excretion (ADME) studies they cannot be taken into consideration regarding metabolism since metabolites were not measured in such studies.

1.2 Biotransformation

The metabolism of mefentrifluconazole in the rat was investigated based on samples obtained in the two aforementioned separate toxicokinetic studies in which three different radiolabels had been used (Wenker, 2015; Fabian & Landsiedel, 2016) but also from the in vivo part of the metabolism study as reported above (Birk, Bogen & Doebbe, 2016). The parent compound and its metabolites were identified by means of tandem high-performance liquid chromatography/mass spectrometry (HPLC–MS/MS) and nuclear magnetic resonance (NMR) analysis. Targets of study were urine, faeces, plasma, tissue samples and bile of animals from different study groups that had received either 5 or 180 mg/kg bw. Confirmatory HPLC–MS was used as a second system for peak assignment. In addition, co-chromatography and comparison of peak patterns were performed.

Metabolism of mefentrifluconazole is extensive (see Annex 1). A total of 68 metabolites were identified in the rat, resulting from both phase I and phase II reactions, along with unchanged parent. As to be expected for such a large number of metabolites, their individual quantitative abundance was low in most groups and matrices, with the exceptions mentioned in the following paragraphs. Metabolic phase I reactions comprised hydroxylation (often accompanied by a chlorine shift within the chlorophenyl ring), methylation, cleavage of the ether group, and cleavage of the triazole ring from the parent molecule. In phase II, metabolites resulting from phase I became subject to conjugation by sulfatation, glucuronidation, or glutathione (GSH) adduction. For visualisation of metabolic pathways of mefentrifluconazole, the applicant provided a number of figures which are shown in Annex 1.

In urine, there was not much difference due to radiolabel, sex or dose. The most abundant urinary metabolite was 1,2,4-triazole (M750F001) accounting for up to 10.5% of the dose in male rats receiving 180 mg/kg bw. In general this metabolite was more abundant in males than in females. Metabolite M750F001 results from cleavage of the triazole ring from its parent mefentrifluconazole. Common minor metabolites in urine were M750F049, M750F050, M750F054, M750F058, M750F071, and M750F081.

In faeces, the parent compound was found in widely differing amounts, ranging from 3 to 35% of the administered dose (AD). The most abundant metabolites were M750F015 (10–41%) and M750F016 /M750F017 (given as sum), which accounted for 15 to 32% of the dose in the various studies and experimental groups. Metabolite M750F003 was found as a minor component in the experiments with triazole or trifluoromethyl labels. Apart from this, neither radiolabel nor sex influenced the metabolite pattern. The metabolites in faeces result from either hydroxylation or, in the case of M750F003, from cleavage of the ether group. The contribution of intestinal bacteria to formation of these faecal metabolites is not known.

In bile, the main metabolites (M750F035, M750F044, M750F045, M750F049, and M750F087) which together account for 22–53% of total AD, are all glucuronides which have been formed subsequent to previous hydroxylation of the parent. Their main precursors (the hydroxy metabolites M750F015, M750F016 and M750F017) were found in bile too, but at amounts between 55 and 12% in the various groups. Again, sex or position of the radiolabel seemed to have little influence on the results.

In tissues (mainly in the liver) and plasma, the parent compound and its primary hydroxylated metabolites were identified, along with minor amounts of glucuronidated or sulfated hydroxylation products. It was noted that the relative ratio of the two isomers of mefentrifluconazole in faeces, remained at 1:1, the same as in the applied doses. In methanolic extracts of liver and kidney, as well as in the plasma, this isomer ratio shifted towards a larger proportion of the R-enantiomer, for reasons that remain unclear (Birk, Bogen & Doebbe, 2016).

A comparative study to investigate the metabolism of mefentrifluconazole in vitro was conducted in human, rat and mouse hepatocytes. The test substance was radiolabelled in the chlorophenyl ring, in the trifluoromethylphenyl ring, or in the triazole moiety. Stock solutions of the test article were made up with acetonitrile. Cryopreserved hepatocytes of different species origin (mixed by sex) were purchased from commercial suppliers and thawed before experimental use. Cells of proven viability were incubated with radiolabelled test material at a final concentration of 1 µM in dimethyl sulfoxide (DMSO) for 10, 30, 60 or 180 minutes before the reaction was stopped by addition of ice-cold ethanol. The specific concentration had been chosen because it offered the highest cell viability (up to 87%). Supernatants were analyzed for radioactivity and abundance of metabolites by LSC and HPLC. Pellet extracts were also analyzed. As reference materials ^{14}C-labelled testosterone and ^{14}C-labelled ethoxycoumarin were

used, of which stock solutions were prepared in either ethanol or DMSO. These control substances were applied to prove metabolic activity of the different hepatocyte batches and gave the expected results.

In contrast to the extensive metabolism of mefentrifluconazole seen in vivo, the number of metabolites observed in vitro was very limited. In human as well as rat hepatocytes only a single metabolite was detected, along with the parent compound. This metabolite had its peak at 7.6 minutes, (m/z = 590), and was the same in both species. So no unique human metabolite was identified in this study. However, metabolism in rat hepatocytes was faster or more pronounced since decreasing amounts of parent mefentrifluconazole were observed after incubation periods of 10, 30, or 60 minutes until its complete disappearance after 180 minutes, at which point metabolism appeared to be complete. The sole metabolite was already present after 10 minutes and the percentage of applied radioactivity that could be allocated to its peak consistently increased. In human hepatocytes, by contrast, the majority of radioactivity was represented by the parent compound at all time points and regardless of which radiolabel was employed. The metabolite was detected for the first time after the longest incubation time (180 minutes), at levels with respect to applied radioactivity ranging from 18.71% with the chlorophenyl label to 21.47% with the trifluoromethyl label.

At the request of JMPR, the sponsor made efforts to better characterize this metabolite and to compare it to the metabolites found in the rat in vivo studies. Because of its very similar and accurately measured mass of 590, it can be reasonably assumed that this human and rat in vitro metabolite was identical to one of three of the bile metabolites (either M750F035, M750F044 or M750F045) the structures of which are depicted in Part 1 of Annex 1. These three in vivo rat metabolites are all glucuronides. Prior to glucuronidation they had all been hydroxylated at different positions in the chlorophenyl ring. They are not only structurally very similar to each other but also had an unlabelled nominal mass of 588.1, equivalent to the measured mass of the in vitro metabolite which was 590.11.

No metabolism was observed in mouse hepatocytes, suggesting that human metabolism in vitro more closely resembles that of the rat, albeit less efficient. However, because of the very limited metabolism in vitro, no firm conclusion can be drawn. It must be emphasized that the fewer metabolites seen in such an in vitro study, compared with in vivo studies, is not entirely unusual. The difference can be attributed to the artificial system and also to the different chromatographic conditions which could have an influence on peak pattern and resolution. Positive controls included in the study showed that the test system, in principle, worked and the study design was appropriate (Funk, Wotske & Glaessgen, 2016).

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

In an acute oral toxicity study carried out according to the "Acute toxic class" method, mefentrifluconazole (Batch no. COD-001740; purity 98.8%) was applied to young adult female Wistar [Crl:WI(Han) SPF] rats. In a first step, a limit dose of 2000 mg/kg bw was administered by gavage to three animals. The test article had been dissolved in corn oil and the dosing volume was 5 mL/kg bw. Since no mortality occurred, treatment at the same dose level was repeated in a second group of same size.

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 just before the animals were killed and subjected to gross examination.

All rats survived until study termination. However, there were clinical signs noted from two hours after dosing onwards in four of the six animals; this lasted for another one to three hours. These signs comprised cowering position, piloerection and the overall poor condition of the animals. However, all animals gained weight over the course of the study and there were no remarkable gross pathological findings in any rat.

In this study, mefentrifluconazole was of low acute oral toxicity. The median lethal dose (LD_{50}) was greater than 2000 mg/kg bw (Hoeger, Becker & Kamp, 2013a).

In an acute dermal toxicity study (limit test) mefentrifluconazole (Batch no. COD-001740; purity 98.8%), as a suspension in corn oil, was applied to the clipped skin of five male and five female Wistar [Crl:WI (Han) SPF] rats at a dose level of 5000 mg/kg bw and covered by a semi-occlusive dressing for 24 hours.

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, just before the animals were killed and subjected to gross examination.

All rats survived until study termination. There were neither signs of systemic toxicity nor of local irritation. As often seen in dermal toxicity studies, body weight tended to stagnate during the first week following treatment in female rats, but thereafter all animals gained weight again. There was no effect on the body weight of male rats. At necropsy, there were no remarkable gross pathological findings in any rat.

In this study, mefentrifluconazole was of low acute dermal toxicity. In both sexes, the LD_{50} was greater than 5000 mg/kg bw (Hoeger, Becker & Kamp, 2013b).

An acute inhalation toxicity study was performed, as a limit test, in five male and five female Wistar (RccHan: WIST) rats. The animals were exposed (head-nose only) for four hours to a dust aerosol of mefentrifluconazole (batch no. COD-001740; purity 98.8%; milled). The nominal test concentration was 5 mg/L whereas an actual concentration of 5.314 mg/L of air was measured. Cascade impactor measurements resulted in particle size distributions with a mass median aerodynamic diameter (MMAD) of 3.8 µm.

After exposure, the rats were observed for 14 days for mortality and clinical signs before they were killed and subjected to gross pathological examination. The animals were weighed prior to treatment, on days 1, 3, and 7 following exposure, and at scheduled termination.

There were no deaths during the study. Accordingly, the median lethal cncentration (LC_{50}) was greater than 5.314 mg/L in both sexes, which is indicates mefentrifluconazole has a low acute inhalative toxicity. However, a number of clinical signs were noted during exposure, lasting until days 10 or 11 of the post-observation period, and suggesting that exposure by inhalation was more challenging for the animals than oral intake. The most common signs (observed in all or nearly all rats of both sexes and not disappearing for a considerable time) comprised abdominal respiration, respiratory sounds, piloerection and hyperexcitability. Laboured respiration was noted in all animals during exposure. Substance-contaminated fur was seen in all animals until post-exposure day 4. More rarely occurring signs were red or colourless discharge from the nose, encrustations around nose or eyes, hunched posture and a poor general state or missed defaecation.

The mean bodyweights had decreased in both sexes on day 1 post exposure, but then increased again in all animals from day 3 onwards. There were no remarkable gross findings at necropsy (Landsiedel & Wittmer, 2014).

(b) Dermal irritation

The skin irritating properties of mefentrifluconazole were examined in an in vitro system (the EpiDerm™ skin corrosion/irritation test) on human epidermis according to Organisation for Economic Co-operation and Development (OECD) test guidelines TG 431 and 439. For this study, only a four-page study report is available and no GLP certificate was provided. However, it was considered to provide reliable supplementary information.

In this assay, 11 mg of mefentrifluconazole (Batch no, L84-176; purity not given in this report but mentioned as 97.7% when used in other studies) was applied as solid, undiluted test substance to reconstituted human epidermis. In the corrosion test, exposure was either for three minutes or for one hour. In the irritation test, one-hour exposure was followed by a 42 hour "post-incubation" period. Subsequently, cell viability was determined in all experiments by dehydrogenase-catalysed conversion of yellow MTT [3-(4,5-dimethyltriazol-2yl)-2,5-diphenyl tetrazolium bromide) which is contained in mitochondria into its blue formazan salt. After isopropanol extraction from the tissue, the extent of this reaction is determined by measuring the optical density in the extracts and comparing this to a negative control (epidermal tissue treated with just deionized water or PBS). Positive control substances

were 8 M potassium hydroxide for the corrosion test and 5% sodium dodecyl sulfate (SDS) in deionized water for the irritation test.

Viability of the cells from reconstituted epidermis was not affected by mefentrifluconazole in any experiment, but was strongly reduced by the positive controls. The conclusion was that the test substance was neither corrosive nor irritant (Remmele, 2012a).

The absence of mefentrifluconazole to display any skin irritating potential was confirmed in an irritation test in New Zealand White rabbits. The test substance (Batch no. COD-001740; purity 98.8%) was applied at 0.5 g to the intact skin of three female animals and kept there under a semi-occlusive dressing for four hours. The test was performed in a stepwise procedure starting with one rabbit before the two remaining animals were treated. Skin reactions were assessed immediately after removal of the patch and at 1, 24, 48 and 72 hours thereafter.

In one rabbit, very slight erythema was visible when the patch was removed but this disappeared within one hour. No signs of irritation were observed in the other animals at any time point. Accordingly, the mean scores for erythema and oedema at 24, 48 and 72 hours were 0.0 for each rabbit. It may be concluded that mefentrifluconazole was not irritating to rabbit skin in this in vivo experiment (Hoeger, 2013).

(c) Ocular irritation

A potential for eye irritation was studied in vitro as well as in a classical in vivo study. In the first in vitro study, two EpiOcular™ tissue samples were incubated with 50 µL bulk volume (about 15 mg) of the test substance (Batch No. L84-176; purity not given in this report but mentioned as 97.7% when used in other studies) for 90 minutes followed by a post-incubation period of 18 hours. In this assay, tissue destruction is determined by measuring the metabolic activity of the tissue after exposure and again afetr post-incubation using a colorimetric test. A reduction of mitochondrial dehydrogenase activity, as indicated by a decrease in formazan production after incubation with a tetrazolium salt (MTT), is the relevant end-point when this method is applied. Extent of formazan production is compared to that of negative control tissues (which are exposed only to de-ionized water) and is expressed then as "relative tissue viability". A viability of less than 50% suggests irritative properties of a test substance.

In this study, mefentrifluconazole treatment gave a viability value that corresponded to 81% of the control. The positive control (methyl acetate) by contrast, reduced the tissue viability to 20% of the negative control value. It was concluded that mefentrifluconazole did not show an eye irritation potential in the EpiOcular™ in vitro eye irritation test. It must be noted that for this study, only a four-page study report was available and no GLP certificate was provided. Nonetheless, this supplementary data is considered reliable (Remmele, 2012b).

In the bovine corneal opacity and permeability (BCOP) test, the substance to be examined is applied to the epithelial surface of isolated corneas from the eyes of freshly slaughtered cattle. Corneal opacity is measured quantitatively as the extent of reduction of light transmission through the cornea. Permeability is measured quantitatively as the amount of sodium fluorescein dye that passes across the full thickness of the cornea. Both measurements are used to calculate an "in vitro irritancy score" (IVIS) which can be used to predict serious eye damage. In addition, cross sections stained with hematoxylin–eosin can be evaluated for histopathological corneal lesions, and may provide evidence of potential corrosivity of a test substance.

In this study, three corneas were treated with 750 µL of a 20% mefentrifluconazole preparation in deionized water. The exposure period was four hours, followed by measurements of opacity and permeability. The histopathological evaluation of all corneas categorized observations as follows: no findings, minimal, mild, moderate or moderate/severe alteration.

Mefentrifluconazole gave a mean IVIS value of -0.4 ± 2.1. The vehicle control value was 5.5 ± 2.5, whereas the positive control substance (20% imidazole in de-ionized water) caused a much higher value of 118.3 ± 3.6. Following the prediction model used, IVIS scores higher than 55 indicate a risk of serious damage to the eyes. The histopathological evaluation did not reveal any evidence of eye damage. It was concluded that mefentrifluconazole was not irritating to the eyes under the conditions of this study. However, only a very brief (five-page) report was available and the study was apparently not performed under GLP. Nonetheless, it is considered suitable to provide valuable additional information (Remmele, 2012c).

Mefentrifluconazole's lack of eye-irritating properties was confirmed in vivo. Around 38 mg of the test substance (Batch No. COD-001740; purity 98.8%) was instilled as a bulk volume of 0.1 ml into the conjunctival sac of the right eye of three female New Zealand White rabbits. Application of the test substance was performed in a stepwise procedure starting with one animal, followed by the other two. About 24 hours after application the eyes were rinsed with tap water.

Ocular reactions were assessed approximately 1, 24, 48 and 72 hours after administration of the test substance. An additional eye examination was performed at 24 and 48 hours by additional instillation of a fluorescein solution, followed by inspection of the cornea or iris using a slit lamp and an otoscope lamp.

There were no signs of corneal opacity or iritis during the study. Slight conjunctival redness was observed in all three animals at 1 and 24 hours. In one rabbit this sign was still apparent 48 hours after instillation. Two animals showed slight conjunctival chemosis and one rabbit was found with conjunctival discharge, but only at the one-hour examination. Additional findings, such as injected scleral vessels in a circumscribed area, were noted until the 24-hour inspection but not later. On balance, all ocular reactions had disappeared within 72 hours of instillation. The individual and overall mean eye irritation scores at 24-, 48- and 72-hour readings were 0.0 for corneal opacity, iritis, and conjunctival chemosis. Corresponding mean conjunctival redness scores for each of the three animals were 0.3, 0.3 and 0.7, respectively. Based on these results, mefentrifluconazole can be regarded as not irritating to rabbit eyes in this study (Hoeger & Lammer, 2013).

(d) Dermal sensitization

The skin sensitizing potential of mefentrifluconazole was investigated in a Guinea pig maximization test according to the protocol of Magnusson and Kligman. The study was run in female Dunkin Hartley (Crl:HA) Guinea pigs of which 10 were allocated to the treatment group and five animals served as negative controls. A positive control was not included but sensitivity of the test system was regularly checked in the performing laboratory.

Intradermal induction was performed with a 5% test substance (Batch No. COD-001740; purity 98.8%) preparation in paraffin oil or in Freund′s complete adjuvant / 0.9% aqueous sodium chloride solution (1:1). The subsequent topical induction one week later was conducted with a 60% test substance preparation in paraffin oil, which represented the maximum concentration that could be prepared and applied in a homogeneous mixture. For the epicutaneous challenge after 21 days after intradermal induction, a 50% test substance preparation in paraffin oil was employed. The various concentrations for the main test were selected based on the results of pretests. In the control animals, the vehicle (with or without the adjuvant) was applied for inductions, with the test substance used only for the challenge.

After intradermal induction all animals of the control and test groups showed graduated slight to moderate erythema (grades 1 and 2) at the different injection sites. On days prior to and after topical induction, slight to moderate erythema and even necrosis were observed on those areas where preparations containing Freund′s complete adjuvant had been injected.

Following challenge, discrete or patchy erythema was noted in six out of 10 test group animals at 24 and/or 48 hours after removal of the patch. Papules were additionally noted in two of the test group animals. No skin reactions were observed in the control group. So mefentrifluconazole exhibited skin sensitizing effects in the Guinea pig maximization test (Vaeth, 2013).

(e) Phototoxicity

In an in vitro phototoxicity test, the test substance (Batch No. COD-001880; purity 98.6%) was applied to mouse embryo fibroblast cell line Balb/c 3T3 at concentrations of up to 100 µg/mL. Higher concentrations (as applied in pretests) were already cytotoxic with and without irradiation at ultraviolet to visible (UV/VIS) wavelengths.

The cells were incubated in the dark with the test substance or the positive control substance (chlorpromazine) for one hour, then, UV/VIS irradiated for 50 minutes. Cytotoxicity was determined by measuring the uptake of Neutral Red dye which is inhibited when cells are damaged. Cytotoxicity

was observed at the maximum concentration of 100 µµg/mL, both with and without irradiation, but not at the next lowest concentration of 46 µg/mL. No difference in cytotoxicity was observed when irradiated and non-irradiated cultures were compared. Thus, under the conditions of this study, mefentrifluconazole was negative for phototoxicity, whereas the positive control substance produced clear cytotoxicity under all conditions but at much lower concentrations with irradiation, as would be expected (Cetto & Landsiedel, 2015).

2.2 Short-term studies of toxicity

(a) Oral administration

Mouse

For this species, two feeding studies are available. In the first one, a 28-day, range-finding experiment, mefentrifluconazole (Batch No. COD-001662; purity 95.5%) was administered to C57BL/6J Rj mice. Groups of five male and five female mice were given the test substance at dietary dose levels of 0, 30, 100, 300 or 1000 ppm. The corresponding mean daily intakes were 0, 4.8. 15.5, 47.9, and 128 mg/kg bw in males, 0, 5.8, 18.5, 51 and 145 mg/kg bw in females. The animals were monitored daily for mortality and clinical signs. Body weight, its gain and food intake were determined regularly. A number of haematological and clinical chemistry parameters were measured prior to scheduled termination. Thereafter, the animals were killed and a selection of organs taken for weighing and subsequent histopathology.

There were no unscheduled deaths and no clinical signs of toxicity in this study. At 1000 ppm, significant reductions in food consumption, mean body weight (–13% compared to the controls in males and –6% in females after four weeks) and mean body weight gain were observed in both sexes. In males, there was even a small net body weight loss (–0.1 g on average) over the course of the study. Apparently, the body weight decreases were due to a markedly lower weight gain in the initial phase of treatment. It is not clear to what extent a reduced palatability of the diet might have contributed to this effect.

The only haematological effects observed were lower red blood cell (RBC) volume at the top dose in both sexes, and a lower mean cellular haemoglobin concentration in high-dose males. These findings were based on calculations and were not considered adverse since all directly measured RBC parameters were not affected. In addition, there was a significant increase over the controls in monocyte count in males, an effect that was seen at all treatment levels in females, but for which a clear dose–response relationship was lacking. On balance, these findings perhaps might suggest a weak impact of the test substance on blood parameters which would be expected to become more pronounced in studies of longer duration if they were of any concern.

A number of alterations in clinical chemistry parameters indicated that the liver was a target organ of mefentrifluconazole although it is difficult to assess the adversity of these effects given the low number of animals on study. Selected biochemical parameters are shown in Table 12. The values in bold are those regarded as of highest concern because of the very marked difference compared to the respective control values and since they were also outside the range of historical control data (HCD).

Table 12. Selected clinical chemistry parameters in the 28-day short-term toxicity study in mice

		Males					Females				
Dose level (ppm)		**0**	**30**	**100**	**300**	**1000**	**0**	**30**	**100**	**300**	**1000**
ALT	(µkat/L)	0.83	0.81	0.97	1.14	**2.08***	1.15	1.03	1.22	1.36	2.67
AST	(µkat/L)	5.18	4.40	4.83	5.11	5.72	5.70	5.86	5.48	4.87	6.46
ALP	(µkat/L)	1.56	1.70	1.82*	1.87*	2.07*	2.28	2.07	2.11	1.98	2.53
Cholesterol	(mmol/L)	2.53	2.01*	1.51**	1.24**	**0.69****	2.04	1.32**	0.87**	0.68**	0.83**

		Males					Females				
Dose level (ppm)		**0**	**30**	**100**	**300**	**1000**	**0**	**30**	**100**	**300**	**1000**
Triglycerides	(mmol/L)	0.98	0.85	0.50*	0.68	0.66	0.82	0.48**	0.39**	0.30**	0.63*
Total protein	(g/L)	49.55	49.89	45.88*	44.56**	49.67	48.29	46.13	**43.41***	**42.71***	**43.22***
Albumin	(g/L)	31.17	31.33	28.63**	27.87**	31.31	31.85	30.64	**28.67***	**28.16***	**27.69***
Globulin	(g/L)	18.38	18.56	17.25**	16.69**	18.37	16.44	15.48	14.74*	14.55**	15.53
Glucose	(mmol/L)	7.54	7.21	6.96	7.29	5.70	7.79	7.90	6.83	5.95	**5.25****

ALT: Alanine transaminase; AST: Aspartate transaminase; ALP: Alkaline phosphatase

* $p \leq 0.05$; ** $p \leq 0.01$; Kruskal–Wallis and Wilcoxon test (two-sided)

Necropsy revealed liver foci at the top dose in two male and in two female mice, as well as in one male receiving 300 ppm but no other noteworthy findings which could be attributed to treatment. It is tempting to assume hepatomegaly, at least in the high-dose group where liver weight was increased by more than 70%, but surprisingly this was not reported. Mean absolute and relative liver weight was increased in both sexes at all dose levels, and the dose-related increment was significant from 100 ppm upwards in all cases. In males the organ weight increase, up to the upper mid dose of 300 ppm, was associated with hepatocellular hypertrophy but with no other histological lesions and was not accompanied by critical clinical chemistry findings (see above). In females receiving 300 ppm, the increase in liver weight by more than 30% was big enough to be considered potentially adverse. Even though it was, again, only associated with hypertrophy, the large reduction in total protein and albumin also suggested a toxic effect on the liver. At the top dose level of 1000 ppm, liver weight increase was highest in both sexes and correlated not only with hypertrophy but also with associated obvious hepatotoxic findings; multifocal liver cell necrosis, hyperplasia of oval cells and bile duct hyperplasia.

Another presumably treatment-related organ weight change was a dose-related and statistically significant increase in absolute and relative thymus weight in female mice from 100 ppm upwards. At the top dose, the upper edge of the HCD was exceeded. The thymus was not examined under the microscope and therefore, in the absence of more information, this organ weight increase must be considered potentially adverse. The study authors arrived at the same conclusion but confined it to the maximum dose level. For a final conclusion to be made, the subsequent 90-day study (Schonmakers, 2015a, see below) must be taken into account.

Based on evidence of liver toxicity, the NOAEL in this study was set at the lowest dose of 30 ppm. Since it was derived from female mice, this dietary level is equal to a mean daily intake of 5.8 mg/kg bw per day. Males were less sensitive and for them the NOAEL could, notionally, be much higher (Stark et al., 2014).

In a 90-day study, mefentrifluconazole (Batch No. COD-001740; purity 98.8%) was administered to groups of 10 male and 10 female C57BL/6J Rj mice at dietary concentrations of 0 (control), 10 (low), 50 (intermediate low), 250 (intermediate high) and 750 ppm (top dose). These doses corresponded to time-weighted mean daily intakes of 2, 11, 58 and 174 mg/kg bw in male mice and 3, 15, 67 and 211 mg/kg bw in females. The study was primarily designed to select appropriate dose levels for a subsequent 18-month mouse carcinogenicity study. Satellite groups of five animals per sex and dose were treated the same way but for only 63 days. From these mice, under isoflurane anaesthesia, blood was taken, for determination of plasma concentrations, from the retro-orbital sinus on days 21, 42, and at termination.

The animals were monitored daily for mortality and clinical signs. Body weight, its gain and food consumption were determined on a weekly basis. A number of haematological and clinical chemistry parameters were measured prior to scheduled termination. Thereafter, the animals were killed, necropsied, a gross examination made and a number of organs taken. Organ weights of adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes with epididymides, thymus and uterus were determined. All those and a number of additional organs were subjected to histopathology but most of them were examined only in the control and high- dose groups. However, histopathology of liver and thyroid was performed on all animals.

No clinical signs of toxicity were observed in any group. In the main groups there were no unscheduled deaths. In the high-dose satellite groups however, four of five males died under anaesthesia upon first blood sampling (day 21). The actual cause of death was not elucidated but this particular pattern of mortality suggests at least the possibility that previous exposure to the test substance might have weakened the isoflurane anaesthesia tolerance of the mice. (It is worth remembering that one high dose male rat also died in the 28-day study during blood sampling under anaesthesia. However, in the whole data package on mefentrifluconazole there is no further data to either substantiate or contravene this hypothesis.) On the other hand, mortality was also seen in male mice in the toxicokinetic study of Fabian & Landsiedel (2014, see above) following blood sampling. In this case however, the mice had been given a single mefentrifluconazole dose of 75 mg per kg body weight, but blood sampling was not performed under anaesthesia. The sex difference with regard to these observations is striking.

Administration of the top dose of 750 ppm caused a significant reduction in body weight gain in male mice. For the whole duration of the study, body weight gain was more than 30% lower than in the control group. However, this effect resulted in a mean final body weight for high-dose males at termination that was only 5% lower than in the control group. Food consumption appeared to be lower in males receiving 250 and 750 ppm and in high-dose females, but this finding was confined to the first week of treatment and might have been due poor palatability.

Haematology revealed some equivocal evidence of haemoconcentration since there were increases in RBC count, haematocrit (Ht) and haemoglobin (Hb) as well as a higher platelet count in both sexes. The differences from the control group achieved statistical significance in male mice at 50 and 250 ppm and in females at 250 ppm. A dose response could not be properly assessed because of the low number of samples (2–4 in males, differing amongst individual parameters, and not more than six in females) available from the high-dose groups. This deficiency was due to technical problems such as blood clotting. The reason for haemoconcentration could not be determined and its relationship to treatment is unknown. In principle, it might be caused by low water consumption, but regular visual inspection of water bottles throughout the study did not suggest such an effect had occurred. Other explanations like a stress response or polycythemia are also unlikely since no hypertrophy of the adrenal cortex or cell proliferation in spleen or bone marrow were observed.

Clinical chemistry confirmed the liver as the main target organ. Respective findings comprised a significant and dosely related reduction in cholesterol in all treated groups, and significantly lower protein concentrations in most of them (see Table 13). Decreases in total protein did not show a dose– response relationship and were apparently due to lower albumin concentrations, whereas globulins were not affected. As in the 28-day study (see Table 12), ALP activity was increased in high dose males but ALT activity was not. The reduction in blood glucose in females that was observed in the preceding study could not be confirmed. By contrast, in this study blood glucose was even seen to increase, but only in males. The scattered changes in electrolytes in female mice were not dose related even though the differences from control values sometimes achieved statistical significance.

Table 13. Selected clinical chemistry parameters in the 90-day feeding study in mice

Parameter	Males (ppm)					Females (ppm)				
	0	**10**	**50**	**250**	**750**	**0**	**10**	**50**	**250**	**750**
ALP (µkat/L)	80	71*	69**	77	100**	117	111	110	113	1.04
Cholesterol (mmol/L)	2.36	2.09**	1.63**	1.33**	1.03**	2.03	1.76**	1.63**	1.01**	1.01**
Total protein (g/L)	52.0	49.0**	48.5**	46.8**	49.3**	49.7	48.5	47.1**	45.1**	47.6*
Albumin (g/L)	29.6	27.8**	27.5**	26.1**	27.0**	29.2	28.9	27.6**	26.0**	26.3**
Glucose (mmol/L)	13.7	17.2**	18.8**	18.0**	13.5	13.8	14.3	15.1	16.8*	14.6

ALP: Alkaline phosphatase * $p = 0.05$; ** $p = 0.01$ Dunnett′s test, based on pooled variance

The analysis of plasma concentrations of mefentrifluconazole is summarized in Table 14. An increase in plasma concentrations with dose can be seen. A five-fold higher dose resulted in an approximately three-fold higher plasma concentration, so absorption was sublinear, confirming the findings in the toxicokinetic study in mice by Fabian & Landsiedel (2014, see above). Some saturation of absorption

at higher doses appears likely. However, as with all toxicokinetic investigations that form part of routine toxicological studies, the impact of the extensive mefentrifluconazole metabolism could not be adequately taken into consideration by this measurement. Surprisingly, mefentrifluconazole was also detected in the plasma of control animals suggesting some carry-over, the precise time point of which is unknown. Maximum mean values in the plasma of untreated animals on study day 63 were nearly 11 ng/mL in males and around 43 ng/mL in females, that is about half of the value in the female group that had received 10 ppm. The analysis was also flawed by the fact that four out of five high-dose males died upon blood sampling, as outlined above.

Table 14. Plasma concentrations of mefentrifluconazole in mice after different periods of feeding

Concentration in diet (ppm)	Sampling time (study day)	Males		Females	
		Samples evaluated	Mean ± SD (ng/mL)	Samples evaluated	Mean ± SD (ng/mL)
10	21	5	185.4 ± 19.4	5	136.0 ± 16.5
	42	5	134.0 ± 24.7	5	88.98 ± 27.2
	63	5	134.8 ± 19.9	5	85.25 ± 12.5
50	21	5	642.8 ± 125.7	5	366.8 ± 98.8
	42	5	458.4 ± 71.9	5	316.1 ± 162.2
	63	5	487.7 ± 120.7	5	346.5 ± 91.9
250	21	5	1791 ± 274.0	5	1108 ± 290.4
	42	5	1433 ± 283.3	5	1652 ± 735.2
	63	5	1389 ± 145.1	5	980.6 ± 282.4
750	21	2	2454 ± 15.1	5	1565 ± 186.9
	42	1	815.8	5	1215 ± 431.1
	63	1	3183	5	1951 ± 328.4

Gross examination at necropsy did not reveal any treatment-related lesions. Regarding organ weights, there was a clear impact on the liver. By contrast, the increase in adrenal weight in high-dose male mice and the decrease in spleen weight in females at the two upper dose levels (Table 15), although statistically significant and dose-related, were of equivocal toxicological relevance since they were not accompanied by any histological findings. An increase in thymus weight (as observed in female mice in the 28-day study) was not confirmed in this study which was of longer duration and employed more animals.

Histopathology revealed what were presumed to be treatment-related findings only in the liver. In male mice centrilobular hypertrophy was observed at 50 ppm and above. From the next higher dose level of 250 ppm upwards, such hypertrophy was associated with cytoplasmic alterations which were mainly characterized by hyaline vacuoles. This finding was interpreted by the study author as an early sign of degeneration. Four and five males were affected in the groups receiving 250 and 750 ppm, respectively. Single cell necrosis was seen in two males from the mid-dose group and was common in the high-dose group, with eight out of 10 animals affected.

Similar findings were noted in females, but hypertrophy was seen to start at 250 ppm and was more diffuse than centrilobular. Multifocal subcapsular necrosis in 6/10 and cytoplasmic changes in 2/10 females were confined to the top dose.

Table 15. Mean weights of selected organs in the 90-day study in mice (n = 10 per sex and dose)

Organ	Males (ppm)					Females (ppm)				
	0	10	50	250	750	0	10	50	250	750
Liver										
Absolute (g)	1.19	1.24	1.38**	1.62**	2.16**	1.10	1.09	1.16	1.36**	1.78**
Relative (%)	4.45	4.69	5.09**	6.12**	8.34**	4.87	4.81	5.03	6.12**	8.12**
Adrenals										
Absolute (mg)	4.1	5.1	4.9	5.5	6.0*	10.0	9.8	11.0	9.8	10.7
Relative (%)	0.015	0.019	0.018	0.021	0.023**	0.044	0.044	0.047	0.044	0.049
Spleen										
Absolute (mg)	61	52	65	63	55	81	78	77	66**	62**
Relative (%)	0.23	0.24	0.24	0.24	0.21	0.36	0.35	0.33	0.30**	0.28**

* $p = 0.05$; ** $p = 0.01$ Dunnett's test, two sided; relative organ weights rounded to two significant figures

Based mainly on histopathology, (toxic liver findings) the NOAEL in this study was 50 ppm (equal to 11 mg/kg bw per day). There were some findings (organ weight increase and hepatocellular hypertrophy in males as well as alterations in a few clinical chemistry parameters) that suggested an impact on the liver at this dose, and perhaps even hinted that the effects had already begun at the lowest dose of 10 ppm. However, these lower dose level findings were not considered adverse but adaptive (Schoenmakers, 2015a).

Rat

Study1

In a range-finding study, mefentrifluconazole (Batch No. L84-176; purity 97.7%) was administered via the diet to groups of five male and five female Wistar rats over a period of four weeks at concentrations of 0, 500, 1500 and 4000 ppm. Mean daily intakes of 0, 47, 135 and 388 mg/kg bw were calculated for male rats, and for females 0, 47, 138 and 334 mg/kg bw. The animals were examined for signs of toxicity or mortality at least once a day. Moreover, detailed clinical examinations were conducted prior to the start of the ingestion period and weekly thereafter. Food consumption and body weights were determined weekly. Haematological and clinical chemistry parameters (including urinalysis) were measured at the end of the ingestion period. On day 15 plasma levels of the test substance were been determined. At scheduled termination, all animals were killed, necropsied and assessed for gross pathology. Selected organs were taken, weighed and prepared for histopathological examination which was mostly confined, however, to the control and high-dose groups.

One high-dose male died during terminal blood sampling. All other animals survived until scheduled termination and there were no clinical signs of toxicity. However, dietary administration of mefentrifluconazole resulted in reduced body weight gain (by about 30%) in both male and females given 4000 ppm, resulting in mean terminal body weights that were by about 15% and 9% lower than in the respective male and female control groups. In high-dose females, but not in males, a concomitant decrease in food consumption was observed.

Haematology and urinalysis did not reveal any evidence of treatment-related effects. Notable clinical chemistry findings were confined to mid- and high-dose females and comprised a significant decrease in albumin and an increase in cholesterol. These alterations suggested an impact on the liver but were accompanied by an organ weight increase and histopathological findings only at the highest dose.

Determination of plasma levels of mefentrifluconazole revealed increase with dose that was initially supralinear but tended to linear as dose increased (Table 16). Remarkably, plasma levels were much higher in female rats than in males but not in the low-dose group. Standard deviation also increased with dose. When plasma concentrations of mefentrifluconazole at 500 ppm in rats are compared to those after administration of 250 or 750 ppm for 21 days to mice (see Table 14), the values for rats appear to be much lower.

Table 16. Mefentrifluconazole plasma concentrations in rats on administration day 15

Dose (ppm)	500	1500	4000	500	1500	4000
	Males			Females		
Plasma conc. (ng/mL) ± SD	182 ± 48	889 ± 458	3131 ± 1250	197 ± 66	2264 ± 594	6298 ± 1487

A slight increase in relative liver weight (23%) was noted in high-dose females. Histopathology revealed centrilobular liver cell hypertrophy of minimal severity in all five females and in two males receiving 4000 ppm. On balance the liver was the only target organ in this study. Effects were more pronounced in females but still considered borderline between toxic and adaptive. On the basis of liver findings in females and lower body weight (gain) in both sexes at the highest dose level, the NOAEL was next lowest dose of 1500 ppm (equal to 135 mg/kg bw per day) (Buesen, 2015a).

Study2

Mefentrifluconazole (Batch No. COD-001662; purity 95.5%) was fed for 94 or 95 days at nominal concentrations of 0, 400, 1200, or 3600 ppm to groups of 10 male and 10 female Crl:WI(Han) Wistar rats. Mean daily intakes of 27, 76 and 256 mg/kg bw were calculated for male rats and of 30, 91, and 314 mg/kg bw for females.

The animals were monitored daily for mortality and clinical signs and were weighed prior to start of treatment and then weekly. Food consumption was also determined on a weekly basis. Once a week a more detailed clinical examination was performed. Ophthalmoscopy took place on day 0 and close to termination on day 91. For investigation of neurotoxicity, a functional observational battery (FOB) and measurements of motor activity were performed prior to commencement of treatment and when it was finished. Urinalysis was performed on day 93 and haematological and clinical chemistry parameters measured in blood samples taken at termination. In addition, blood samples (about 200 µL) were taken from all animals, under isoflurane anaesthesia, on study days 24, 45, and 66 to determine plasma concentrations of mefentrifluconazole. Blood was also sampled on day 31 for subsequent measurement of thyroid hormones which was, however, not performed since it was optional depending on thyroid weight and histopathology, according to the study plan. At the end of the study period the animals were killed and necropsied. Organ weights of adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes (with epididymides), thymus, thyroid (with parathyroids) and uterus were determined. All these and a number of additional organs were subjected to histopathology, but mostly organs were examined only in the control and high-dose groups. However, a histopathological examination was made of adrenals, kidneys, liver, spleen and thyroid from all animals, as well as of all gross lesions observed. In male rats, special staining for the presence of renal tubular proteins was carried out. Additionally, with a few males an immunohistochemical stain was used to detect α_{2U} globulin.

All animals survived until scheduled termination and there were no clinical signs which could be attributed to treatment. Ophthalmoscopy and neurological examinations by means of FOB or motor activity measurement did not reveal any evidence of substance-related effects in any group. However, body weight and body weight gain were compromised in both sexes at the top dose, whereas food consumption was not affected. At the end of the in-life period, males and females fed 3600 ppm weighed 6.5% less and nearly 9% less than controls, respectively. The overall body weight gain during the administration period of high-dose males and females was by about 11% lower and 20% lower than for their respective control groups.

There were no remarkable haematological or urinalysis findings in this study. In contrast, some blood clinical chemistry parameters were altered at the high dose, suggesting an impact of treatment on the liver. Males and females given 3600 ppm showed increased levels of serum ALP activity. In high-dose females increased cholesterol and decreased albumin levels were also found. An increase in γ-glutamyl transpeptidase (GGTP) activity in the top-dose female group was still within the historical control range but since it was clearly above the study's own control value, and since GGTP is regarded as a slow reacting but sensitive parameter of disturbed liver function, this finding was also considered adverse.

Plasma levels of mefentrifluconazole increased with dose but tended to decline with ongoing duration of the study. It is not known to what extent the latter observation might be due to metabolism.

There were no gross lesions at necropsy which could be attributed to substance administration. Some organ weight changes were noted but, with the presence of concomitant alterations in clinical chemistry parameters and histopathological findings, only the dose-related increase in mean relative liver weight was considered potentially adverse. The respective increases by 6% and 11% in mid- and high-dose males and by 13% in high-dose females were statistically significant. Histopathological examination revealed centrilobular hypertrophy of low severity (grade 1) in the liver cells of 3/10 mid-dose and 8/10 high-dose males, and in three high-dose females, this being consistent with the relative organ weight increase.

In contrast to the liver findings, a significant increase in relative kidney weight in females at all dose levels was not dose-related, was not accompanied by histopathological findings, therefore was not regarded as treatment-related. It is worth also noting that histochemical staining of the kidneys in male rats did not reveal a difference between treated and control animals. The significant decrease in adrenal absolute weights, by about 20% in both sexes at the top dose, was consistent with correspondingly lower body weights since relative organ weights were unaltered. Also, there was no histopathological correlate in the case of kidneys. Mean relative thyroid weights were increased in high-dose females but absolute organ weights were not, and histopathology did not reveal any evidence that the thyroid might have been affected by treatment.

The mid dose of 1200 ppm (equal to 76 mg/kg bw per day) was the NOAEL in this study. It was based on reductions in body weight and its gain and on limited evidence of liver toxicity in both males and females at the high dose level (Buesen, 2015b).

Dog

A range-finding experimant, a 90-day and an one-year study are available in which the test substance (Batch No. COD-001880; purity 98.6%) was administered to male and female beagle dogs once a day by gelatin capsule.

Study1

In the preliminary range-finding study with three animals per sex and group, originally planned for a duration of four weeks, the intended dose levels in males were 300 and 1000 mg/kg bw per day but these had to be reduced due to overt toxicity, such as vomitus or unsteady gait. These findings were observed within two days of treatment. Food intake was immediately reduced and the general condition of the animals impaired. The dogs in both treated groups were affected and there was not much difference between those receiving the low and the high dose. After an interruption by a treatment-free interval of five days, administration was continued until termination on days 35 or 36, but dose levels were reduced to 125 and 250 mg/kg bw per day.

Female dogs were initially given doses of 300 or 500 mg/kg bw per day, apparently based on the experience with male dogs. Since similar signs to those seen in males had already occurred subsequent to first dosing, administration was discontinued for two days. From study day 3 onwards reduced doses of 125 or 250 mg/kg bw per day (as for males) were given to the female groups, until termination on study days 29 or 30. Control groups consisting of three animals per sex received "empty capsules" for four weeks.

The animals were monitored daily for clinical signs; body weight and food consumption were determined weekly. A detailed clinical examination also took place once a week. Haematological and blood clinical chemistry examinations as well as urinalysis were performed prior to commencement of treatment and after more than three weeks of continuous substance administration. On study day 23, blood samples were taken before dosing and at several time intervals thereafter to determine plasma concentrations of mefentrifluconazole. At terminal kill, animals were necropsied and a comprehensive set of organs taken, weighed and preserved. Histopathology of the liver (and, if present, any gross lesions) was performed for all dogs, whereas the remaining tissues were examined microscopically only in the case of control and high-dose animals.

Thanks to immediate cessation of administration of the obviously excessive initial doses, all animals survived until scheduled termination. Following dose reduction, clinical findings at the low dose of 125 mg/kg bw per day were confined to a reduced food intake on some treatment days, and to one male dog with vomitus occurring on two days during the rest of the study. At the high dose of

250 mg/kg bw per day, by contrast, two of the three dogs from each sex consistently showed a markedly reduced food intake. In addition, vomiting was occasionally observed at least in males. Body weight development was clearly affected in the high-dose males and females and still albeit to a lesser extent at the low dose. The terminal body weight of high-dose group dogs was 12–13% lower than for the control groups. In both sexes it was lower at the end of the treatment period than near the beginning. At the low dose mean terminal body weights in male and female dogs were by 6% and 9% lower, respectively, than in the control groups even though there was no weight loss when compared to the pretreatment figures. The variation in body weight in the individual animals over the course of the study was remarkable, most likely reflecting the strong oscillations in food consumption and perhaps also pointing to differences in individual susceptibility.

Haematology did not reveal clear evidence of treatment-related effects since the low number of animals on study prevented meaningful statistical evaluation and all individual values were within the historical control range. Alterations in clinical chemistry parameters were more suggestive of an impact due to the test substance. Cholesterol was decreased in both sexes at both dose levels and, since the individual values were also below the historical control range, this effect was probably treatment-related. Reduced cholesterol in the dog is usually considered to indicate either liver disease, anorexia, malabsorption or hormonal disturbance and therefore may be adverse (Neumann, 2014). In this case, liver toxicity or reduced food intake might be the most likely explanations. A possible effect on liver function was also indicated by increases in the enzyme activities of AST and ALT in all treated groups. In females these changes were partly above the upper limit of the historical control range. In all treated animals, urea tended to be increased compared with control values, but values were still within the historical control range. Urinalysis suggested a lower urine volume and, consistent with this, an increase in the specific gravity of urine in female dogs in both dose groups.

An overview on plasma concentrations in the third week of continuous treatment with the reduced doses is provided in Table 17. Even though mean values in male and female dogs were of similar magnitude, marked interindividual differences became obvious. Maximum concentration was reached after four hours (the T_{max}) in low-dose males and females and after eight hours in the high-dose groups.

Table 17. Mean plasma concentrations of mefentrifluconazole at different time points after dosing (study day 23) in the range-finding study in dogs

	Mefentrifluconazole plasma concentration (ng/mL)			
	Males		**Females**	
Test group	**1**	**2**	**1**	**2**
Dose level (mg/kg bw per day)	**125**	**250**	**125**	**250**
t = 1 hour	15 789 ± 3740	16 552 ± 12 575	13 292 ± 9058	14 085 ± 3192
t = 2 hours	19 238 ± 3967	19 763 ± 13 438	16 030 ± 10 148	17 442 ± 3323
t = 4 hours	23 455 ± 4787	23 554 ± 10 692	17 496 ± 9715	19 995 ± 5169
t = 8 hours	21 936 ± 1173	25 515 ± 10391	17 010 ± 9986	21 254 ± 5177

Occasional gross lesions such as red liver foci in one male or focal red discoloration in the jejunum of one female were not attributed to test substance intake but to hyperemia related to terminal procedures. Some variation in organ weights was observed (Table 18). A higher liver weight at both dose levels, though not strictly dose-related, was attributed to treatment since it was accompanied by changes in clinical chemistry parameters and by histopathological findings comprising centrilobular hypertrophy of minimal severity in five of six dogs, and of moderate severity in one high-dose male, eosinophilic cytoplasm and a reduction in vacuolation compared with the controls. The higher thyroid weights, in contrast, were not likely to be treatment-related since a dose–response realtionship was completely lacking and there was no histological correlate. The markedly lower uterus weight may be explained by generally large variation in sexually immature females but presumably also by a delay in maturation due to general toxicity. However, there were no remarkable histopathological findings for the uterus. , There was no convincing explanation for the lower thymus weight and histopathology revealed nothing remarkable.

Table 18. Selected organ weight changes (% of control) in the range-finding study in dogs

Organ	Absolute weight				Relative weight			
	Males		Females		Males		Females	
	LD	HD	LD	HD	LD	HD	LD	HD
Liver	129	120	115	116	139	136	125	131
Thymus	92	38	47	47	102	43	51	56
Thyroid	148	125	116	112	158	142	129	128
Uterus	-	-	29	18	-	-	32	19

LD: Low dose; 300↓125 mg/kg bw per day in both males and females, see text for regime

HD: High dose; 1000↓250 mg/kg bw per day in males and 500↓250 mg/kg bw per day in females, see text for regimes

In conclusion, a NOAEL could not be derived in this study, mainly due to adverse clinical findings, a strong impact on food consumption and some evidence of liver toxicity. The early-onset toxicity in this study might support the need to establish an ARfD (Keller et al., 2015a).

Study2

In a three-month study, mefentrifluconazole was administered to groups of five male and five female beagle dogs for at least 91 days at dose levels of 0, 15, 90 or 180 mg/kg bw per day. The animals were monitored daily for mortality and signs of toxicity, including a detailed clinical examination at weekly intervals. Ophthalmoscopy was performed prior to and at the end of the treatment period. The dogs were weighed once a week and food consumption also determined on a weekly basis. Haematological and clinical chemistry analyses including urinalysis were performed before commencement of treatment, after around 6 weeks of substance administration and towards scheduled termination. On day 83, blood samples were taken twice for determination of mefentrifluconazole in plasma, just before and six hours after capsule administration.

At termination on days 92–98, the dogs were killed and necropsied. In total, 11 or 12 organs per sex were weighed and a wide selection of organs and tissues were taken from all animals and prepared for subsequent histopathological examination.

There were no unscheduled deaths in this study. Treatment-related clinical findings were confined to the top dose of 180 mg/kg bw per day, with the possible exception of transiently reduced food intake which as an isolated finding was not considered adverse. Lowered food intake on some days throughout the study was observed in four high-dose females and one high-dose male, in three mid-dose females and in one female receiving the low dose, but also in one control female. Clinical signs other than lower food intake were vomitus in one high-dose male and two high-dose females on study days 1 or 2, that is just after the first administrations. Body weight gain over the whole treatment period was significantly reduced in male dogs receiving the high dose and in high-dose females during most of the study (days 0 –77). This decrease resulted in a slightly lower total body weight at termination but the difference from the control group did not achieve statistical significance (Table 19). Ophthalmoscopy did not uncover any notable eye findings.

Haematology did not reveal any evidence of treatment- or dose-related effects. Concerning clinical chemistry parameters, a dose-related increase in ALP activity was observed in male dogs at the mid and high dose. However, the mean value for mid-dose males was still within the historical control range. A decrease in total protein in male (significant after six weeks only) and female dogs in the top-dose groups was also considered an effect due to treatment, which supported the assumption of an impact on the liver. A lower bilirubin concentration in high-dose males and females might be indicative of increased metabolic demand, but in contrast to an increase, it is not considered adverse. Urine parameters were not altered.

Plasma concentrations of mefentrifluconazole on day 83 were lower than in the 28-day study in which higher doses had been administered, but were clearly related to dose. Six hours after application of the gelatin capsules concentrations were three to five times higher in both males and females receiving 15 or 90 mg/kg bw per day than at the time of dosing, and were doubled at the top-dose level. Interindividual variation appeared smaller than in the 28-day study.

There were no gross lesions which could be attributed to mefentrifluconazole administration but there were some changes in organ weights which warranted more detailed assessment. Liver weight was increased in male dogs at the two upper dose levels but there was no clear dose–response relationship. On the other hand, the lower terminal body weight must be taken into account. An increase in liver weight in top-dose females did not achieve statistical significance. The organ weight increase was accompanied by a slight (grade 1) histopathological findings affecting mainly the cytoplasm of centrilobular hepatocytes. This finding was described as "eosinophilic change" and reflected a lower vacuolation than in the control animals. It might be indicative of decreased glycogen storage and could also result from reduced nutritional state. On balance, the liver weight increase was considered treatment-related and potentially adverse. A summary of liver findings is provided in Table 19.

Reduced uterus weights are also shown in this table since the respective observation in the 28-day study was confirmed even though the applied statistical test did not reveal a significant difference from the control group. In addition, an increase in testis weight was observed at the two upper dose levels reaching statistical significance for the relative organ weight at the top dose. However, changes in the uterus and testis weight were not accompanied by histopathological findings. Variability might be explained by relative sexual immaturity of the animals. In accordance with that, no such findings were observed in the one-year feeding study of Keller et al. (2016) described next.

Table 19. Selected organ weights and liver findings in the three-month study in dogs

	Males (*n* = 5 per group)				**Females (*n* = 5 per group)**			
Dose (mg/kg bw per day)	**0**	**15**	**90**	**180**	**0**	**15**	**90**	**180**
Terminal body weight (kg)	13.52	13.64	13.88	12.72	10.94	10.80	10.50	10.28
Mean absolute uterus weight (g)	-	-	-	-	8.8	13.3	7.3	4.9
Mean relative uterus weight (%)	-	-	-	-	0.08	0.10	0.07	0.05
Mean absolute weight of testes (g)	17.7	19.6	22.9	22.7	-	-	-	-
Mean relative weight of testes (%)	0.13	0.14	0.16	0.18**	-	-	-	-
Mean absolute liver weight (g)	377.5	372.0	477.2**	429.1	341.0	304.9	332.1	378.6
Mean relative liver weight (%)	2.8	2.7	3.4	3.4**	3.1	2.8	3.2	3.7
Eosinophilic change in cytoplasm (number of dogs affected)	0	2	5	5	0	0	2	5
ALP activity (µkat/L), week 13	1.13	1.14	2.08*	3.27*	1.18	1.08	1.73	2.20*
Total protein (g/L), week 13	55.37	53.35	53.80	51.34	54.84	51.66	53.36	50.95*

ALP: Alkaline phosphatase

* $p < 0.05$, ** $p < 0.01$ Kruskal-Wallis and Wilcoxon tests, 2-sided; weights rounded to one or two significant figures

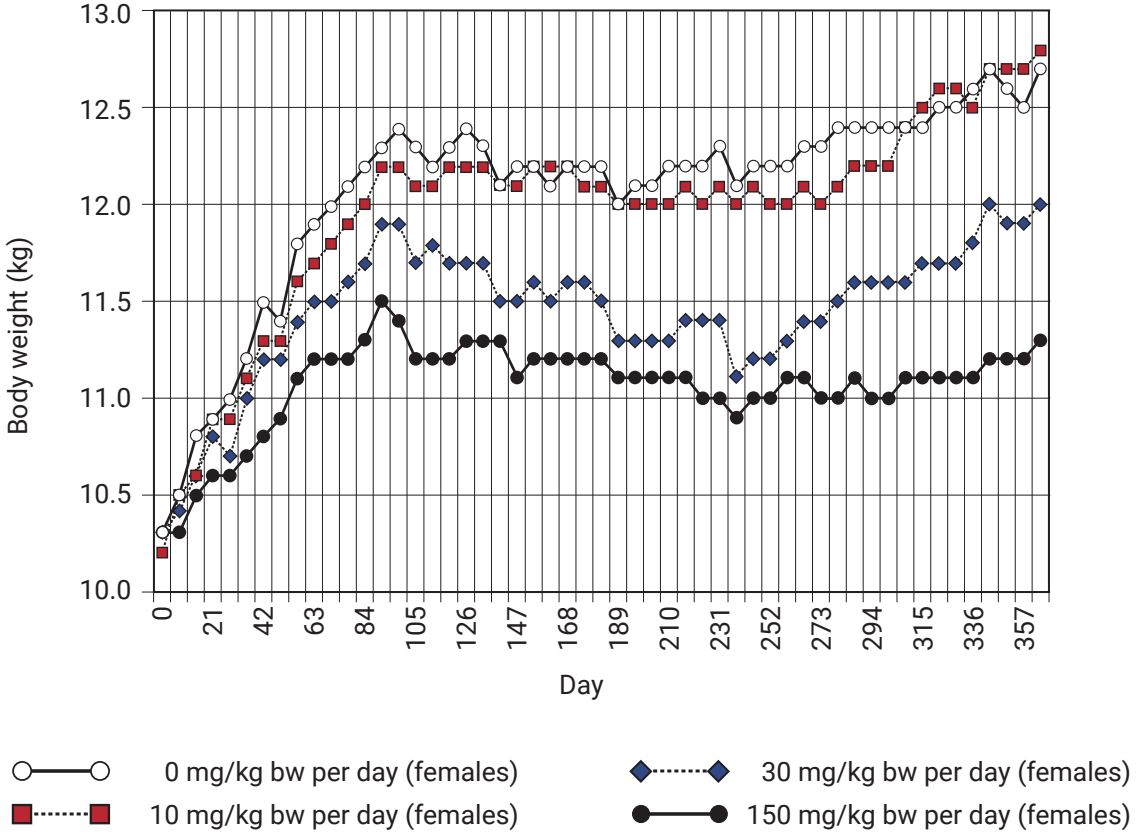
Based on liver effects in male dogs, a NOAEL of 15 mg/kg bw per day had been proposed. However, as demonstrated in a recent analysis (Yokoyama et al., 2019), an increase in ALP activity is common in dogs and, when occurring in the absence of other findings, may be not sufficient to indicate hepatotoxicity. Likewise the increase in absolute and relative liver weights was not accompanied by adverse histological changes since the relevance of the low degree eosinophilic cytoplasmic change is not known. A reduction in body weight gain in both sexes was confined to the top dose level and was at best marginal. No significant difference in terminal body weight between the groups was noted. Organ weight changes in reproductive organs, in both sexes, were considered equivocal in this study and were eventually outweighed by the results of the subsequent one-year study in which no such effects were observed. On balance, the Meeting concluded that there might be treatment-related findings at lower dose levels but no adverse effects were observed up to the highest dose level of 180 mg/kg bw per day so that is the NOAEL (Keller et al., 2015b).

Study3
In this one-year study, mefentrifluconazole was administered to groups of five male and female beagle dogs at daily dose levels of 0, 10, 30, or 150 mg/kg bw. The animals were monitored daily for mortality and signs of toxicity, including a detailed clinical examination at weekly intervals. Ophthalmoscopy was performed prior to and at the end of the treatment period. The dogs were weighed once a week and food consumption was also determined on a weekly basis. Haematological and clinical chemistry analyses including urinalysis were performed before the beginning of treatment period, after three- and six-month intervals and immediately prior to termination.

At terminal kill, the dogs were necropsied and a gross inspection carried out. Organ weights were determined and a wide range of organs and tissues were subject to histopathological examination. Slides were peer-reviewed internally. Special stains were applied to better detect glycogen or fat in vacuoles in the liver and kidneys.

There were no unscheduled deaths and no clinical signs of toxicity in this study. While food consumption was not affected by treatment, body weight and its gain tended to be lower in high-dose males. These latter parameters were still more strongly affected in high-dose females, with reductions in total mean body weight of greater than 11% at termination and a total weight gain that was by more than 60% lower than in the control group. A similar effect but much less pronounced was observed in mid-dose females, where body weight was 5.7% lower than for the controls and body weight gain was reduced by 32.8%. Body weight development in female dogs over the course of the study is depicted in Fig. 4.

Figure 4. Body weight development in female beagle dogs in the one-year study with mefetrifluconazole



Since it is widely acknowledged that individual values and findings in studies of this type may be more relevant than mean values or statistical analysis, due to the low number of animals per group, individual body weights in female dogs at different time points were examined in greater detail (Table 20).

Table 20. Individual body weights (kg) for female dogs over the course of the one-year study

Study day	0	182	364
Control group			
No. 123	12.0	12.2	12.5
No. 129	10.2	11.7	12.3
No. 131	10.5	12.1	13.1
No. 171	9.5	11.5	11.6
No. 173	8.5	13.3	14.0
Low dose group; 10 mg/kg bw per day			
No. 121	13.1	14.0	14.4
No. 141	10.8	11.8	12.7
No. 155	9.8	12.4	12.2
No. 158	9.0	9.7	11.6
No. 168	8.4	12.5	12.9
Mid-dose group; 30 mg/kg bw per day			
No. 128	11.2	11.6	12.5
No. 138	9.1	10.2	10.4
No. 139	12.1	12.2	12.5
No. 157	8.5	10.4	11.5
No. 171	10.8	12.9	13.0
High-dose-group; 150 mg/kg bw per day			
No. 130	10.0	9.9	10.3
No. 136	11.8	11.9	12.0
No. 140	12.4	12.2	11.9
No. 167	9.3	11.8	11.8
No. 170	8.2	10.4	10.3

In the control group, all females gained considerable weight over the course of the study but there was one exception in which total weight gain was only 0.5 kg. As to be expected, the majority of the weight increment for nearly all animals, was observed in the first half year of the study period. Virtually the same situation was found in the low-dose group where the lowest weight gain over the study was 1.3 kg. Remarkably, and for unknown reasons, one female slightl lost weight during the second half of the study. In the mid-dose group, the lowest total body weight gain was 0.4 kg, similar to that in the control group. It might be worth noting that the two animals with very low weight gains (control animal 123 and mid-dose animal 139) both had the highest individual starting weights in their respective group when treatment commenced. Based on individual animal data, the indications of a substance effect on body weight development at the mid-dose level of 30 mg/kg bw per day (as suggested by Fig. 4) could not be confirmed. By contrast, at the high dose, an adverse effect due to mefentrifluconazole is much more likely because of a net body weight loss over the course of the study in one female (No. 140) and during the first half of treatment in another (No. 130) and because of low total weight gains in two animals (Nos 130 and 136). However, it also seems clear that not all animals were affected in the same way since the two remaining dogs experienced a good weight gain even though this seems to have stopped in the second half of the treatment period.

Haematological findings were not consistent. The study authors reported a decrease in lymphocyte count in high-dose males as potentially adverse, but the change was statistically significant only after three months. Clinical chemistry findings of concern were confined to the highest dose level of 150 mg/kg bw per day and comprised a significantly higher ALP activity in both sexes at all time points of measurement, lower total protein and lower albumin concentrations. So the evidence from previous

observations in the 28-day and the 90-day studies was confirmed. In addition, there was some evidence of a decrease in calcium in top-dose males and females even though this finding gained statistical significance in male dogs only after three months and in females after three- and six months of treatment. Urinalysis revealed a more frequent occurrence of squamous epithelial cells in urine sediments from female dogs receiving 30 or 150 mg/kg bw per day. The relevance of this finding is equivocal.

There were no gross lesions which could be attributed to treatment. Organ weight analysis revealed a significant increase in absolute and relative liver weight in high-dose males, in relative adrenal weight in high-dose females and in relative kidney weight in both sexes at the top dose. Increases in absolute liver weight by 17% and in relative liver weight by 31% confirmed a similar trend in female dogs even though statistical significance was not achieved. A non-significant increase in absolute adrenal weight (by 25%) in high-dose females suggested a possible effect on this organ too. By contrast, the higher relative kidney weight observed may just reflect a lower terminal body weight. It is worth noting that the higher testis and lower uterus weights as observed in studies of shorter duration were not confirmed.

Histopathology of the liver revealed centrilobular or diffuse hypertrophy in all animals receiving the high dose and centrilobular hypertrophy in three male dogs and two females at the mid-dose level. Similar to that seen in the 90-day study, eosinophilic, finely granular cytoplasm and a lower degree of vacuolation than in the control animals was observed in all treated groups (in three low-dose, three mid-dose and in all five high-dose males, and in two, three, or five females in the different dose groups). Periodic acid–Schiff staining, however, revealed no difference in glycogen storage which contradicted the previous hypothesis. On balance, the eosinophilic change remains of unknown nature and equivocal toxicological relevance.

In female dogs, cytoplasmic vacuolation of the kidney was slightly reduced in mid- and high-dose animals suggesting a decrease in lipid storage but the toxicological relevance of this finding is considered low.

The increase in adrenal weight in high-dose females had no morphological correlate but was considered treatment-related by the study authors.

The NOAEL in this study was 30 mg/kg bw per day, based on a lower body weight and body weight gain in females at the top dose, supported by limited evidence of liver toxicity in both sexes (Keller et al., 2016).

(b) Dermal application

In a four-week study, mefentrifluconazole (Batch No. COD-001880; purity 98.6%) was dissolved in 0.5% aqueous CMC and dermally applied to groups of 10 male and 10 female Crl:WI(Han) Wistar rats at dose levels of 0, 100, 300 and 1000 mg/kg bw per day. The animals were treated for six hours per day on five days per week.

The rats were monitored for mortality and clinical signs of toxicity. Food intake and body weight development were observed. An FOB, haematological and clinical chemistry examinations were performed towards termination. At the end of the study the animals were necropsied and gross examination carried out. A range of organs was taken, weighed and prepared for histopathological examination.

There were no unscheduled deaths and no clinical signs could be attributed to treatment. No signs of irritation were observed. There were no notable changes in any parameter under investigation. Thus, both the systemic and the local NOAELs in this study were 1000 mg/kg bw per day, the highest dose tested (Buesen, 2015c).

(c) Exposure by inhalation

No study available or warranted.

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

A carcinogenicity study was performed in C57BL/6JRj mice over a duration of 18 months. In the main study, groups of 50 animals per sex were fed mefentrifluconazole (Batch No. OD-001740; purity 98.8%) at concentrations of 0, 20, 50 or 200 ppm for male mice (high dose 250 ppm for females). These dietary doses were equal to mean daily intakes of 3.5, 9.1 or 36 mg/kg bw in male mice and 4.9, 12.6 or 61.5 mg/kg bw in females. Satellite groups of seven animals per sex received the same doses to provide blood samples for the determination of plasma levels after 1, 4, 13, 26, and 52 weeks, but were terminated after the last sampling time without further examination.

The mice were observed daily for mortality and any occurrence of clinical signs. Food consumption and body weight were determined on a weekly basis for the first three months of the study and every four weeks thereafter. Blood smears were prepared after 12 and 18 months from all animals, but changes in total and differential white blood cell count and for visible changes in red blood cells were only investigated in smears from the control and high-dose groups, plus any mice which had to be killed intercurrently for humane reasons. Full haematological analysis was not performed because it is not compulsory in studies of this type. In contrast to many other long-term studies in mice, clinical chemistry parameters in blood or urine were not determined. The absence of such data might have an impact on the NOAEL since some presumably treatment-related alterations had been noted in the 90-day study in mice (see Schoenmakers 2015a in previous section).

All animals surviving until scheduled termination after 78 weeks and all available mice which were found dead or were intercurrently killed over the course of the study were necropsied and gross examination carried out. Adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes (with epididymides), and uterus were weighed. All these and an extensive selection of further organs from both the control and high-dose groups were subjected to histopathological examination. In addition, liver and thyroid were microscopically examined in all animals from the mid- and low-dose groups, along with kidneys from all males and adrenal glands from all females.

Survival was excellent in all male groups and sufficient in females (see Table 21). In females, a number of animals (1, 2, 7, and 3 in the control, low-, mid-, and high-dose groups respectively) had to be killed during the study because of skin lesions. These lesions were characterized by alopecia and wounds and are quite common in this mouse strain. The latter claim was substantiated by a summary of historical control data (Groeters & Mitkus, 2018). This data had been compiled at the request of the United States Environmental Protection Agency (US EPA) and Health Canada, and was also made available by the sponsor to JMPR. To conclude, there were no deaths and no clinical signs which could be attributed to treatment.

Mean body weight (see Table 21 for terminal values) and its gain were significantly reduced in top-dose females over the whole duration of the study and in male mice receiving 200 ppm for the majority of the treatment period. In females, transient decreases were also seen at the mid dose. At least in females, the extent of the difference between the high-dose group and the control groups was large enough to consider the maximum tolerated dose (MTD) to be reached since mean body weight was 10–14% lower after 26, 54 and 78 weeks, whilst body weight gain was reduced by up to 33%. In males, however, body weight effects were less pronounced with reductions of up to 15% at the top dose. Food intake was transiently compromised during the first third of the study in high-dose females but occasionally tended to be higher during subsequent stages. In males, food consumption was not altered at all. This data suggests that the adverse effects on body weight and its gain cannot be explained by a decrease in food intake and should be considered a clear sign of toxicity.

The limited haematological information obtained from blood smears did not point to adverse effects. The only finding worth noting was a significant increase in neutrophil granulocytes in high-dose males at termination, accounting for 33.2% of all white blood cells compared to 28.7% in the control group. Such an increase might indicate an inflammatory process but, in this case, cannot be interpreted with any certainty. Plasma concentrations of mefentrifluconazole, as measured in the satellite groups, were clearly dose-related but sometimes sublinear (underproportional) suggesting either saturated absorption or a more efficient elimination and/or metabolism. Male mice tended to have higher concentrations in plasma, even when groups receiving the slightly different maximum doses (200 compared with 250 ppm) were compared.

No gross lesions at necropsy could be attributed to treatment. However, there were some dose-related organ weight changes (shown in Table 21), but these findings should be regarded in the context of histopathological findings as reported in Tables 22 and 23 that follow.

Table 21. Mean organ weights in the carcinogenicity study in mice (termination at 78 weeks)

	Males				Females			
Dietary dose (ppm)	**0**	**20**	**50**	**200**	**0**	**20**	**50**	**250**
Number examined	45–49	47/48	47–50	50	46	46	41	42
Terminal body weight (g)	36.1	34.2	34.8	33.6*	34.0	33.0	31.6	29.5**
Liver, absolute (g)	1.34	1.42	1.53**	1.78**	1.38	1.43	1.68	1.94**
Liver, relative (%)	3.73	4.19**	4.40**	5.28**	4.17	4.39	5.45*	6.53**
Kidneys, absolute (g)	0.52	0.49*	0.48**	0.45**	0.44	0.44	0.58	0.42
Kidneys, relative (%)	1.45	1.46	1.39	1.35*	1.32	1.35	1.85	1.43
Adrenal glands, absolute (mg)	3	2.8	3.3	3.8**	10.7	9.1	10.1	11.6
Adrenal glands, relative (%)	0.0083	0.0084	0.0096	0.012**	0.033	0.028	0.033	0.040*
Uterus, absolute (g)	-	-	-	-	0.12	0.13	0.13	0.13
Uterus, relative (%)	-	-	-	-	0.35	0.40	0.42	0.47*

* $p < 0.05$, ** $p < 0.01$, Dunnett′s test; organ weights rounded to not more than two significant figures

The dose-related and mostly statistically significant liver weight increase in mid- and high-dose males and females was accompanied by microscopic findings which were indicative of a substance-related adverse effect. In contrast, in the absence of statistically significant histopathological changes, the increase in relative liver weight in low-dose males might simply have resulted from the lower mean body weight. A significant increase in macrovesicular fatty change was observed in mid- and high-dose males and in high-dose females. It was noted that background incidence of this finding in control groups was also quite high in both sexes (see Table 22) but the difference in the number of animals affected was statistically significant. The severity of fatty degeneration of different types is reported in Table 23 and discussed below. In the high-dose male group, in addition, 38 animals exhibited centrilobular eosinophilic inclusions which were not seen in any other male group nor amongst females. Single cell necrosis, by contrast, was observed in ten out of 50 top-dose females and in one mid-dose female. Even though the latter finding were exhibited only to a minimal degree they were considered adverse. A number of mechanistic studies were performed to further investigate the MOA behind the liver effects and their potential relevance to humans, and these are reported here in section 2.6(d).

The lower mean absolute kidney weight seen in all male dose groups partly reflected their lower body weight. This became clearest in the low-dose group in which relative kidney weight was nearly identical to the control group. At the two upper dose levels, in particular with the high dose, a lower incidence of tubular vacuolation might have also contributed to reduced organ weight. Vacuolation is a common finding in untreated male mice of this strain and was noted in 42 control males and 42 low-dose males. However, it was seen at the mid dose level in only 34 male mice and in only six of those receiving 200 ppm. The toxicological relevance of this reduced vacuolation is uncertain. However, taking a precautionary approach, at least the very pronounced change in high-dose males is regarded as potentially adverse. It is worth noting that a similar effect was described in the one-year dog study of Keller et al. (2016). Cytoplasmic alterations consisting mainly of reduced vacuolation were observed in the kidneys of female dogs and in the livers of both sexes.

The increase in absolute and relative adrenal weights in high-dose males was not accompanied by any histopathological findings and was covered by recent historical control data. In four 18-month studies in the same mouse strain which had been initiated in the same test facility in 2013 or 2014, the range in mean absolute adrenal weights in untreated control males was 3.0 to 3.7 mg, with a mean of 3.4 mg. The mean relative organ weights in these studies varied between 0.008% and 0.014% and the mean was 0.0098%. To conclude, the increase in adrenal weight in male mice in this study with mefentrifluconazole is at least of questionable relation to treatment and cannot be considered unequivocally adverse.

The situation with female groups was quite different; all mean absolute and most mean relative adrenal weights in the control and treated female groups were clearly outside the historical control range. This is exemplified by the highest mean absolute adrenal weight in female mice from the same historical database as above which was 8 mg, that is, lower than in all groups including the control in the current study. Therefore, HCD is of no help in assessing the apparent increase in adrenal weight in female mice. Of course, the significantly higher relative organ weight can be considered to reflect a lower terminal body weight. However, also reported was a microscopic finding that was unique to high-dose females. This cytoplasmic change was described as diffuse eosinophilic appearance of cells in all cortical zones, along with a slight increase in cell size. It was seen in 20 females in the 250 ppm group, compared to only two similarly affected control females. This finding was interpreted by the study author, with some reservations, as a stress response. Its toxicological relevance is equivocal and its contribution to the organ weight increase unknown.

The increase in relative uterine weight, in the absence of histological changes, may be simply explained by the lower terminal body weight of the female mice and does not suggest a specific effect on the organ.

The only organ which was not weighed but displayed remarkable histopathological findings was the thyroid. In high-dose males there was a significant increase in focal follicular hyperplasia (Table 22). The incidence in that group was clearly above the historical control range and was considered adverse.

Table 22. Summary of selected histopathological findings in the 18-month study in mice

	Males				Females			
Dietary dose (ppm)	**0**	**20**	**50**	**200**	**0**	**20**	**50**	**250**
Number examined	50	50	50	50	50	50	50	50
Number killed at scheduled termination	49	48	50	50	46	46	41	42
Neoplastic findings								
Liver, adenoma	1	2	3	0	0	0	0	0
Liver, carcinoma	1	1	1	0	1	0	0	0
Liver, Ito cell[a] tumour	0	0	0	0	1	0	0	0
Thyroid, follicular cell adenoma	0	0	0	2[#]	1	0	1	3[#]
Non-neoplastic findings								
Liver: macrovesicular fatty change, all grades	23	16	35*	46**	21	16	22	39**
Liver: centrilobular eosinophilic inclusions	0	0	0	38**	0	0	0	0
Liver: increase single cell necrosis (minimal degree)	0	0	0	0	0	0	1	10*
Kidney: tubular vacuolation	42	42	34	6**	Incidence not reported for females			
Thyroid: follicular cell hyperplasia (focal or multifocal)	21	16	17	37**	19	14**	8*	26
Adrenals: cytoplasmic eosinophilic change in cortex	0	NE	NE	0	2	6	3	20**

[a] Fat-storing cells usually located in the space of Disse; NE: Organ not microscopically examined in that group

* $p < 0.05$, ** $p < 0.01$, Fisher′s exact test, one-sided; [#] Significant trend in Cochran–Armitage test

The only histopathological finding that was also significantly more rponounced at the mid-dose level (at least in male mice) and for which a dose–response relationship was apparent was macrovesicular fatty liver change, and this was subject to further analysis for severity of the effect. In addition, diffuse fatty change was taken into consideration by the Meeting with regard to its degree even though the total incidence of this finding did not differ among the groups. The results of this analysis for the degree of fatty change are compiled in Table 23. In male mice, moderate and marked fatty degeneration of

the macrovesicular type was seen only at the mid- and high-dose levels, along with a larger number of animals exhibiting a slight change (grade 2). Similar observations were made in females, with an outstandingly high number of affected mice in the high-dose group. No differences with regard to severity were seen between the control and the low-dose groups, suggesting a treatment-related effect at the two upper dose levels.

With regard to the generally even commoner diffuse fatty degeneration, severity was increased in both sexes at the mid- and high-dose levels too, and was most pronounced, once again, in high-dose females. There was no difference between the control and low-dose groups of female mice, whereas the finding of grade 3 fatty change was more than double in low-dose males compared to the controls. However, considered against the high background incidence of this lesion in all groups and because macrovesicular fatty change was not increased in this group, any difference might be treatment-related but was not considered adverse.

Table 23. Severity of fatty liver change in male and female mice in the long-term study with mefentrifluconazole

Sex	Males				Females			
Dietary dose (ppm)	**0**	**20**	**50**	**200**	**0**	**20**	**50**	**250**
Number examined	50	50	50	50	50	50	50	50
Macrovesicular fatty change: all grades	23	16	35*	46**	21	16	22	39**
Minimal	20	14	8	13	14	13	7	3
Slight	3	2	14	16	7	3	12	2
Moderate	0	0	11	13	0	0	3	11
Marked	0	0	2	4	0	0	0	23
Diffuse fatty change: all grades	48	46	47	48	40	44	38	43
Minimal	7	5	2	1	10	11	5	3
Slight	30	15	3	3	29	32	21	6
Moderate	10	24	33	35	1	1	11	8
Marked	1	2	9	9	0	0	1	26

* $p < 0.05$, ** $p < 0.01$, Fisher′s exact test, one-sided

The total incidence of neoplastic findings was relatively low in this study. There was no dose-related increase in tumour frequency in any organ, the thyroid being the only exception. A slight increase in liver adenoma in male mice is apparent when the control group is compared to the mid-dose group, but this was eventually discounted as not dose-related since no liver tumours were seen in the highest dose group. An overview of selected tumour and on non-neoplastic findings of the liver and thyroid is also shown in Table 22.

The small increase in follicular cell adenoma of the thyroid was not significant in Fisher′s exact test which is, however, not very sensitive for rare tumours. A test for trend had not been performed as part of the original study. Therefore, during review for JMPR 2021, an asymptotic one-sided Cochran–Armitage trend test was applied. In its simplest version, it resulted in a statistically significant increase in thyroid adenoma in males ($p = 0.0283$) but not in females. If the (non-linear) doses are given their appropriate weight however, the one-sided test gives a lower p value of 0.0082 for males and revealed a significant increase also for females ($p = 0.0347$). For interpretation of such findings, HCD may be helpful. Unfortunately, it must be acknowledged that the database of suitable studies as submitted by the sponsor was small and therefore of limited value. The database comprised only four studies of the same mouse strain initiated in the same test facility in 2013 or 2014 (years the study under review was itself performed). In these studies, follicular cell adenoma incidence in male mice was either 0 or 1 out of 50, with a mean of 1.5%. In females, there was a range from 0 to 6%, with a mean of 1.2%. So the actual study incidence in the top-dose group was at its upper edge in females (6%) but fell above it in males (4%). Other HCD provided with the study report might possibly suggest a higher incidence in untreated mice of the same strain of up to 6% in males and 8% in females. However, this HCD cannot be used since those studies had been performed between 1998 and 2007, and in a different laboratory.

It seems likely that the higher incidence of thyroid adenoma in the high-dose group was related to the increase in focal hyperplasia as observed in both sexes (Table 22) even though statistical significance was only achieved for males. Both lesions are often considered a pathological continuum since hyperplasia may progress to benign tumours.

The lowest dose of 20 ppm (equal to a mean daily intake of 3.5 mg/kg bw) was the NOAEL in this study. It is based on higher liver weights in both sexes and on a statistically significant increase in macrovesicular fatty liver change in male mice receiving 50 and 200 ppm and in high-dose females. Also, at the mid and high doses, a higher grade (severity) of macrovesicular and diffuse fatty degeneration was noted in both sexes. Moreover at the maximum dose level other observations included reductions in body weight and body weight gain in males and females, further microscopic liver findings such as centrilobular eosinophilic inclusions in males or single cell necrosis in females and follicular hyperplasia of male thyroids. Effects of less certain toxicological relevance comprised a reduction in tubular vacuolation in kidneys of males and an increase in adrenal weight in both sexes accompanied by cortical eosinophilic change in females. Some progression of non-neoplastic thyroid findings to benign tumours was seen at the top dose in both sexes and was reflected in a positive test for trend. This last effect was considered treatment-related. Accordingly, the NOAEL for oncogenicity was the mid dose of 50 ppm (equal to 9.1 mg/kg bw per day) (Schoenmakers, 2015b).

Rat

In a long-term, combined chronic toxicity and carcinogenicity study, groups of 50 Wistar [Crl:WI(Han)] rats per sex received mefentrifluconazole (Batch No. OD-001740; purity 98.8%) at dietary concentrations of 0, 100, 600, or 3600 ppm for 24 months. For male rats mean daily intakes of 4, 25 and 163 mg/kg bw were calculated. In females, dietary intake corresponded to mean daily doses of 6, 38 and 302 mg/kg bw. In addition, satellite groups of 10 animals per sex were given the same dietary doses but only for 12 months and were then terminated. With the exception of high-dose females, and as might be expected, the mean daily intakes in these groups were slightly higher than for the main study groups that received doses over a longer administration period.

Animals were monitored daily for mortality and the occurrence of clinical signs. A detailed clinical examination of all animals took place once a week. Ophthalmoscopy was confined to satellite animals and was performed prior to commencement of treatment in all, and at its end in control and high-dose animals. Food consumption was determined once per week for a day on a cage basis (five animals were housed together) but only for the first three months. Thereafter food intake was observed at four-week intervals until termination. A similar approach was taken for body weight determination. The rats were weighed on the day of first substance administration and once a week for the first three months, then monthly and eventually at terminal kill, prior to necropsy.

Blood sampling for haematology and clinical chemistry and urinalysis was performed in the satellite groups after 3, 6, and 12 months. At termination (also when animals intercurrently died or had to be killed for humane reasons) the rats were necropsied and assessed for gross pathology. In both main study and satellite groups, organ weights of adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes (with epididymis) and uterus were determined. In addition the thyroid was weighed in the satellite groups after one year. A wide range of organs and tissues was prepared for subsequent histopathology. Histopathological examination of females was confined to the control and high-dose groups, with the exception of liver and thyroid which were examined in all groups, as were all gross lesions. In males, by contrast, full histopathology comprised all animals on study. This decision was taken to clarify the incidence of tumours of the lymphoreticular system for which a non-significant increase had been observed in male rats (see below). In selected animals, immunohistochemical stains were applied to the liver and the pituitary in order to detect basophilic or eosinophilic foci if they were present.

In general, the animals tolerated the administration of mefentrifluconazole quite well. There were no unscheduled deaths and no clinical (including ophthalmoscopic) signs of toxicity could be attributed to treatment. In the satellite groups, during 12 months of treatment, there was no mortality at all. In the main study, survival was satisfactory in all groups and was apparently not affected by the test substance. In male rats, mortality was highest in the low-dose group (24%) but much lower than among animals receiving 3600 ppm (6%). It was remarkable (but is not completely unknown) that no control animal died during the whole two-year study period. In females mortality varied between 10% and 24%

with the lowest percentage noted at the top-dose level. An advantageous effect due to lower body weight in this group might have contributed to this finding.

Mean body weights were consistently lower in high-dose males and females from the beginning of the administration period onwards, reaching their maxima (–13% in males and –22% in females) towards termination. Consistent with this, body weight gain was significantly reduced in these groups. The adverse effect on body weight and its development at the top dose cannot be attributed to a lower intake of energy and nutrients since food consumption was unaltered. In mid-dose females body weight tended to be lower during the treatment period and the difference to the control group was statistically significant at termination (Table 25). However, since this difference was less than 10% compared to the control group mean, the reduction was not considered biologically relevant.

Haematology did not reveal any changes in red or white blood cell parameters that could be attributed to treatment. However, activated partial thromboplastin time (APTT) was reduced in mid- and high-dose males and in high-dose females (Table 24). In contrast to an increase in this parameter, a reduction does not necessarily suggest an adverse effect on blood coagulation and is sometimes observed as a result of ongoing inflammatory processes. The study authors speculated about a possible increase in the production of coagulation factors but did not substantiate this hypothesis. In addition, platelet count was significantly decreased in high-dose males after 12 months. No explanation is offered for this finding but the reduction was not severe enough to postulate any adversity.

There were occasional and transient alterations in some clinical chemistry parameters but very few were presumably treatment-related (Table 24). These findings comprised increases in the activity of certain enzymes suggesting an effect on the liver which was, however, not necessarily adverse but should be interpreted in the context of additional findings, in particular with regard to organ weight and histopathology. The increase in ALP and (even though not statistically significant) GGTP activities were in line with similar observations in the 90-day study in rats reported above. Lower glucose and transiently higher cholesterol concentrations in both sexes, although not always achieving statistical significance, might also indicate liver toxicity or at least a disturbed liver function at the two upper dose levels. In addition, there was a remarkable decrease in bilirubin which might be treatment-related but, in contrast to an increase, is not considered an adverse effect. Urinalysis did not reveal any changes that were attributable to substance administration.

Table 24. Selected haematological and clinical chemistry findings in rats from satellite groups treated with mefentrifluconazole for one year (mean values, n = 10 per sex and dose)

	Males				Females			
Dose (ppm)	**0**	**100**	**600**	**3600**	**0**	**100**	**600**	**3600**
Activated patial thromboplastin time (s)								
Day 94	21.4	21.3	20.1	18.8**	19.9	19.7	19.6	18.5*
Day 185	19.7	19.3	18.8*	17.9**	18.5	18.7	18.4	17.3
Day 365/367	20.3	19.8	19.2*	18.4**	19.1	19.6	19.3	17.9*
Platelets (10^9/L)								
Day 94	771	727	719	667	819	796	750	781
Day 185	693	661	663	663	747	724	691	709
Day 365/367	724	661	680	640**	716	717	666	691
Alkaline phosphatase (µkat/L)								
Day 94	1.08	1.12	1.38**	1.59**	0.48	0.61	0.56	0.88**
Day 185	0.92	0.98	1.24**	1.33**	0.34	0.47**	0.43	0.63**
Day 365/367	0.92	1.00	1.28**	1.36**	0.33	0.50*	0.44	0.72**
γ-Glutamyl transferase (nkat/L)								
Day 94	0	1	1	0	8	8	12	17
Day 185	0	0	0	0	0	0	0	3
Day 365/367	0	0	1	2	0	0	0	3

	Males				Females			
Dose (ppm)	**0**	**100**	**600**	**3600**	**0**	**100**	**600**	**3600**
Glucose (mmol/L)								
Day 94	6.95	6.54	6.38	5.49**	5.46	5.40	5.50	4.86*
Day 185	6.88	6.53	6.29	5.76**	5.63	5.26	5.19	5.05
Day 365/367	6.59	6.43	6.22	5.79**	6.10	5.91	5.64	4.90**
Cholesterol (mmol/L)								
Day 94	1.63	1.88	1.92*	2.02*	1.39	1.40	1.29	1.95**
Day 185	1.79	2.05	2.12	2.29**	1.60	1.53	1.45	1.96
Day 365/367	2.33	2.47	2.57	2.77	2.18	2.09	1.97	2.37
Bilirubin (µmol/L)								
Day 94	1.96	1.83	1.76	1.28**	2.00	1.90	1.81	1.23**
Day 185	1.54	1.69	1.56	1.21	3.08	2.98	2.45*	1.71**
Day 365/367	1.33	1.51	1.48	1.15	2.39	2.18	1.96	1.28**

* $p < 0.05$, ** $p < 0.01$, Kruskal–Wallis & Wilcoxon test

Gross pathological examination did not detect treatment-related adverse findings. A higher incidence of macroscopic foci was noted in the testes of high-dose males (10 compared with only two affected animals in the control). Unfortunately these foci were not described in any more detail. In females, an increase in foci of the adrenal cortex was observed in all treated groups but was not dose-related. Since both types of foci, the testicular as well as the adrenal, had no histological correlate they were regarded as incidental. Absolute organ weights were not affected or were lower in high-dose males and females, in agreement with the reduced absolute body weights shown in Table 25 for liver and kidneys. Increases in the relative weights of many organs, some reaching statistical significance, may also find explanation in the lower terminal body weights achieved. On balance, all these changes were at most indirectly attributable to treatment. The increase in relative liver weight in high-dose males and females, however, was consistent with histopathological and clinical chemistry findings and therefore was considered potentially adverse.

Table 25. Mean organ weights in the long-term study in rats (termination after 24 months)

	Males				Females			
Dietary dose (ppm)	**0**	**100**	**600**	**3600**	**0**	**100**	**600**	**3600**
Number examined	50	38	39	47	38	40	41	45
Terminal body weight (g)	559.6	557.3	548.6	492.3**	320.5	319.9	296.4**	245.6**
Liver, absolute (g)	11.66	11.39	12.27	10.94	6.67	6.99	7.10	6.34*
Liver, relative (%)	2.08	2.04	2.21	2.22**	2.09	2.20	2.44**	2.58**
Kidneys, absolute (g)	2.94	2.97	2.92	2.74*	2.00	2.02	2.00	1.78**
Kidneys, relative (%)	0.53	0.54	0.54	0.56**	0.63	0.64	0.68*	0.72**

* $p < 0.05$, ** $p < 0.01$, Kruskal–Wallis & Wilcoxon test; organ weights rounded to not more than two significant figures

Histopathology revealed centrilobular hypertrophy of hepatocytes as the only remarkable finding that could be clearly attributed to treatment. However, it was confined to the highest dose level at which 15/50 male rats and 7/50 females were affected. Hepatocellular hypertrophy was not noted in the control or in any of the other treatment groups receiving lower doses. Dilatation of the ductus choledochus was more frequently seen in high-dose females compared to the respective control group (5/50 compared with 1/50). This (non-significant) finding might also point to liver toxicity. However, there was no increase in hyperplasia or tumours of the liver.

Despite an apparent numeric increase in endometrial adenocarcinoma it exhibited no dose–response relationship. This tumour was observed in one of 50 control animals and occurred at incidences of 7/38, 3/37 and 5/50 females in the low-, mid- and high-dose groups. Differences from the control group did not gain statistical significance and there were no further histological findings that might suggest the uterus to be an additional target organ. Furthermore, all group incidences were covered by appropriate

HCD. The same holds true for malignant lymphomas in male rats which were seen only in the treated groups and not in controls. The incidence of this tumour type was 4% in the low- and mid-dose groups and 6% (3/50) at the high dose, but the increase was not statistically significant. The historical control range from 12 studies in Wistar rats which were initiated between 2003 and 2013 varied from 0 to 6% with a mean of 2.5%. On balance there was no convincing evidence that these tumour findings could be attributed to treatment.

The NOAEL in this study was 600 ppm (equal to 25 mg/kg bw per day). This was mainly based on the adverse effect on body weight and its development in both sexes at the top dose of 3600 ppm. A higher relative organ weight, hepatocellular hypertrophy and a number of clinical chemistry findings suggested an effect on the liver, at least in the highest dose group, however in the absence of further microscopic findings, these effects were not regarded as adverse. There was no evidence of carcinogenicity due to mefentrifluconazole in rats under the conditions of this study (Buesen et al., 2016a).

2.4 Genotoxicity

Mefentrifluconazole was extensively tested in a battery of appropriate studies which clearly revealed that the substance was devoid of a genotoxic potential in vitro. However, all these studies made apparent the marked cytotoxicity of this compound, both towards bacteria and mammalian cells, and this avoided, in many cases, the need for testing at higher concentrations. Because of the consistently negative results of the in vitro studies, it was considered acceptable that in vivo testing was confined to a single study that was also negative. An overview of the available genotoxicity studies is given in Table 26.

Table 26. Summary of studies on the genotoxicity of mefentrifluconazole

Study type	Test system	Concentration range	Result	Reference
		In vitro		
Gene mutation in bacteria (Ames test)	*S. typhimurium* strains TA1535, 1537, 98, 100 and *E. coli* strain WP2uvrA both ± S9 mix	0–333, 1000, 5000 µg/plate (depending on strain and test method)	Negative	Woitkowiak, 2014
		0–333, 1000, 5000 µg/plate (depending on strain and test method)	Negative	Woitkowiak, 2015a
Gene mutation in mammalian cells (mouse lymphoma assay, *tk* locus)	Mouse lymphoma L5178Y cells, ± S9 mix, 4 h or 24 h exposure	0–45, 60 µg/mL (depending on the experiment, limited by cytotoxicity)	Negative	Wollny, 2015a
		0–50, 62.5 µg/mL	Negative	Wollny, 2015b
Chromosome aberration test	Human lymphocytes, ± S9 mix, 4 h or 22 h exposure	0–33.3, 60, 100 µg/mL (depending on the experiment, limited by cytotoxicity)	Negative	Naumann, 2019
Micronucleus assay (cytokinesis block method)	V79 Chinese hamster lung cells (fibroblasts), ±S9 mix, 4 h or 24 h exposure	0–50 µg/mL	Negative	Schulz & Landsiedel, 2014
	Human lymphocytes, ± S9 mix, 4 h or 24 h exposure	0–8.2 µg/mL	Negative	Sokolowski, 2015a
		In vivo		
Micronucleus assay in mouse bone marrow	Male NMRI mice, single oral application, animals killed 24 h or 48 h post dosing	0, 375, 750, 1500 mg/kg bw	Negative	Schultz, Becker & Landsiedel, 2014

(a) In vitro studies

In an Ames test in four strains of *Salmonella typhimurium* and one of *Escherichia coli*, mefentrifluconazole (Batch No. OD-001880; purity 98.6%) was tested by means of both the pre-incubation and the plate incorporation methods. All tests were performed with and without metabolic activation by S9 mix. The test item, as well as the positive control substances, was dissolved in DMSO which was also used as the negative control substance. In the absence of S9 mix, the following positive control substances were employed: *N*-methyl-*N'*-nitro-*N*-nitroso guanidine at a concentration of 5 µg/plate for strains TA100 and TA1535; 9-aminoacridine at 100 µg/plate for TA 1537; 4-nitro-*o*-phenyldiame at 10 µg/plate for TA98; and 4-nitroquinolone-*N*-oxide at 5 µg/plate for *E. coli* WP2 uvrA. In all the activation experiments, 2-aminoanthracene was used at concentrations of 2.5 µg/plate (all *Salmonella* strains) or 60 µg/plate (*E. coli*).

The maximum amounts of mefentrifluconazole in the various experiments differed greatly, depending on their toxicity towards the bacteria. In the plate incorporation assay a range of seven concentrations from 0 to 5000 µg/plate was applied (with or without metabolic activation) to *E. coli* WP2 uvrA as well as to all *Salmonella* strains except TA1537. When TA1537 was treated in the absence of S9 mix, 333 µg/plate was the maximum concentration. In the pre-incubation assay however, seven concentrations from 0 to 1000 µg/plate were applied to all the *S. typhimurium* strains, with and without activation, whereas the *E. coli* strain was only exposed to the limit concentration of 5000 µg/plate.

Some cytotoxicity was observed from concentrations of 100 µg/plate (pre-incubation test, TA1537) or 333 µg/plate (plate incorporation test, TA1537) onwards, but the various strains differed with regard to their sensitivity. Precipitation started, with all strains, at 1000 µg/plate. These findings were independent of the presence or absence of metabolic activation.

No increase in revertants was observed with any of the tested concentrations of mefentrifluconazole. The positive control substances, in contrast, gave the expected clear responses.

Under the conditions of this study, mefentrifluconazole was not mutagenic to bacteria in the Ames test. The highest attainable concentrations, if not the limit of 5000 µg/plate, were tested. (Woitkowiak, 2014)

A very similar study of the same type was performed with another batch of mefentrifluconazole (Batch No. 01651-181; purity 97.9%) . Again, the pre-incubation and plate incorporation methods were followed, both in the presence and absence of S9 mix for metabolic activation. The same strains of *Salmonella typhimurium* or *Escherichia coli* as in the previous study were tested. The vehicle and positive control substances were also as in Woitkowiak (2014). In the plate incorporation assay, concentrations of up to 5000 µg/plate were applied, with TA1537 being the only exception; for this strain, 1000 µg/plate was the maximum concentration. In the pre-incubation test, all strains except TA1537 could be exposed to concentrations up to 1000 µg/plate. With TA1537, the maximum test concentration was 333 µg/plate. All individual experiments were carried out in triplicate.

The negative outcome of the previous study with regard to mutagenicity was confirmed. There was no increase in revertant colonies at any tested concentration of mefentrifluconazole whereas the positive control substances produced the appropriate responses. Cytotoxicity and precipitation were observed from 100 or 333 µg/plate upwards with TA1537 again, being the most sensitive strain. Previous metabolic activation did not alter these effects (Woitkowiak, 2015a).

Mefentrifluconazole (Batch No. OD-001740; purity 98.8%) was tested for its ability to induce forward mutations in mammalian cells by assessing the mutation at the thymidine kinase (*tk*) locus in mouse lymphoma L5178Y cells. Two independent experiments were conducted in the presence or absence of metabolic activation with two parallel cultures each. The solvent was DMSO. Based on the results of a preliminary cytotoxicity assay, concentrations up to 60 µg/mL were used in the main experiment since cytotoxicity and precipitation had been observed at concentrations of 62.9 µg/mL and above. The treatment intervals for both experiments in the presence and absence of metabolic activation were generally four hours, except in experiment II (in the absence of metabolic activation) where a treatment interval of 24 hours was applied. Methyl methanesulfonate (MMS) and cyclophosphamide (CPA) served as positive controls in the experiments with and without metabolic activation, respectively.

After the incubation period, treatment media were replaced by culture medium in both experiments and the cells were incubated for 48 hours for expression of mutant cells. This was followed by incubation of cells in selection medium containing 5-trifluorothymidine (TFT) for 10–15 days.

No substantial and reproducible dose-dependent increase in mutant colony numbers was observed in either main experiment. No relevant shift of the ratio of small versus large colonies was observed up to the highest concentration of test substance. It was noticed that the maximum concentration of 60 µg/mL was already cytotoxic and the treated cultures could be evaluated only in one experiment (the trial in which cells were exposed for 24 hours without S9). The reference mutagens MMS and CPA produced a distinct increase in mutant colonies, indicating that the tests were sensitive and valid.

Under the conditions of this study, mefentrifluconazole was negative in the mouse lymphoma assay, with and without metabolic activation (Wollny, 2015a).

In a similar study, another batch of mefentrifluconazole (Batch No. 01651-181; purity 97.9%) was tested for mutagenicity in a mouse lymphoma assay. The method was essentially the same as in the study of Wollny (2015a) described above, but this time three independent experiments were performed both in the absence and presence of metabolic activation, with two parallel cultures each. The maximum tested concentration was 62.5 µg/mL, the same magnitude as before. The same positive control substances were employed.

In most experiments, concentrations up to 50 µg/mL could be evaluated. The negative results of the first study were confirmed since no increase in mutant frequency was observed in any experiment at any concentration. Mefentrifluconazole proved negative under the conditions of this study. It may be concluded from both mouse lymphoma assays that the substance is devoid of mutagenic potential in mammalian cells but that its cytotoxicity is quite high (Wollny, 2015b).

Mefentrifluconazole (Batch No. COD-001740; purity 98.8%) was tested for its potential to induce micronuclei in V79 Chinese Hamster lung fibroblasts as an indication of clastogenic or aneugenic activity. Two independent experiments were carried out with and without the addition of liver S9 mix from induced rats, for exogenous metabolic activation. The vehicle DMSO was also used as a negative control, ethyl methanesulfonate (EMS) served as positive control in the absence of metabolic activation and CPA as positive control in the presence of metabolic activation. Cells were incubated with the test item at concentrations in the range 0.39–50 µg/mL. The maximum concentration had been selected on the basis of a preliminary cytotoxicity assay. Two independent experiments were performed where the cells were incubated for four hours (with and without S9 mix) or 24 hours (without S9 mix). In the experiments with four hour exposure treatment was followed by recovery times of 20 or 40 hours, respectively. Cells were harvested either 24 or 44 hours after the application of test substance. In the experiment with prolonged (24 hour) exposure, the cells were then immediately harvested. Following exposure to the test or control substance, cell cultures were incubated with cytochalasin B, then fixed and DNA and cytoplasm stained. Cytotoxicity parameters and the number of micronucleated cells were determined in at least 1000 binucleated cells per culture, making 2000 cells for each test group since the experiments were run in duplicate.

Cytotoxicity was indicated by a dose-dependent decrease in proliferative activity of treated cells. Looking at the actual results, the (expected and common) relative increase in cell count, cytokinesis-block proliferation index and replicative index were each clearly reduced in all experiments at the highest applied concentration of mefentrifluconazole, but sometimes even at lower concentrations. In addition, dose-related effects on cell attachment and morphology, as well as precipitation in the culture medium were observed.

Despite the above, the test substance did not lead to a biologically relevant increase in the number of micronucleated cells either with or without metabolic activation. An increase in the frequencies of micronuclei induced by the positive control substances EMS and CPP clearly demonstrated the sensitivity of the test system and of the metabolic activity of the S9 mix.

In conclusion, mefentrifluconazole did not exhibit clastogenic or aneugenic activity under the conditions of this in vitro study in V79 cells, neither in the absence nor in the presence of metabolic activation (Schulz & Landsiedel, 2014).

A second in vitro micronucleus assay with another batch of mefentrifluconazole (Batch No. 01651-181; purity 97.9%) was performed in peripheral human lymphocytes. These cells had been obtained from a 31-year-old man (Experiment I) and from a 32-year-old woman (Experiment II). Both donors were considered healthy, were non-smokers and did not receive any regular medication. Prior to exposure, the cell cultures were stimulated for 48 hours by phytohaemagglutinine.

The test substance was dissolved in DMSO and applied at concentrations of 2.0, 4.1, or 8.2 µg/mL. These rather low concentrations had been selected on the basis of preliminary cytotoxicity testing. In experiment I, the exposure time was four hours, both in the absence and presence of S9 mix. In experiment II, cells were also exposed for four hours with metabolic activation but for 24 hours in its absence. In all cultures receiving four-hour treatment, exposure was followed by a 16-hour recovery period. At its end, or immediately following the 24-hour exposure period, cytochalasin B was applied for a further 20 hours before harvesting.

Whereas just the vehicle was used for negative control purposes, different positive control substances were employed. In the absence of metabolic activation, mitomycin C was applied in the case of four hour exposures but demecolcin used for 24 hour ones. With S9 mix, cyclophosphamide was used as the reference mutagen.

All tests were performed in duplicate. Following cell preparation and staining, 1000 binucleated cells per culture were evaluated for cytogenetic damage. This was apparently done in a "blind" manner since slides had been coded beforehand.

Some cytotoxicity was seen at all concentrations in both experiments but all the cultures could be assessed and a clear dose–response relationship became apparent. There was no increase in micronucleated cells at any concentration of the test item, neither with nor without metabolic activation. By contrast, the positive control substances gave the expected results. To conclude, mefentrifluconazole was devoid of clastogenic potential in this in vitro micronucleus test in human lymphocytes. In addition, the study author stated there was no evidence of aneugenic activity (Sokolowski, 2015a).

The micronucleus assay is now considered the preferred method to detect clastogenic activity in vitro and may give indications of aneuploidy. Nevertheless, a classical chromosome aberration test was also performed. Mefentrifluconazole (Batch No. OD-001740; purity 98.8%) was applied to human lymphocytes, with or without metabolic activation, in three independent experiments. To obtain the cells, blood samples had been drawn from two donors, (28-year-old woman and a 35-year-old man, both healthy non-smokers). The test item was dissolved in DMSO which also served as a negative control. Positive control reagents were EMS for the non-activated experiments and CPP when S9 mix was present.

Exposure times were 4 and 22 hours without activation and just four hours in the presence of S9 mix. The four-hour exposures were followed then by an 18-hour recovery period. Accordingly, all cells were harvested at 22 hours after treatment had begun. Three hours prior to sampling, 0.2 µg/mL of colcemid was applied for spindle inhibition. A number of concentrations were tested but, due to cytotoxicity and precipitation, the maximum concentrations that could be used for cytogenetic evaluation were as follows: 33.3 µg/mL in the experiment with four-hour exposure without activation, 100 µg/mL in the four-hour exposure experiment with activation and 60 µg/mL in the cultures which were treated for 22 hours in the absence of S9 mix (see also Table 24).

Duplicate cultures were run for each concentration and condition. Following harvest, preparation, staining and coding of slides, 1000 cell per culture were counted to determine the mitotic index as a measure of cytotoxicity. Finally 150 well spread metaphases per culture were evaluated for structural chromosomal aberrations.

Some cytotoxicity and precipitation were apparent at the highest concentrations that were used for cytogenetic evaluation as described above, but cytogenetic assessment was possible. In the experiments with four-hour exposure to mefentrifluconazole, with and without metabolic activation, no increases in structural chromosome aberrations were observed. In the experiment in the absence of metabolic activation, but with longer duration of treatment (22 hours), a positive trend with regard to aberrant cells (including gaps) was noted (Table 27). However, the increase over the control level was very slight and the percentages of aberrant cells were covered by the historical control range of the

performing laboratory. The HCD was based on 22 studies from 2016 and 2017 (0.0–2.0% including and 0.0–1.9% excluding gaps). On balance, the finding is more likely here to be incidental than genuinely treatment-related. The positive control substance produced the expected increases in aberrant cells. No evidence of an increase in polyploid metaphases was seen in any experiment at any concentration.

Table 27. Percentages of aberrant cells (mean of two cultures, including and excluding gaps) in three experiments from the chromosome aberration study with mefentrifluconazole

Treatment	Exp. I (4 h, −S9 mix)		Exp. II (4 h, +S9 mix)		Exp. III (22 h, −S9 mix)	
Aberration	Incl. gaps	Excl. gaps	Incl. gaps	Excl. gaps	Incl. gaps	Excl. gaps
Solvent (DMSO)	0.3	0.3	0.3	0.3	1.0	0.7
EMS	8.3	8.3	ND	ND	25.0	25.0
CPP	ND	ND	13.3	13.3	ND	ND
10.9 µg/mL	1.0	1.0	ND	ND	ND	ND
19 µg/mL	1.0	1.0	1.0	0.7	ND	ND
33.3 µg/mL	0.0	0.0	1.3	1.3	ND	ND
40 µg/mL	ND	ND	ND	ND	1.0	1.0
50 µg/mL	ND	ND	ND	ND	1.3	1.3
60 µg/mL	ND	ND	ND	ND	1.7	1.3
100 µg/mL	ND	ND	1.7	1.0	ND	ND

ND: Not determined

The conclusion can be drawn that mefentrifluconazole did not increase the frequency of chromosome aberrations in this in vitro study (Naumann, 2019).

(b) In vivo studies

Because of the overall negative outcome of the in vitro test battery for genotoxicity, conduct of an in vivo study, strictly speaking, was not needed. However, a bone marrow micronucleus assay with oral administration in mice is available which had been performed in 2014 when only a few of the in vitro studies had been run.

In this study, mefentrifluconazole (Batch No. OD-001740; purity 98.8%) was suspended in a mixture of DMSO and corn oil and administered by oral gavage to groups, each of five male NMRI mice. The dose levels were 0, 375, 750 or 1500 mg/kg bw and the dosing volume 10 mL/kg bw. The animals were killed 24 hours later. An additional high-dose group and a second control group were, however, terminated after 48 hours.

Two positive control groups received CPA (20 mg/kg bw) by oral gavage or vincristine sulfate (0.15 mg/kg bw) by intraperitoneal injection. Both reference compound were dissolved in deionized water. Termination of these groups took place after 24 hours. Cyclophosphamide was expected to cause clastogenic effects and vincristine sulfate was supposed to act as a spindle poison.

Femoral bone marrow was prepared and after staining, 2000 polychromatic erythrocytes per animal were evaluated for the presence of micronuclei. In addition, blood samples taken at terminal kill were analyzed by liquid chromatography–mass spectrometry (LC/MS) for plasma levels of mefentrifluconazole to demonstrate bioavailability of the test substance and verify that the target tissue had been exposed.

Clinical signs of toxicity comprised piloerection, hunched posture, irregular respiration and lacrimation which occurred at all dose levels of mefentrifluconazole. The general condition of all animals was impaired but no animal died prematurely.

There was no significant or dose-related increase in micronucleus formation at any of the dose levels of mefentrifluconazole. Administration of the two positive control substances led to strong and statistically significant increases. Vincristin sulfate, in addition, produced the expected increase in large micronuclei which is indicative of an aneugenic effect.

In the animals killed after 24 hours, there was no change in the ratio of polychromatic (PCE) to normochromatic (NCE) erythrocytes. However, a lower PCE : NCE ratio in the additional high-dose group (kept alive for 48 hours) compared to the concurrent control group (1.47 compared to 2.26), though not statistically significant, suggested a possible adverse effect on erythropoiesis of at least the high dose. With regard to plasma analysis, it was reported that mefentrifluconazole had been detected in all samples from treated animals from all groups but not in the controls. However, the concentrations were not quantified.

Under the conditions of this study, the test substance proved negative in the micronucleus assay and may be considered to be devoid of a clastogenic potential in vivo. It can be reasonably assumed that the bone marrow was exposed to mefentrifluconazole. This study confirms the negative in vitro results with regard to genotoxicity (Schulz, Becker & Landsiedel, 2014).

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Mefentrifluconazole (Batch No. OD-001740; purity 98.8%) was administered over two generations to groups of 25 male and 25 female healthy young Crl:WI (Han) Wistar rats. One litter was produced per generation. The test substance was homogenously admixed to the diet in differing concentrations which were weekly adjusted to obtain target dose levels of 0, 25, 75 and 200 mg/kg bw per day. Over the course of the study the calculated mean daily intakes were close to these nominal dose levels. The approach taken in this study was more suited to ensuring similar intakes in relation to body weight throughout the various reproductive phases than is the standard procedure of maintaining dietary concentrations over the whole study.

At least 75 days after the beginning of treatment, F0 parental animals from the same dose groups were mated to produce an F1 generation. Based on the information provided by the supplier of the rats, sibling mating was avoided. Following successful mating, test substance administration to pregnant and lactating dams was continued. After delivery, culling to four male and four female pups per litter (if numbers allowed) was carried out and the females were left to rear their pups until postnatal day (PND) 21 when 25 male and 25 female pups per dose group were randomly chosen for further breeding. An attempt was made to include each litter. Post weaning, these F1 groups were offered diets which were adjusted to contain the same target dose as their parents had received. F0 parental animals and the remaining F1 pups were killed. The F1 young adults selected for breeding to produce the F2 litter were administered mefentrifluconazole for at least 74 days before their mating period started. Sibling mating was once again avoided. During gestation and lactation, dietary treatment was continued as before. Following parturition and culling the F2 pups were reared until PND 21. The study was terminated then and F2 weanlings and F1 parental animals all killed.

F0 and F1 parental animals as well as their pups were monitored daily for mortality and clinical signs of toxicity. The adult rats were examined for their mating and reproductive performance. Food consumption and body weight were determined on a weekly basis. During gestation and lactation, F0 and F1 females were weighed on gestation days (GDs) 0, 7, 14 and 20, and on PNDs 1, 4, 7, 14 and 21. Blood samples for haematological and clinical chemistry investigations were withdrawn from 12 selected F0 and F1 parental animals per sex and group shortly before scheduled termination. Following terminal kill, all adult parental animals were necropsied and assessed for gross pathology. Several organ weights were determined. The control and high-dose animals from the F0 and F1 generations were subjected to an extensive histopathological examination, with special attention being paid to the organs of the reproductive system. In male rats the livers were examined under the microscope in all groups after hypertrophy had been detected in high-dose animals (see below). In addition, a quantitative assessment of primordial and growing follicles in the ovaries was performed, also by immunohistochemical staining, in all control and high-dose F1 parental females.

Estrous cycle data were evaluated for all parental females over a three-week period prior to mating until evidence of copulation occurred. Moreover, the estrous stage of each dam was determined on the day of scheduled sacrifice. Various sperm parameters (motility, sperm head count, morphology) were assessed in the F0 and F1 generation males at scheduled termination.

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 recorded. At necropsy all pups were examined macroscopically. Organ weights of brain, spleen and thymus were determined in one pup per sex and litter. All surviving pups were examined for the presence or absence of the first signs of appearance of nipple/areola on PND 12 and re-examined towards the end of the lactation period (PND 20). Anogenital distance measurements (defined as the distance from the centre of the anal opening to the base of the genital tubercle) were conducted in all liveborn pups, in a blind randomized fashion on PND 1 using a measuring ocular.

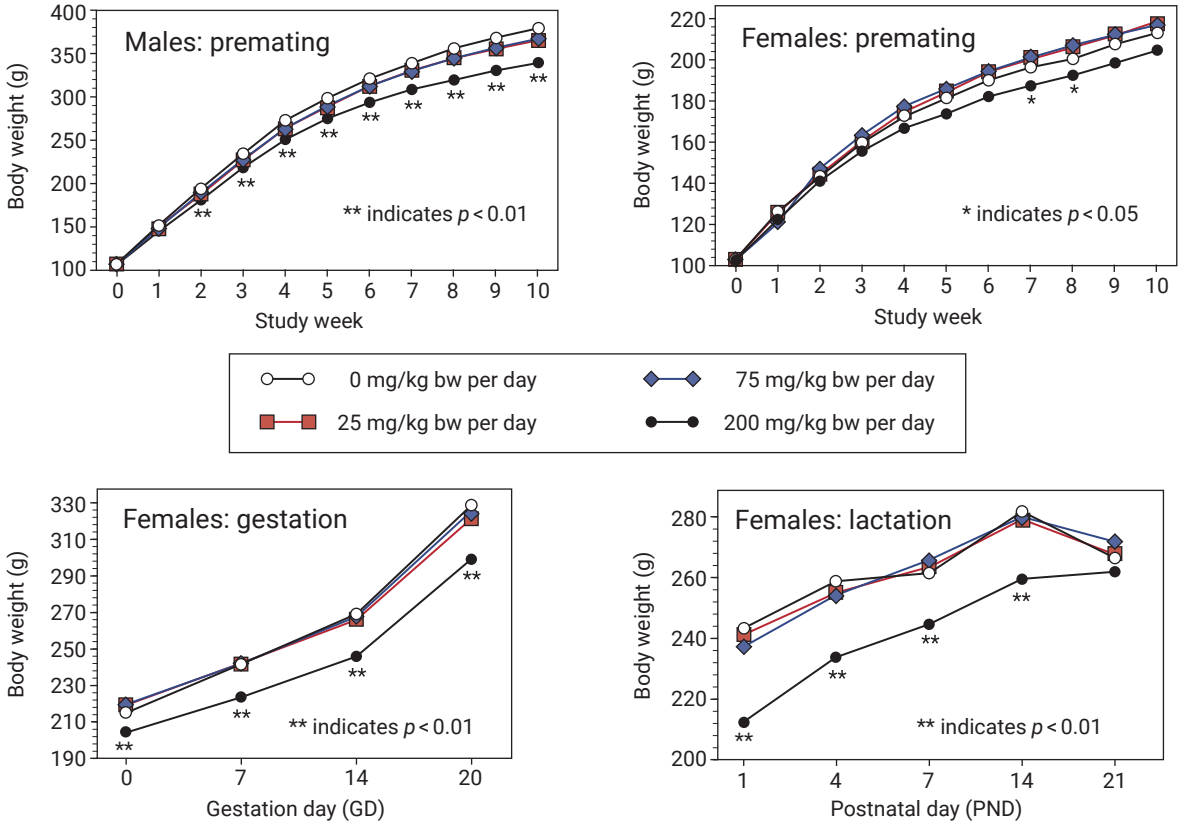
Sexual maturation was investigated in all F1 pups which had been selected to become F1 parents by recording the day of vaginal opening or of preputial separation.

No premature deaths or clinical signs in parental animals could be attributed to treatment, neither in the F0 nor F1 generations. In high-dose animals however, food intake was decreased in both males and females. In F0 males an intake reduction by up to 11% compared to the control group was noted during the premating period, mating days and thereafter. In F0 females receiving 200 mg/kg bw per day, food consumption was decreased throughout the whole study, most severely during gestation (up to 15% below controls) and lactation (about 12% below controls). Similar reductions in food intake were observed at the highest dose level in the F1 generation, reaching 10% in males and even 20% in females.

Consistent with the lower food consumption, body weight and its gain were decreased in both generations and both sexes at the top dose. The effects on body weight during different phases of the study are depicted for the F0 generation in Fig. 5. In high-dose males, mean body weights were lower from premating day 13 onwards until the end of the study (up to 11% below controls) and body weight gain was decreased by up to 33% during major parts of the study. In high-dose females lower body weights were noted throughout the study. Reductions were most severe during gestation (up to 9% below controls) and early lactation (up to 13% below controls) and body weight gain was depressed during major parts of the gestation period by up to 24% compared to the controls.

Similar adverse effects on body weight and its development were observed in F1 males and females but once again, confined to the highest dose level (see Table 29). Mean body weights were depressed in males from premating day 1 onwards until the end of the study (up to 12% below controls) and body weight gain even more so, by up to 58% lower than in the control group during major parts of the study. In females body weights were decreased throughout the study, most severely during gestation (up to 16% below controls) and early lactation (by up to 17%). Body weight gain was mainly reduced during the gestation period (by up to 32% below controls).

Figure 5. Body weight development of F0 parental animals administered mefentrifluconazole



Haematology did not reveal any significant differences between the groups. Alterations in clinical chemistry parameters of adult animals resembled those observed in the 90-day and the two-year studies in rats reported in Table 28. Dose-related and statistically significant increases in the activities of ALP in both generations and of GGTP in the F0 generation were considered adverse findings and indicative of liver toxicity. An impact on the liver was also suggested by the higher cholesterol concentrations which were, in addition, supported by an increase in triglycerides in female rats at the two upper dose levels. As in the long-term study, bilirubin levels were lower, but such reductions are not considered adverse, as is the case with the not clearly dose-related decrease in triglycerides in males. The changes in glucose suggested a tendency at least towards a decrease which was consistent with previous results, but this finding was of equivocal toxicological relevance.

Table 28. Clinical chemistry findings in the two-generation study in rats

		Males				Females			
Dose level (mg/kg bw per day)		**0**	**25**	**75**	**200**	**0**	**25**	**75**	**200**
Alkaline phosphatase (µkat/L)	F0 gen.	1.23	1.45	**2.03****	**2.26****	0.77	0.74	**0.96***	**1.15****
	F1 gen.	1.24	1.55	**1.95****	**2.36****	0.89	0.86	0.84	**1.26****
γ-Glutamyl transferase (nkat/L)	F0 gen.	0	0	2	4	5	5	6	14*
	F1 gen.	0	0	0	0	0	0	0	2
Glucose (mmol/L)	F0 gen.	6.52	6.12	5.95	5.89	6.31	5.65	5.98	5.67
	F1 gen.	6.64	6.14*	6.32	5.97**	5.08	5.26	4.75	4.61
Triglycerides (mmol/L)	F0 gen.	0.95		0.76	0.80	0.99	0.88	1.12	1.47
	F1 gen.	1.19	0.87*	0.89*	0.81*	0.72	0.94	**0.98***	**1.09****
Cholesterol (mmol/L)	F0 gen.	1.87	2.11	**2.30***	**2.56****	2.05	2.10	1.95	2.21
	F1 gen.	2.13	2.35	2.21	2.45	1.99	2.11	1.86	2.10
Total bilirubin (µmol/L)	F0 gen.	2.11	2.02	1.58**	1.58**	3.04	3.16	3.25	3.38
	F1 gen.	1.52	1.37	1.21**	1.11**	1.69	1.55	1.44	1.24*

* $p \leq 0.05$; ** $p \leq 0.01$ Kruskal-Wallis & Wilcoxon test, two-sided

Gross examination of parental animals at necropsy did not reveal indications of treatment-related findings. Many organ weights, absolute and relative, were significantly altered in both sexes and both generations at the highest dose level. With exception of the liver, however, all these changes could be attributed to the lower terminal body weight in the top-dose groups. The increase in liver weight at the two upper dose levels (Table 29), by contrast, was considered treatment-related and adverse since it was accompanied by clinical chemistry and, even though in males only, by histopathological findings.

Table 29. Terminal body weight and liver weights in the two-generation study in rats

Parameter / Dose	F0		F1	
	Males	**Females**	**Males**	**Females**
Terminal body weight (g)				
0 mg/kg bw per day	397.7	227.5#	383.8	228.6
25 mg/kg bw per day	384.5	224.7	384.6	219.3
75 mg/kg bw per day	385.4	220.5	386.1	219.1
200 mg/kg bw per day	**354.1****	**213.2****	**339.3****	**202.5****
Absolute liver weight (g)				
0 mg/kg bw per day	9.17	7.29	9.18	7.60
25 mg/kg bw per day	9.01	7.18	9.48	7.25
75 mg/kg bw per day	9.32	**7.84***	9.74	7.69
200 mg/kg bw per day	9.10	**8.03***	9.13	7.39
Relative liver weight (%)				
0 mg/kg bw per day	2.30	3.20	2.39	3.32
25 mg/kg bw per day	2.34	3.20	2.46	3.30
75 mg/kg bw per day	**2.42****	**3.60****	**2.52****	**3.51***
200 mg/kg bw per day	**2.57****	**3.77****	**2.69****	**3.63***

* $p \leq 0.05$; ** $p \leq 0.01$ (Kruskal–Wallis & Wilcoxon test, two-sided

The histopathological liver findings attributable to treatment were confined to minimal grade centrilobular hypertrophy in 15 high dose males in both the F0 and F1 generations, but not in the mid- or low-dose groups. No such morphological change was seen in female rats at any dose.

Estrous cycle length was not affected in the F0 generation. In F1 females, average estrous cycles were prolonged in the high-dose group by half a day compared to the control group. However, this (significant) difference was still within the historical control range and apparently had no impact on reproductive performance or success.

Sperm parameters were altered in neither F0 nor F1 males.

As demonstrated for F1 females, the differential follicle count in the ovaries of control and high-dose females showed no significant difference between the groups with regard to either primordial or growing follicles.

Mefentrifluconazole did not affect the mating index, the male and female fertility index, or the gestation index in the F0 generation (Table 30). Implantation rate and postimplantation losses were not compromised. A significant increase in the duration of gestation was noted at the top dose but the difference compared to the control group was less than half a day and was still within the historical control range of the performing laboratory. Accordingly, there was no impact of substance administration on reproductive performance.

The viability index among F1 pups was not significantly altered by treatment, nevertheless pup mortality during PNDs 1 to 4 was highest at the top dose; 18 dead pups compared with three in the control group. This was mainly due to a single dam which experienced a complete litter loss by PND 2. In fact, 11 out of the 18 pups which died were from this litter. There was no milk in the stomach of one of these pups and this was interpreted as a possible sign of insufficient nursing. The relevant dam,

however, did not exhibit an particularly low body weight, so this litter loss cannot be attributed to maternal toxicity. On balance it cannot be excluded that this postnatal litter loss was related to treatment but it is not clear whether it should be regarded as an indication of reproductive or offspring toxicity.

Otherwise pup survival was not affected since the lactation index in F1 pups did not differ between groups. Mean pup body weights in the high-dose group however were depressed (about 10% below controls) as was body weight gain (up to 15% below controls) during lactation. The lower absolute organ weights of brain, spleen, and thymus in this group were considered secondary to the reduction in pup body weights since the relative organ weights were either not affected or, in the case of the brain, were even higher. No effects on pup development were observed in the low- or mid-dose groups.

There were no gross necropsy findings in the F1 pups that could be attributed to treatment.

Some findings in the F1 generation warrant more detailed consideration with regard to reproductive toxicity. Two high-dose females did not become pregnant despite evidence of successful mating. No explanation for this failure could be given but the study authors considered this finding to be within the normal biological variation. The same conclusion was drawn with regard to the lower numbers of implantation sites and delivered pups per dam in this group. These arguments are supported by the HCD as submitted by the sponsor and reported below in Table 30.

Another remarkable finding was the detection of one dam which apparently had only a single fetus and that had been resorbed. Accordingly, the number of litters in this high-dose F1 group was further reduced to 22, and this contributed greatly to the relatively small total number of delivered pups which was lower than in any other group.

One high-dose female showing piloerection and a distinctly poor general state around birth had only stillborn pups. A further dam suffered a complete litter loss by PND 3. In this case, the pups exhibited signs of insufficient nursing since their nutritional condition was severely impaired and in six out of eight pups there was no milk in their stomachs at necropsy. On balance, these perinatal losses might be attributed to maternal toxicity rather than to reproductive or offspring toxicity. Indeed, individual body weights for these dams were the lowest of the whole group on lactation day 1.

In summary, the viability index in the high-dose group was markedly lowered even though the difference did not gain statistical significance. Nonetheless, a similar observation in the F0 generation (F1 pups) was confirmed.

Table 30. Reproduction and offspring parameters in the two-generation study in rats

Parental generation	**F0**				**F1**			
Dose level (mg/kg bw per day)	**0**	**25**	**75**	**200**	**0**	**25**	**75**	**200**
Females mated	25	25	24	25	25	25	25	25
Female mating index (%)	100	100	100	100	100	100	100	100
Females pregnant	25	25	24	25	25	25	25	23
Female fertility index (%)	100	100	100	100	100	100	100	92
Mean precoital interval (days)	2.8	2.4	3.0	2.8	3.0	3.0	2.5	2.8
Mean duration of gestation (days)	22.1	22.2	22.2	22.4*	22.2	22.0	22.0	22.2
Implantation sites, total	307	284	288	295	300	285	308	229
Implantation sites per dam	12.3	11.4	12.0	11.8	12.0	11.4	12.3	10.0*
Postimplantation loss (%)	3.9	5.5	1.3	5.2	2.4	5.0	7.1**	8.9 (4.8)[a]
Number of litters	25	25	24	25	25	25	25	22
Females with liveborn pups	25	25	24	25	25	25	25	21
– with some stillborn pups	1	1	0	1	1	3	2	3
– with all stillborn pups	0	0	0	0	0	0	0	1
Gestation index (%)	100	100	100	100	100	100	100	91.3

Parental generation	F0				F1			
Dose level (mg/kg bw per day)	0	25	75	200	0	25	75	200
Pups delivered	297	267	284	277	298	269	285	217
– per dam	11.9	10.7*	11.8	11.1	11.9	10.8	11.4	9.9**
– liveborn	296	266	284	274	295	263	283	208
– stillborn	1	1	0	3	3	6	2	9
Live birth index (%)	99.7	99.6	100	98.9	99.0	97.8	99.3	95.9
Viability index, PND 4 (%)	99.1	99.4	100	93.5	99.7	99.6	99.0	93.6
Lactation index (%)	100	100	99.0	100	100	99.5	100	99.4
Sex ratio (% live males), PND 0	47.7	49.5	51.3	49.2	49.4	44.4	52.8	50.2
Sex ratio (% live males), PND 21	47.6	50.7	47.8	50.2	48.9	48.0	50.8	48.7

PND: Postnatal day * $p < 0.05$, ** $p < 0.01$ Wilcoxon with Bonferoni–Holm correction, one-sided;

[a] Higher implantation rate was due to one female with only one resorption site but no further implants resulting in a 100% resorption rate in this dam which contributed a lot to the higher mean, number in brackets was the resorption rate after exclusion of this animal

Historical control data (33 studies run 2008–2015 at the same test facility in Wistar rats of same origin)

– Duration of gestation: 21.8–22.9 days;

– Gestation index: 87.5–100%;

– Implantation sites/dam: 9.4–14.0

– Postimplantation loss (mean %): 0.9–17.7;

– Pups delivered/dam: 9.2–13.4

– Live birth index (mean %): 92.1–100

The lactation index indicating pup survival between PNDs 4 and 21 was not affected by treatment. As in the previous generation, F2 pup body weights were depressed compared with controls at the top dose (by about 14%) and so was body weight gain (up to 19% below control) during lactation, causing secondary effects on pup organ weights. Dilated renal pelvis was observed in 23 high-dose pups compared to only three cases in the control group. This finding was considered by the study authors to support the hypothesis of general developmental delay at the highest dose, consistent with lower pup body weight. No effects on pup development were observed in the low- or mid-dose groups.

Sex ratio was not skewed and the anogenital distance unaltered in the F1 and F2 pups. Male pups had no areolae. With regard to landmarks of sexual maturation, there were some significant intergroup differences in the time of vaginal opening (Table 31) which were not really severe enough to substantiate a specific effect, and were still within the historical control range. On balance these were either incidental or due to the adverse effect of pup weight on development. In high-dose male pups, preputial separation occurred even at a lower body weight and practically on the same day as in the control group, suggesting the lower body weight had no serious effects on sexual development.

Table 31. The effects of mefentrifluconazole on sexual maturation in F1 pups in the two-generation study in the rat

Dose (mg/kg bw per day)	0	25	75	200
Mean time of vaginal opening in females (postnatal day)	30.0	30.9*	30.2	31.8**
Mean body weight on day of vaginal opening (g)	91.6	93.5	90.8	91.9
Mean time of preputial separation in males (postnatal day)	43.1	42.7	43.2	43.5
Mean body weight on day of preputial separation (g)	182.3	177.3	183.6	167.9**

* $p < 0.05$, ** $p < 0.01$, Dunnett′s test, two-sided

The NOAEL for parental toxicity, under the conditions of this two-generation reproduction toxicity study, was 25 mg/kg bw per day, the lowest dose tested. It was mainly based on clinical chemistry findings that suggested liver toxicity and a higher liver weight in adult animals at the two upper dose

levels. These effects were consistent with what had been observed in the short- and long-term studies in this species. At the top dose, decreased food consumption and lower body weight and body weight gain were observed in male and female rats of both generations.

Even though reproductive performance was not significantly altered, a lower fertility in females of the F1 generation and perinatal litter losses in both generations suggests reproductive toxicity at the highest dose cannot be completely excluded. Therefore, the mid dose of 75 mg/kg bw per day is the NOAEL for reproductive toxicity.

The NOAEL for offspring toxicity was 75 mg/kg bw per day, based on effects on pup body weight gain and associated weak developmental delay (Schneider et al., 2015).

(b) Developmental toxicity

Rat

Mefentrifluconazole (Batch No. L84-176; purity 97.7%) was administered by oral gavage to three groups of 25 time-mated Crl:WI(Han) Wistar rat females, once a day, from day 6 of presumed gestation to day 19. The dose levels were 50, 150 or 400 mg/kg bw per day. A control group of the same size received the vehicle (1% aqueous solution of CMC). The dosing volume was 10 mL/kg bw. It was noted that while the study had been conducted in 2012 it apparently took much longer to complete the evaluation phase and study report.

The dams were observed daily for mortality, behavioural changes and signs of overt toxicity. Food consumption was recorded for three-day intervals throughout the study. The animals were weighed four times during pretreatment, on GDs 8, 10, 13, 15, 17, 19 and at scheduled termination on day 20. Following terminal kill, the dams were necropsied and assessed for gross pathological changes. The uteri and ovaries were removed and corrected body weight gains calculated from:

(terminal body weight − gravid uterus weight) − body weight on GD 6.

The number of corpora lutea was determined in the ovaries. In the uteri, 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. Conception rate and preimplantation and postimplantation losses were calculated.

All fetuses were weighed, sexed (by external appearence, confirmed later by internal examination) and evaluated for external anomalies. Fetuses were then killed and half of their number per dam prepared for visceral examination, the others for external examinations. Placentas were also weighed.

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 gross necropsy findings. However, three females, one each from the control, mid- and high-dose groups, were not pregnant and therefore, were excluded from all calculations.

Food consumption in high-dose females over the whole treatment period was by 8% lower than in the control group. In the intervals from GDs 8 to 10 and from GDs 17 to 19 the difference was even greater (12–14% below controls). Consistent with reduced food intake, body weight gain for the entire treatment period was about 17% lower than in the control group. Whereas the mean gravid uterus weight was not altered, net body weight at the top dose was 7% lower than in the control group. No effects on food consumption or body weight were noted in the low- and mid-dose groups.

Reproductive parameters, (conception and implantation rate, preimplantation and postimplantation losses, number of resorptions and viable fetuses) did not differ among the groups. Placental weight was significantly increased in high-dose females (Table 32). The toxicological relevance of this finding is equivocal.

Sex distribution of the fetuses and mean fetal body weight were not altered by treatment even though in female fetuses at the high dose the mean body weight was 6% lower than for the control group. However, the mean weight of 3.4 g in that group was just equal to the mean taken from the available HCD. A direct adverse effect on pup weight is not likely since male fetuses were not affected. For the same reason, an indirect effect due to lower maternal body weight is not a convincing hypothesis.

There were two fetuses with external malformations of the skull. One mid-dose fetus exhibited mandibular micrognathia. Findings in one high-dose fetus comprised cleft palate, microphthalmia and a malformed mandible. No distinct pattern of malformations was apparent and, taking into account their isolated occurrence, these findings were considered incidental. There was no dose-related increase in visceral or skeletal malformations.

A soft tissue variation of the urinary tract (dilated renal pelvis) was observed more frequently in the high-dose group (Table 32) but the difference in frequency did not achieve statistical significance in Fisher′s exact or in the Wilcoxon test and was within the historical control range even though the means of 2.4% (for fetuses) and 10.9% (for litters) were exceeded in the high-dose group. Based on 76 studies in total, the HCD fetal range was 0–11.8% and the litter range 0–57.1%.

In contrast, an increase in the litter incidence of two minor skeletal variations was statistically significant and could be more likely attributed to treatment. Supraoccipital holes are a common finding. Based on a total of 76 studies from the same laboratory, the historical control range of 0–52.3% of fetuses obtained from untreated dams, with a group mean of 12.4%, is reported in the applicant′s dossier (Stinchcombe & Sauer, 2019). The control litter incidence in the HCD studies ranged from 0–50.8% with a mean of 12.2%. In the study with mefentrifluconazole the fetal incidence was well within the historical control range, but the litter incidence at the two upper dose levels exceeded HCD levels. On the other hand, there was no clear dose–response relationship in this study. The toxicological relevance of this findings is very limited since the skeletal variations were small and most likely not detrimental to postnatal development.

The increase in misshapen sacral vertebrae was dose-related and fetal incidence was at the upper limit of the historical control range. In the same HCD database as above the mean fetal incidence was 3.3% (range 0–10.2%) whereas the mean litter incidence was 14.8% (range 0–41.7%). However, misshapen sacral vertrebrae are usually considered a minor variation with no serious effects on postnatal survival or health. Thus the variation is of equivocal toxicological relevance and does not necessarily qualify as an adverse finding.

Table 32. Placental and fetal weight and fetal visceral and skeletal variations in the developmental study with mefentrifluconazole in rats

Dose (mg/kg bw per day)	0	50	150	400
Number of pregnant dams	24	25[a]	24	24
Mean placental weight (g)	0.44	0.44	0.46	0.50**
Number of live fetuses / litters	223 / 24	221 / 24	230 / 24	222 / 24
Mean fetal weight, males (g)	3.7	3.8	3.8	3.6
Mean fetal weight, females (g)	3.6	3.5	3.6	3.4*
Dilated renal pelvis; fetuses affected (percentage)	2 (1.9%)	1 (0.9%)	3 (2.7%)	8 (7.6%)
litters affected (percentage)	2 (8.3%)	1 (4.3%)	1 (4.3%)	5 (21%)
Supraoccipital holes; fetuses affected (percentage)	8 (6.8%)	6 (5.2%)	21 (18%)	17 (15%)
litters affected (percentage)	6 (25%)	5 (21%)	15 (63%)[##]	13 (54%)[#]
Missshapen sacral vertebrae; fetuses affected (percentage)	3 (2.6%)	5 (4.3%)	7 (5.8%)	12 (10%)
litters affected (percentage)	3 (15%)	3 (13%)	5 (21%)	9 (38%)[#]

[a] One pregnant dam with all fetuses resorbed

* $p < 0.05$; ** $p < 0.01$, Dunnett′s test, two-sided; [#] $p < 0.05$, [##] $p < 0.01$, Fisher′s exact test

To conclude, the maternal NOAEL in this oral developmental study in Wistar rats was the mid-dose of 150 mg/kg bw per day, based on reduced food consumption and body weight gain in high-dose dams which were both considered treatment-related and potentially adverse. In addition, mean placental weight was higher in this group.

Mefentrifluconazole was not teratogenic to rats. It was presumed that treatment-related effects on fetuses were confined to increases in skeletal variations (misshapen sacral vertebrae, supraoccipital holes) at the highest and partly also at the mid-dose levels. However, these findings were regarded by the Meeting as not adverse. Therefore, the highest dose level of 400 mg/kg bw per day was the developmental NOAEL in this study (Schneider, 2015).

Rabbit

In the main study, mefentrifluconazole (Batch No. OD-001662; purity 95.5%) was administered daily by stomach tube to four groups of 30–33 inseminated and presumably pregnant New Zealand White rabbits from day 6 of presumed gestation to day 28. The dose levels were 0, 5, 15 and 25 mg/kg bw per day. The control group received only the vehicle which was a 1% aqueous solution of CMC. The dosing volume was 10 mL/kg bw.

Dose levels had been selected on the basis of two preliminary range-finding experiments. Full reports on these studies were apparently not available, but the information on their results as provided in the sponsor′s dossier (Stinchcombe & Sauer, 2019) as well as in a supplement and addendum to the main study (Schneider, 2016, described here) is sufficient and reliable.

In the first of these preliminary studies, three non-pregnant female rabbits per group received daily doses of 50, 150 or 400 mg/kg bw, that is the same doses as given in the developmental study in rats by Schneider (2015) reported above. At all dose levels the rabbits showed a marked reduction in food intake, decrease in defaecation and a steady body weight loss. These signs were observed from the very outset or, at latest from the second day of dosing. These findings were clearly indicative of an acute effect. One rabbit receiving the high dose was already found dead on day 2 and the two remaining animals from that group had to be killed in a moribund state the same day. As a result the next lowest dose of 150 mg/kg bw per day had to be reduced to 15 mg/kg bw per day on day 3, but the animals did not recover and had to be killed for humane reasons the next day. Necropsy findings in these early decedents comprised empty small intestines, large intestines with watery contents, empty rectum, and stomach erosions. In the group receiving the low dose of 50 mg/kg bw per day, treatment could be continued for longer, but two of them had to be killed in a moribund state on days 14 and 20.

As a follow-up to this range-finding study, a further treatment group of three non-pregnant females received 25 mg/kg bw per day and a second control group was included. The results of this second part of the study were inconclusive; one rabbit again showed reduced food intake during the first three days of dosing, reduced or no faeces and body weight loss, and had to be killed on study day 17. If these signs were in fact treatment-related, they cannot be regarded as an acute effect at this relatively low dose. By contrast the two remaining females tolerated the treatment very well and no differences from the parallel control group became obvious.

In the second separate range-finding study, groups of five pregnant rabbits received doses of 0, 5, 10, or 20 mg/kg bw per day and were observed for maternal toxicity. Since no effects were seen, a steep dose response was assumed and 25 mg/kg bw per day was selected as the maximum dose for the full-scale developmental study, representing half a lethal dose.

During the main study does were observed daily for mortality, behavioural changes and clinical signs of toxicity. Food consumption was recorded daily throughout the study. The animals were weighed frequently, (on GDs 0, 2, 4, 6, 9, 11, 14, 16, 19, 21, 23, 25, 28) and at scheduled termination on day 29. On the morning of that last day blood was taken from the ear veins of fasted animals to determine a number of haematological and clinical chemistry parameters. This additional investigation was considered an interesting approach, but is unusual in studies of this type.

Following terminal kill, does were necropsied and assessed for gross pathological changes. The uteri and ovaries were removed. The number of corpora lutea was determined and uteri were weighed, opened and the number of implantation sites and their distribution recorded. Live and dead fetuses were counted as well as resorption sites. An attempt was made to distinguish between early and late resorptions. Conception rate and preimplantation and postimplantation losses were calculated. Placentas were also weighed.

All fetuses were weighed and evaluated for viability and external anomalies. Fetuses were then killed and abdomen and thorax opened. The hearts and kidneys were sectioned in order to assess their internal structure. The sex of the fetuses was determined internally by examination of the gonads. The fetuses were then prepared for skeletal examination. Particular attention was paid to the heads which were severed from the trunk in one half of the total number and assessed according to Wilson′s method by performing 10 transverse sections.

There were four unscheduled deaths during the study. One control female and one doe from the high dose group died because of a gavage error and one rabbit each from the low- and mid-dose groups were killed ahead schedule following abortion. The distribution of these premature losses did not provide any evidence that they might have been treatment-related. Similarly, no clinical signs could be attributed to the test substance administration.

Total food consumption over the entire study was not affected but there was a significantly lower food intake in high-dose females on GDs 6 to 7, just after the first dose of mefentrifluconazole. For this interval mean food consumption of 153.6 g per animal in the high-dose group was 13% lower than in the control group (177.4 g). However, since this transient reduction in food intake did not affect body weight gain it was considered a treatment-related effect but not adverse. During the whole study period there was no significant impact in any dose group on body weight nor on its gain. Nor was the corrected body weight gain, (terminal body weight − gravid uterus weight) − body weight on GD 6, affected by treatment.

Haematological parameters were not affected. Clinical chemistry revealed significantly lower ALT activity in the high-dose group, and lower AST activity at the two upper dose levels, also a lower globulin concentration in the group receiving 25 mg/kg bw per day. All of these findings may be attributed to treatment, but reduced liver enzyme activity, in contrast to an increase, is usually not considered adverse. Lowered globulin concentration may have different causes but, as an isolated finding in particular, it is of equivocal toxicological relevance. Necropsy of the does did not reveal any findings that could be attributed to treatment.

Reproductive parameters were not affected. In the control, low-, mid- and high-dose groups, 25, 21, 23 and 23 females respectively, were found, in practice, to be pregnant. In the mid-dose group, the mean number of implantation sites was lower and preimplantation losses were higher than in the controls, low- and high-dose groups, resulting in a lower number of fetuses available for evaluation. Since this difference was not related to dose however, it cannot be attributed to treatment. The number of litters with viable fetuses and the number of live fetuses (see Table 33), fetal weight and sex ratio did not differ significantly between groups.

There was no increase in external, visceral or skeletal malformations or variations in any of the dose groups compared with the control. The only skeletal variation revealing a numeric but not statistically significant increase (Fisher′s exact test) was a minor one described as “fused sternebra with unchanged cartilage”. The fetal and litter incidences for this variation are given in Table 33. At the maximum dose incidence was clearly above the historical control range. In 12 studies in the same strain of rabbit from the same laboratory, fused sternebrae without cartilaginous alterations were observed in 0–1.9% of the fetuses and in 0–14.3% of the litters. The HCD mean values had been 0.9% (fetuses) or 6.3% (litters). This finding was discussed in detail by Schneider & Stinchcombe (2017). The variation itself would be of low impact on postnatal survival and health. Therefore, and without being of statistical significance, this isolated finding might or might not be attributable to treatment but is not considered adverse. It is known that the development of the sternum (including fusion of sternebrae and ossification) is extremely variable both in humans (O′Neal et al., 1998) and rabbits (Kamal Rashed & Erasha, 2016) and is usually completed after birth.

Table 33. Fetal and litter incidences of the skeletal variation “fused sternebra” in the developmental study with mefentrifluconazole in rabbits

Dose (mg/kg bw per day)	0	5	15	25
Number of live fetuses/litters	194 / 23	161 / 20	135 / 21	176 / 22
Fused sternebra; fetuses	1 (0.5%)	3 (1.9%)	1 (0.7%)	7 (4%)
litters	1 (4.3%)	2 (10%)	1 (4.8%)	4 (18%)

To conclude, the highest dose of 25 mg/kg bw per day in this main developmental study in rabbits was the NOAEL for both maternal and developmental toxicity. Mefentrifluconazole was not teratogenic in rabbits.

The range-finding studies reported here clearly revealed that the rabbit is much more sensitive to this compound than other animal models examined. Clinical signs, mortality and pathological findings were attributed to a treatment-related entity that was described as "mucoid enteropathy". There was a steep dose–response relationship. Because of the early onset of clinical signs from a dose of 50 mg/kg bw per day onwards, setting of an ARfD is necessary and it may be derived on the basis of the NOAEL as obtained in the main study (Schneider, 2016).

A review of reproductive and developmental studies with mefentrifluconazole was recently published. The limited reproductive and developmental effects of this compound, in particular in comparison to other triazole fungicides, was explained by its low potential to inhibit aromatase (Tesh et al., 2019).

2.6 Special studies

(a) Neurotoxicity

In an acute neurotoxicity study, mefentrifluconazole (Batch No. OD-001880; purity 98.6%) was administered by oral gavage to groups of 10 male and 10 female Crl:WI(Han) Wistar rats. The dose levels were 0 (control group receiving the vehicle, 1% aqueous CMC), 200, 600, and 2000 mg/kg bw.

The animals were observed for two weeks after dosing. They were monitored daily for mortality and clinical signs of toxicity and were weighed seven days prior to dosing, on the day of substance administration (day 0), and on days 7 and 14 thereafter. At the same time points, all rats were subjected to a functional observation battery (FOB) and motor activity assessments. At terminal kill, five animals per sex and dose were fixed, under deep isoflurane anaesthesia, by in situ perfusion and prepared for neuropathological examination. For this purpose, these rats were necropsied and their brain removed for organ weight determination. Following gross examination, from control and high-dose groups, the brain, eyes (with retina and optical nerve), parts of the cervical and lumbar cord, trigeminus ganglia with nerve and the gastrocnemius muscle were subjected to histopathology. Respective samples and cross-sections or longitudinal sections from low- and mid-dose animals were embedded and preserved in case they might be needed later.

The limit dose of 2000 mg/kg bw was toxic to male and female rats but no animal died before scheduled termination. Toxicity was apparent from a reduction in body weight gain compared with controls of 29% in males and 20% in females during the first week after dosing. Thereafter, recovery was noted in both sexes since body weight gain was similar to that in the control groups. The transient decrease was not sufficient to significantly alter total body weight. Body weight development was not affected in the low- or mid-dose groups.

Even though there were no obvious clinical signs of systemic toxicity in any group, FOB revealed transient impairment of neurological functions just after dosing. Unsteady gait was noted on day 0 in five high-dose males and in three-high dose females but in no other animals. Even in the affected high-dose animals the impairment of co-ordination was no longer visible on days 7 or 14. In addition, forelimb grip strength was reduced (by 22% on average) in high-dose males on day 0 and the landing foot splay test revealed an increased distance between the hind limbs. These findings, once again transient, were regarded as indicative of a lower body tension. Overall motor activity was reduced in high-dose males and females on the day of dosing but not thereafter.

Gross examination and neurohistopathology did not reveal any changes which could be attributed to treatment. Brain weights were not affected. In one high-dose male, ocular inflammation and degeneration of the cornea were noted, but because of their unilateral occurrence in a single animal, they were considered incidental.

The NOAEL in this acute oral neurotoxicity study was 600 mg/kg bw per day, based on a reduced body weight during the first week following dosing and transient alterations in FOB parameters in high-dose males and females. Nonetheless, there was no evidence of mefentrifluconazole having specific neurotoxic potential (Buesen et al., 2015a).

A specific neurotoxicity study with repeated administration was not submitted.

(b) Immunotoxicity

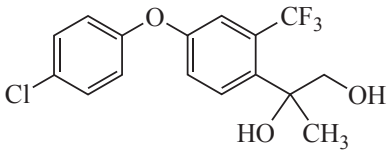
No specific study is available, but no concerns arise from routine studies which which have investigated a number of potential immune-related endpoints, such as haematological parameters like white blood cell count, spleen and thymus weights or histopathology of the spleen, thymus, lymph nodes and bone marrow. An increase in mean thymus weight in the 28-day study in mice was not reproducible in studies of longer duration.

(c) Studies on metabolites

Metabolite M750F022 (synonym Reg. No. 6011210)

Toxicological studies were confined to the important livestock metabolite M750F022 for which a separate evaluation might be warranted. This metabolite, 2-[4-(4-chlorophenoxy)-2-(trifluoromethyl) phenyl]propane-1,2-diol (see Fig. 6)**,** was not detected in rat metabolism even though it was predicted to occur as a potential intermediate (see Annex I). However, it represents a major proportion of residues in hen matrices (together with its three fatty acid conjugates, see below) and was also found in goat kidney, as well as in muscle, liver, fat, and milk.

Figure 6. Structure of metabolite M750F022 (synonym No. 6011210)



The available studies with this metabolite are summarized in Table 34 and briefly reported below. To summarize, it may be concluded that M750F022 was equally or less toxic than the parent and that the reference doses established for mefentrifluconazole are also applicable to this metabolite.

Table 34. Toxicological, genotoxicity and mechanistic (endocrine disruption potential) studies with mefentrifluconazole metabolite M750F022

Study type	Test system	Doses/concentrations	Results	Reference
Acute oral toxicity	Female Wistar rat	2000 mg/kg bw	LD_{50} > 2000 mg/kg bw; no mortality but transient clinical signs	Hoeger & Lammer, 2013
28-day feeding study	C57BL/6 mouse (males/females)	0,87, 872, 2500 ppm	NOAEL = 87 ppm (equal to 20/32 mg/kg bw/day in males/females), based on liver effects; bw reduced at the top dose	Buesen et al., 2016b
Mutagenicity in bacteria (Ames test)	Four strains of *S. thyphimurium*, one *E. coli* strain (± S9 mix)	Up to 5000 µg/plate (evaluation confined to 100–1000 µg/plate due to cytotoxicity)	Not mutagenic; bacteriotoxic effects from 33 or 100 µg/plate upwards	Woitkowiak, 2015b
Mouse lymphoma assay	Mouse lymphoma L5178Y cells, with/ without metabolic activation, treatment 4 h or 24 h exposure	Up to 200 µg/mL (evaluation possible up to 25–50 µg/mL)	Not mutagenic; Relatively strong cytotoxicity	Schulz & Landsiedel, 2015
In vitro micronucleus assay	Human lymphocytes	Up to 100 µg/mL (evaluation possible up to 18.7 µg/mL without S9 or 32.7 µg/mL with S9 mix)	Not clastogenic	Sokolowski, 2015b
Human recombinant aromatase assay	Insect cells (not further specified)	Not applicable	Inhibition of aromatase activity in vitro much lower than with the parent	Mentzel, 2016a

A Derek Nexus (provided by Lhasa Limited) analysis was run for both parent mefentrifluconazole and metabolite M750F022. They are clearly structurally dissimilar mainly because of cleavage of the triazole ring from the parent molecule to form the metabolite. Alerts were found for neither of the two compounds with regard to a number of health-related end-points including chromosome damage and bacterial mutagenicity. Mefentrifluconazole triggered an alert for carcinogenicity and hepatotoxicity but not so the metabolite. In contrast to this, M750F022 triggered an alert for nephrotoxicity which was not observed with the parent molecule. However, since it was a rapid prototype alert, this finding was considered equivocal (Woolley, 2015).

An acute oral toxicity study was performed in female Wistar rats according to the "Acute Toxic Class" method. Two groups of three animals received the test item (Reg. No. 6011210, Batch No. L85-116; purity 99.0%) at a dose level of 2000 mg/kg bw as a single gavage application. The vehicle for the suspension was corn oil and the dosing volume was 5 mL/kg bw.

Following treatment, the rats were observed for 14 days for mortality and clinical signs of toxicity. They were weighed prior to dosing and weekly thereafter. At termination the animals were necropsied and examined for gross pathological changes.

There were no unscheduled deaths in this study but the animals exhibited impaired general state and piloerection which disappeared at latest on day 3. In the second dosing group, in addition, cowering position, apathy and dyspnea were observed on the first day after treatment. All animals gained weight and there were no remarkable gross findings at necropsy.

Metabolite M750F022 was of low acute oral toxicity under the conditions of this study. The LD_{50} was above 2000 mg/kg bw. The clinical signs, except dyspnea, resembled those reported in a similar study with parent mefentrifluconazole (Hoeger, Becker & Kamp, 2013a) but lasted a bit longer (Hoeger & Lammer, 2015).

In a 28-day feeding study, the metabolite (under the Reg. No. 6011210; Batch No. L85-116; purity 99.0%) was administered to groups of five male and five female C57BL/6J Rj mice at dietary concentrations of 0, 87, 872 or 2500 ppm. The low and mid concentrations had been chosen to achieve equimolar concentrations to the dietary doses of 100 and 1000 ppm in the 28-day study with mefentrifluconazole in mice (Stark et al., 2014), taking into account the difference in molecular weights (346.73 and 397.78). The actual mean daily intakes of M750F022 were 20, 180 and 587 mg/kg bw in males, 32, 249 and 718 mg/kg bw in females.

The animals were monitored daily for mortality and clinical signs. Food consumption and body weight were assessed on a weekly basis. Haematological and clinical chemistry parameters were examined in blood samples drawn towards the end of the treatment period. In addition, plasma concentrations of M750F022 were determined after 14 days of substance administration. At termination the animals were necropsied and organs weighed, followed by histopathological examinations. Kidneys, liver, ovaries, uterus (with cervix) and vagina from all groups were subjected to histopathology whereas the other organs were only examined under the microscope if they were from the control and high-dose groups. Particular attention was paid to the thymus, spleen, mesenteric and axillary lymph nodes and Peyer′s patches (jejunum), as well as the bone marrow, since histological changes to these might have indicated immunotoxicity.

Plasma kinetics revealed that the metabolite was bioavailable upon dietary exposure since it was detected at all dose levels. However, the actual concentrations were less than proportional to dose, suggesting either saturation of absorption, enforced elimination or metabolism. Individual values from low-dose males varied between 84 and 252 ng/mL whereas values between 143 and 395 ng/mL were measured in high-dose males. Similar overlapping between the dose groups was noted in females with an extreme variation from 85 to 2470 ng/mL in the high-dose group.

Two control group animals (one male and one female) died during blood sampling on day 14. In contrast to what was seen with the parent mefentrifluconazole, the metabolite did not cause a higher mortality under isoflurane anaesthesia. All animals from the treatment groups survived until scheduled termination and no clinical signs were observed that could be attributed to treatment. However, a marked reduction in food consumption was noted in high-dose females on day 7. In both sexes, transient body weight losses and, in consequence, lower terminal body weights were observed at the top dose.

Females were more severely affected and this can be partly explained by their lower food intake.

No significant and/or dose-related changes in haematological parameters were noted. Clinical chemistry revealed some indications of liver toxicity. Alkaline phosphatase activities were increased in high-dose males and females, and so was the activity of ALT in male mice receiving 2500 ppm. Another treatment-related and potentially adverse finding was a decease in triglyceride concentration in mid- and high-dose males.

Gross examination at necropsy revealed dark brown discoloration of the liver in all high-dose males, in four out of five high-dose females and in two females receiving the mid dose of 872 ppm. The liver weight was increased in both sexes at the two upper dose levels and this observation was consistent with hepatocellular hypertrophy as well as with the clinical chemistry findings. Because of its magnitude, the organ weight increase was considered adverse. Liver toxicity was also indicated by multifocal necrosis whereas the reduction in fatty change can be assumed to be due to the lower body weight. Likewise, a decrease in kidney weight and the findings in female reproductive organs were attributed to lower body weight. Histopathology revealed marginal kidney findings in male mice which might confirm the positive alert for nephrotoxicity from the Derek Nexus analysis (Woolley, 2015). A summary of body weight, organ weights and histopathological findings is provided in Table 35.

Table 35. Selected parameters from the 28-day study with M750F022 in mice

	Males				Females			
Dose (ppm)	**0**	**87**	**872**	**2500**	**0**	**87**	**872**	**2500**
Terminal body weight (g)	21.35	20.88	20.74	19.44	17.88	17.14	17.78	15.08*
Mean absolute liver weight (g)	973.0	916.2	1033.6	1347.8*	838.0	770.4	966.2*	1155.8*
Mean relative liver weight (%)	4.56	4.39	4.99*	6.94*	4.68	4.50	5.44*	7.67*
Kidney weight:								
mean absloute weight (mg)	319.0	288.0	286.6	264.8*	251.5	233.4	261.2	226.4
mean relative weight (%)	1.50	1.38	1.38	1.36	1.41	1.36	1.47	1.49
Liver: hypertrophy	0	0	5	5	0	0	5	5
Liver: fatty degeneration	4	5	0	0	4	5	0	0
Liver: multifocal necrosis	0	0	2	4	0	0	0	1
Kidney: basophilic tubules	1	1	2	5	3	0	1	2
Uterus atrophy and absence of Corpora lutea	-	-	-	-	0	0	0	3

* $p < 0.05$, ** $p < 0.01$, Kruskal–Wallis and Wilcoxon tests, two-sided; weights rounded to one or two significant figures

Because of the liver effects at the mid and high doses, the low dose of 87 ppm (equal to 20 mg/kg bw per day) was the NOAEL in this subacute study. The liver findings were similar to those observed with parent mefentrifluconazole in the 28-day study of Stark et al. (2014) and in the 90-day study in mice by Schoenmakers (2015a) suggesting a similar toxicological profile, but the metabolite had to be administered at higher doses to achieve similar responses. As in the studies cited, male mice appeared more vulnerable than females with regard to liver toxicity. In contrast, females were more sensitive when it came down to effects on food intake and body weight. On balance, it may be concluded that M750F022 is of similar or lower oral short-term toxicity to mefentrifluconazole (Buesen et al., 2016b).

The metabolite M750F022 was tested for genotoxicity in a battery of in vitro tests and was found consistently negative. Accordingly, no further studies or clarification in vitro were needed.

In an Ames test in four strains of *Salmonella typhimurium* (TA98, 100, 1535, 1537) and in one *Escherichia coli* strain (WP2 uvrA), the metabolite Reg. no. 6011210 (Batch No. L85-106; purity 98.3%) was tested by means of the plate incorporation method in two separate experiments; the pre-incubation method was used in a third study. All tests were performed with and without metabolic activation by S9 mix. Three plates were treated per dose and activation condition in all trials. The test item, as well as the positive control substances, were dissolved in DMSO which was also used as the negative control substance. In the absence of S9 mix, the following positive control substances were employed for the

different strains: *N*-methyl-*N*′-nitro-*N*-nitroso-guanidine, 9-aminoacridine, 4-nitro-*o*-phenyldiame and 4-nitroquinolone-*N*-oxide. In all the activation experiments, 2-aminoanthracene was used at concentrations of either 2.5 µg/plate (all *Salmonella* strains) or 60 µg/plate (*E. coli*).

No precipitation was observed but the maximum amounts that could be applied or that allowed meaningful evaluations in the various experiments differed a lot, depending on their toxicity to the bacteria. Toxic effects were observed in the plate incorporation assays from a concentration of 33 µg/plate upwards and in the pre-incubation assay at 100 µg/plate and above. The maximum concentrations that could be evaluated varied in the plate incorporation assay between 100 and 1000 µg/plate for the different strains. In the pre-incubation test, 100 or 333 µg/plate were the highest assessable concentrations with the *Salmonella* strains whereas *E. coli* could be tested up to 1000 µg/plate. In general, it appears that *E. coli* was less sensitive.

No increase in revertants was observed at any of the tested concentrations. The positive control substances, by contrast, gave the expected clear responses.

Under the conditions of this study, the metabolite M750F022 was not mutagenic to bacteria in the Ames test. There was however a clear toxicity to bacteria resembling a similar observation made with the parent compound mefentrifluconazole (Woitkowiak, 2015b).

M750F022 (batch L85-115, purity: 99.0%) was tested for its ability to induce forward mutations in mammalian cells by assessing the mutation of the thymidine kinase (*tk*) locus in mouse lymphoma L5178Y cells. Two independent experiments were conducted in the presence or absence of metabolic activation by rat liver S9 mix with two parallel cultures each. The solvent was DMSO. Based on the results of a preliminary cytotoxicity assay, concentrations up to 200 µg/mL were used in the main experiment. In the first experiment he treatment interval was four hours both in the presence and absence of metabolic activation. The maximum concentration was 200 µg/mL, but because of cytotoxicity, evaluation was possible only at a lower concentration, that is at 50 µg/mL. In the second experiment cultures were treated for 24 hours without activation and the highest concentration applied was 100 µg/mL. Evaluation was possible up to 25 µg/mL. In the presence of activation, treatment with up to 150 µg/mL was terminated after four hours. A concentration of 37.5 µg/mL was the highest that could be evaluated. After the incubation period, treatment media were replaced by culture medium and the cells were incubated for 48 hours for expression of mutant cells. This was followed by incubation of cells in selective medium for another 10 days. Methyl methanesulfonate was the positive control substance in the experiments without activation. In the presence of S9 mix 7,12-dimethylbenz[a]anthracene (DMBA) and CPA served as positive control substances.

No substantial and reproducible dose-dependent increase in mutant colony numbers was observed in any experiment. The reference mutagens showed a distinct increase in mutant colonies, indicating that the tests were sensitive and valid.

Under the conditions of this study, the metabolite M750F022 was negative in the mouse lymphoma assay, with and without metabolic activation (Schulz & Landsiedel, 2015).

An in vitro micronucleus assay with M750F022 (Batch No. L85-106 purity 98.3%) was performed in peripheral human lymphocytes. These cells had been obtained from a 32-year-old woman (experiment I) and a 28-year-old woman (experiment II). Prior to exposure, the cell cultures were stimulated for 48 hours with phytohaemagglutinine.

The test substance was dissolved in DMSO and applied, in a pretest, at concentrations of up to 2035 µg/mL, both with and without metabolic activation by rat liver S9 mix. Because of high cytotoxicity, a concentration of 100 µg/mL was chosen as the highest for the subsequent main experiments I and II. In experiment I, the exposure time was four hours, both with and without S9 mix. In experiment II, cells were also exposed for four hours with metabolic activation, but for 24 hours without S9. In all cultures with four-hour treatment exposure was followed by a 16-hour recovery period. At its end, or immediately following the 24-hour exposure period, cytochalasin B was applied for a further 20 hours before harvest.

The vehicle was used for negative control purposes, but different positive control substances were employed. In the absence of metabolic activation, mitomycin C was applied for four hours but demecolcin for 24 hours. In experiments with S9 mix, CPA was used as the reference mutagen.

All tests were performed in duplicate. Following cell preparation and staining, 1000 binucleated cells per culture were evaluated for cytogenetic damage using coded slides. The cytokinesis-block proliferation index was determined for 500 cells per culture to calculate % cytostasis as a measure of cytotoxicity.

Because of the extent of cytostasis, only concentrations up to 18.7 µg/mL could be evaluated in the experiments without S9 mix. Under activation conditions, the highest assessable concentration was 32.7 µg/mL. Up to these concentrations no increase in micronucleated cells was observed in any experiment. By contrast, the positive control substances gave the expected results. To conclude, the metabolite M750F022 was devoid of a clastogenic potential in this in vitro micronucleus test in human lymphocytes (Sokolowski, 2015b).

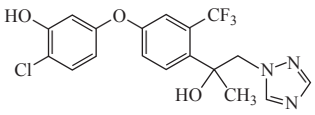
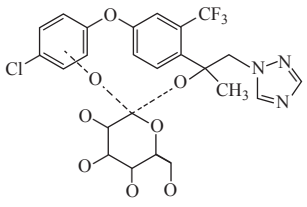
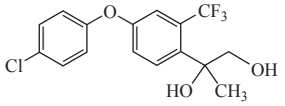
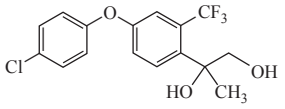
A comparative in vitro study into aromatase inhibition is briefly reported in the next subsection. It was concluded that there was no evidence that the metabolite M750F022 might have a relevant inhibiting effect on aromatase since inhibition was only seen at an exaggerated concentration (Mentzel, 2016a).

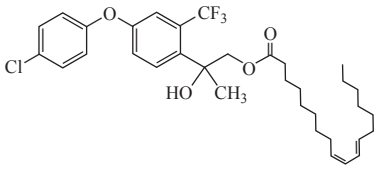
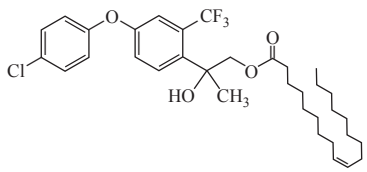
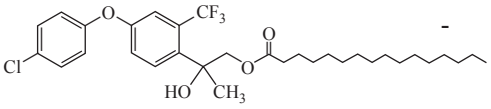
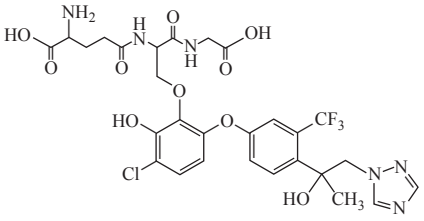
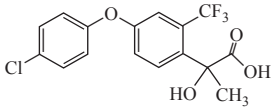
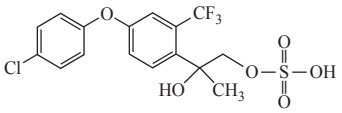
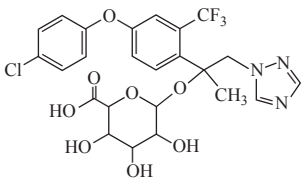
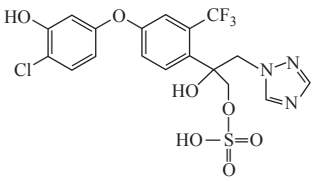
Other metabolites

A large number of mefentrifluconazole metabolites beyond M750F022 (No. 6011210) have been identified not only in the rat (see subsection 1.2 above and Annex I) but also in plants and livestock. An overview is provided in Table 36 of any of these metabolites that may have potential relevance to the residues section.

In contrast to M750F022, no toxicological or genotoxicity data is available for any of these other metabolites. To support their preliminary assessment and at the request of JMPR 2021, the sponsor provided QSAR analyses for genotoxicity for a number of metabolites and also for the parent compound mefentrifluconazole.

Table 36. Main mefentrifluconazole metabolites in plant and livestock matrices and their abundance in rat metabolism

Metabolite	Structure	Maximum of total radioactive residues [TRR] in:		Abundance in rat metabolism (% of dose)
		plant commodities	livestock matrices	
M750F016 (ruminant liver)		-	4–15% (0.13–0.21 ppm); 25–123% of parent	Up to 1.3% in urine in females, 15–32% in faeces and up to 2.1% in liver[b]; up to 2.3–5-5% in bile[c]
MF750F019 (grape leaves)		15–21% (1.1–1.6 ppm); 21–35% of parent	-	-
M750F022 (milk and all ruminant tissues)		-	1–11 % (0.001–0.10 ppm); 3–24% of parent	Possible intermediate, see Annex, Part 6 and 8)
M750F022 (eggs and all poultry tissues)			25–77% (0.02–0.37 ppm); 400–1000% of parent	

Metabolite	Structure	Maximum of total radioactive residues [TRR] in:		Abundance in rat metabolism (% of dose)
		plant commodities	livestock matrices	
M750F023 (eggs and all poultry tissues)			2–2.7% (0.004–0.25 ppm); 40–1000% of parent	-
M750F024 (poultry liver, kidney [C-label], eggs and fat [TFPM-label])[a]		-	0.8–9% (0.003–0.07 ppm); 14–82% of parent	-
M750F025 (poultry liver, kidney [C-label], eggs and fat [TFPM-label])[a]		-	0.6–16% (0.003–0.14 ppm); 5–132% of parent	-
M750F034 (poultry liver)		-	20% (0.12 ppm); 500% of parent	-
M750F038 (ruminant liver, kidney)		-	6–14% (0.06–0.15 ppm); 14–30% of parent	May occur according to Annex 1 schemes; no quantitative data available
M750F043 (milk)		-	14–25% (0.004–0.016 ppm); 29–55% of parent	May occur according to Annex 1 schemes; no quantitative data available
M750F068 (ruminant liver, kidney)		-	4–18% (0.03–0.06); 6–64% of parent	-
M750F072 (milk, ruminant kidney)		-	3–6 % (0.002–0.013 ppm); 6–13% of parent	-

[a] In the study with chlorophenyl label, these two metabolites co-eluted (in a single peak) following analysis of eggs, muscle and fat; they accounted for 11–21% (0.006–0.14 ppm); 200–500% of parent.

[b] Co-eluted with M750F0107

[c] Co-eluted with M750F015, B750F017, M750F075; together with conjugates, up to 72% in bile estimated

In silico analyses were run for the main metabolites M750F015, M750F016, M750F017, M750F022, M750F038 and M750F039, along with the parent. In each case, two "state of the art" QSAR models were used: the rule (expert knowledge)-based Derek Nexus system and a statistical model, for example CaseUltra. The end-points assessed were Ames mutagenicity, structural (clastogenicity) and numerical chromosome aberrations (aneugenicity). For parent mefentrifluconazole and M750F022, the robustness and reliability of these in silico predictions could be validated by comparison with standard genotoxicity assays which have been described earlier in this monograph.

With Derek Nexus all six metabolites and the parent were predicted negative (inactive) for Ames mutagenicity. It is important to emphasize that they were all in the in the domain of the model. Also, no alerts for chromosomal aberration in vitro or in vivo were given for any of these compounds, even though no clear statement was given whether they also were all in the domain for these endpoints.

With CaseUltra, all compounds were predicted to be negative in the Ames test since the GT1_BMUT model gave calculated probabilities for a positive outcome of only 1.3–5.4% which is far below the baseline probability of the model (22.9%) or its classification threshold (50%). For the other end-point, the GT3_MNT model did not give alerts of a positive response for the in vitro micronucleus assay for any of the compounds. The in vitro micronucleus assay would be able to detect both structural and numerical chromosome aberrations. The highest calculated probability for a positive outcome of 15.8% was well below the model′s classification threshold of 30%. All metabolites and the parent were in the domains of both models.

The overall negative predictions in silico agreed with the negative results in appropriate genotoxicity studies with mefentrifluconazole and with M750F022 (Ehnes & Urbisch, 2021).

In a separate document, an explanation was provided that for all metabolites in Table 36 genotoxicity was covered either by tests or by the negative in silico predictions (M750F022). For the main metabolite M750F016 and its co-elutes (M750F015 and M750F017) predictions were negative. In addition, these metabolites, their conjugates and some intermediates account for a considerable amount of rat metabolism based on excretion in bile (estimates up to 72%). In rat faeces, the three hydroxylated metabolites had been found at rates between 10% and 41%. Altogether, the abundance of M750F015, M750F016 and M750F017 suggest it could be considered that toxicity has been adequately co-tested for in the rat. Accordingly, reference values for mefentrifluconazole are applicable to all three metabolites.

M750019 is an *O*-conjugated glucosyl conjugate of M750F016 or M750F017, and may also be considered covered by the available information. Accordingly, the reference values for mefentrifluconazole are applicable to this metabolite as well.

For M750F022, it has already been concluded that it was not genotoxic and that the reference values for the parent were applicable.

M750F023, M750F024 and M750F025 are fatty acid esters of M750F022. Based on negative experiments with the latter, genotoxicity of these metabolites is very unlikely. However, there is no data to conclude on toxicity. Accordingly, the reference values for mefentrifluconazole are not applicable. Instead, the threshold of toxicological concern (TTC) approach for non-genotoxic compounds (Cramer class III) seems appropriate and should be taken if dietary risk assessment is needed. The appropriate TTC value here is 1.5 µg/kg bw per day.

M750F043 is a sulfate conjugate of M750F022 for which appropriate negative tests and negative predictions of genotoxicity are available. Because it is chemically derived from a well investigated metabolite to which it is structurally similar, this conjugate can be regarded as sufficiently covered as well. It is presumably non-genotoxic and the reference values for the parent compound are applicable.

For M750F038, negative in silico predictions have been made. It is an oxidation product of M750F022 (see Appendix I, Part 8); similar toxicity may be assumed. If dietary risk assessment must be performed, the reference values for mefentrifluconazole are applicable, taking the same approach as for M750F022.

For M750F034, no in silico predictions have been performed since they were not considered necessary by the sponsor. The metabolite was assumed to be an unstable intermediate and expected to rearrange to hydroxy metabolites such as M750F015, M750F016 or M750F017. On the other

hand, it has been shown to occur at a significant level in poultry liver. There is no data to conclude on genotoxicity or general toxicity of this metabolite. A possible need for further investigations will depend on the final residue evaluation.

Despite its detection in ruminant liver and kidney, no systemic exposure of humans to M750F068 is expected since it will presumably be deconjugated in the gastrointestinal tract yielding once again the parent molecule. Based on this expectation its genototoxicity and general toxicity may be considered covered. Reference values for mefentrifluconazole are applicable should such a risk assessment be needed.

The metabolite M750F072 is a sulfate conjugate of mefentrifluconazole. In the gastrointestinal tract one might expect it to become biotransformed by cleavage to M750F039, which is essentially the parent mefentrifluconazole but hydroxylated at its methyl group. For this metabolite, negative in silico predictions are available. In the absence of further data for M750F072, the TTC approach for non-genotoxic compounds of Cramer class III should be used for risk assessment if needed (Stinchcombe, 2021).

It should be emphasized here that the triazole ring-derived metabolite 1,2,4-triazole (accounting for up to 20% of the dose in rats) and its downstream biotransformation products (triazole alanine, triazolyl acetic acid and triazolyl lactic acid) have been subject to previous evaluations by JMPR and/or EFSA since they are common to other triazole fungicides. The last three metabolites occur in plants, following treatment with mefentrifluconazole, but have not been detected in rat metabolism. If needed, the respective reference doses as previously established by JMPR should be used for risk assessment purposes.

(d) Mechanistic studies

Liver enzyme induction and cell proliferation

The liver was identified as the main target organ of mefentrifluconazole in various species and efforts were taken to further investigate the nature of liver changes and their potential relevance to humans. For this purpose studies on liver enzymes and cell proliferation induction were carried out in C57BL/6J mice (both wild-type and Pxr/Car knock-out strains) and in Wistar rats, after dietary exposure to dose levels used in the carcinogenicity studies. In addition, in vitro investigations were carried out using primary hepatocyte cultures from human donors and from wild-type and knockout mice.

Study 1

In a non-GLP study, groups of ten male and ten female wild-type mice received via their diet the same dose of mefentrifluconazole but for different treatment intervals. The dietary dose for male mice was 200 ppm but 250 ppm for females, the same nominal doses used in the long-term study in mice by Schoenmakers (2015b) detailed above. Depending on duration of treatment, the corresponding dietary intakes in the different groups varied from 28.4 to 31.7 mg/kg bw in male mice and between 30.9 and 52.6 mg/kg bw in females. The individual groups were terminated after 3, 7, 14, or 28 days of treatment. Control groups of same size were kept for the same time periods but on a control diet. In order to enable further immunohistochemical investigations, osmotic pumps had been surgically implanted, under anaesthesia in the animals' subcutis seven days before scheduled termination. These pumps contained 200 µL of bromodeoxyuridine (BrdU) solution which was continuously released.

Body weight and food consumption were measured on a weekly basis. Following termination, plasma samples were taken for clinical chemistry analysis, liver weights were determined and liver samples taken. After preparation for Taqman® mRNA analysis, Western blotting for protein analysis, and measurement of Cytochrome P450 enzyme activites, a number of biochemical parameters were investigated.

Apart from the premature death of one male mouse from the group treated for three days, all animals survived until scheduled termination. Food consumption was not altered and nor was body weight in male mice. In females, by contrast, mean body weights tended to be higher in all treatment groups. Increases in absolute and relative liver weight (1.2- to 1.6-fold) were observed in both sexes at all time points. Clinical chemistry revealed significant increases in ALT activity and occasional non-significant increases in ALP levels in males and females.

Induction of a variety of CYP enzymes was observed in both sexes with CYP2b10 and, to a lesser extent CYP3a11 being most affected. pentoxyresorufin-*O*-deethylase (PROD) and pentoxyresorufin-*O*-dealkylase (BROD) activities were increased. From these findings and their pattern, the author concluded that liver effects of mefentrifluconazole were CAR receptor-mediated, but could not rule out involvement of the PXR receptor. By contrast, Ah and Ppar receptors were, most likely, not affected (Elcombe, 2015a).

Fixed tissue samples from the above study (liver; jejunum and thymus but the latter only for control purposes) in wild type mice were shipped to the sponsor to investigate liver cell proliferation by measuring the percentage of hepatocytes in S phase. Such cells can be characterized by uptake of BrdU in vivo as made visible postmortem by a colour reaction due to subsequent binding of a primary (mouse anti-BrdU) and a secondary (goat anti-mouse) antibody, in the presence of a chromogen substrate. An increase in proliferation becomes apparent from a higher labelling index. This index was defined as the ratio of BrdU-labelled hepatocytes to total number of assessed cells (being around 3000 per liver) multiplied by 100. In addition, histopathological examination of the livers were performed. The results are summarized in Table 37.

Table 37. Selected findings from subsequent analysis of mouse liver samples after treatment with mefentrifluconazole for up to 28 days

	Males		Females	
Dose	**Control**	**200 ppm**	**Control**	**250 ppm**
Day 3				
Labelling index	0.45	3.25*	1.44	5.47*
Hypertrophy, centrilobular	0/10	**8/9**	0/10	0/10
Hypertrophy, diffuse	0/10	0/9	0/10	**10/10**
Decreased vaculolation	0/10	0/9	0/10	**10/10**
Day 28				
Labelling index	0.79	0.45	1.00	0.69
Hypertrophy, centrilobular	0/10	**10/10**	0/10	0/10
Hypertrophy, diffuse	0/10	0/10	0/10	**10/10**
Decreased vaculolation	0/10	0/10	0/10	**10/10**

* $p < 0.01$, Wilcoxon test, one-sided

In male and female mice, a significant increase in hepatocytes in S phase was observed in the livers taken after three or seven days of treatment, but not in those of the animals which had been administered mefentrifluconazole for 14 or 28 days. After treatment for 28 days, the number of proliferating cells was even decreased when compared to the control animals. In addition to this transient increase in cell proliferation, microscopic changes such as hepatocellular hypertrophy and a decrease in centrilobular vacuolation confirmed previous findings from the routine toxicological studies, also confirming differences that had been seen between male and female animals. It became apparent that these effects occur very early and may be considered acute (Marxfeld & van Ravenzwaay, 2016a).

Study 2

In a second in vivo mechanistic study in the mouse, mefentrifluconazole was administered via the diet for seven days to groups of 10 male and 10 female wild-type C57BL/6 mice to further investigate liver proliferation and enzyme induction. The dose levels in this non-GLP (but well described) study were 0, 20, 50, and 200 (males) or 250 (females) ppm. For comparison, genetically modified (Pxr and Car receptor knock-outs) mice were administred the same maximum doses to elucidate whether the hepatic effects were mediated via these receptors. Treatment groups again comprised 10 mice of each sex, and separate control groups of the same size were included. All animals on study had been equipped, seven days before termination, with osmotic pumps containing BrdU as described above.

There were no unscheduled deaths and no clinical signs of toxicity were reported. Food consumption and body weight were not altered by treatment. Mean daily intakes of mefentriflucoanzole

were 3, 6.4 and 36 mg/kg bw in wild-type males and 31 mg/kg bw in knockout mouse males. In females, mean daily intakes in the wild-type groups amounted to 4.9, 112, and 59 mg/kg bw, whereas due to slightly lower total food consumption by knockout mouse females their intake was only 50 mg/kg bw per day.

Absolute and relative liver weights were increased in wild-type groups in both sexes at 50 and 200/250 ppm. In the knockout mice, there was also a significant increase in liver weights over the respective control groups but the organ weight change was less pronounced than in the wild-type animals. Clinical chemistry revealed, as the only presumably treatment-related change, higher ALT activitiy in high-dose wild-type males and females, but not in the knockout mice or at lower dose levels.

Clear differences between wild type and knockout mice were observed with regard to a number of biochemical parameters measured in the liver. Activity of PROD (as a marker for CYP2b induction) and BROD (as a marker for both CYP2B and CYP3a induction) were increased in liver microsomes from wild-type males and females even though, for PROD, there was no clear dose–response relationship in males. In knockout mice, by contrast, activities of PROD and BROD were either not affected or they even decreased.

Effects on CYP1a enzymes were inconclusive in the wild type as well as in knockout mice, whereas CYP4a was induced in the latter but not in the wild-type groups. Total cytochrome P450 levels were increased in high-dose wild-type males and in wild-type females at all dose levels but not in the knockout animals. Uridine diphosphate glucuronosyltransferase (UDP-GT) activity was not affected in Car/Pxr knockout males and females or in wild-type males. In wild type females, there was a small reduction at the top dose.

The author concluded that liver effects were most likely Car receptor-mediated. This view seems plausible but it seems less clear that the Pxr receptor was not involved. Apparently, mefentrifluconazole did not increase thyroxine glucuronidation (Elcombe, 2015b).

Study 3

Subsequent to the seven-day study (*Study 2*, above), liver samples from wild-type and knockout mice were shipped from the performing laboratory to the sponsor to investigate liver cell proliferation by measuring the percentage of hepatocytes in S phase and for additional histopathological examination. The methodological approach was the same as in the follow-up investigations to the 28-day study in mice by Marxfeld & van Ravenzwaay (2016a) reported above.

Following seven-day treatment of wild-type male mice, hepatocellular hypertrophy was noted in virtually all animals from all three dose groups, with severity increasing with dose. In wild-type females, diffuse hypertrophy was seen in all high dose (250 ppm) animals, but not at lower dose levels. It was accompanied by a decrease in centrilobular vacuolation. In male or female Pxr/Car knockout mice, treated either with 200 or 250 ppm of mefentrifluconazole, no microscopic findings were observed confirming that the histological liver changes were mediated by one or both of these nuclear receptors.

The number of liver cells in S phase, expressed by the BrdU labelling index, is shown in Table 38. Liver cell proliferation was stimulated by mefentrifluconazole in wild-type but not in knockout mice.

Table 38. BrdU labelling index in wild type and Pxr/Car knockout mice after seven days dietary administration of mefentrifluconazole

Group	Wild-type males	Knockout males	Wild-type females	Knockout females
Control; 0 ppm	0.67	0.82	2.23	1.09
20 ppm	1.71**	-	1.42	-
50 ppm	1.29**	-	3.60*	-
200 / 250 ppm	3.35**	0.88	5.87**	1.08

* $p < 0.05$, ** $p < 0.05$, Wilcoxon test, one-sided Source: Marxfeld & van Ravenzwaay, 2016b

Study4
Groups of 10 male and 10 female Wistar rats received mefentrifluconazole via their diet for 3, 7, 14 or 28 days before they were killed. Dosing commenced in a stepwise manner to ensure that the animals were all killed at the same time. The dietary dose in all groups was 3600 ppm, corresponding to mean daily intakes in the range from 228–279 mg/kg bw for males and 197–302 mg/kg bw for females. This nominal dietary dose was identical to the maximum dose in the two-year study in rats (Buesen et al., 2016a; see section 2.3). A control group of 10 rats was fed an untreated diet for 28 days. All animals had been implanted subcutaneously seven days before termination with osmotic pumps releasing BrdU.

Rats were monitored daily for mortality and signs of toxicity. Food consumption and body weight were determined weekly. At terminal kill, the animals were necropsied and their liver weights recorded. Liver sections were prepared for subsequent histopathology (see study below) and samples were taken for quantification of BrdU-labelled hepatocytes to elucidate S phase response and thus infer the liver cell proliferating activity of the test substance.

The observed effects were clearly dependent on the duration of treatment. After three days, no findings or changes were seen that could be attributed to feeding of mefentrifluconazole. After seven days body weight gain in male rats was by 22% lower than in the control group but no liver findings (including cell proliferation) were noted. When rats were treated for 14 or 28 days, body weight gain was lower in males and females even lost weight. In addition there was a significant increase in absolute and relative liver weight in females but no increase in liver cell proliferation was observed (Buesen et al., 2015b).

Liver samples obtained in this same study in Wistar rats were investigated for cytochrome P450, mRNA and protein levels, and enzyme activity. On most parameters under investigation in this ex vivo study, mefentrifluconazole had no impact, or at most only slight impact. Effects were clearly less pronounced than those in mice discussed above. In both sexes, CYP1A1 and CYP2B mRNA and protein levels were slightly to moderately induced. Induction of CYP3A mRNA and protein levels was more pronounced in females than in males. CYP4A markers were not affected. Total hepatic microsomal cytochrome P450 level was increased in males and females, after 28 days of treatment, by a maximum of 1.4- or 2-fold compared to the control group (Elcombe, 2015c).

Study 5
The impact of mefentrifluconazole on enzyme activities and DNA synthesis was examined in two in vitro studies in hepatocytes of both mouse and human origin. In the first study, the potential of mefentrifluconazole to activate the Car and the Pxr receptors and to stimulate cell proliferation was investigated in cultured hepatocytes obtained from both wild type C56BL/6 and from genetically modified (Pxr / Car knockout) mice. Due to somewhat high cytotoxicity, 10 µM was the maximum (but already slightly toxic) concentration that could be assessed. Lower test concentrations of mefentrifluconazole were 0.3, 1, and 3 µM. Phenobarbital and epidermal growth factor were used as positive control substances.

In hepatocytes from wild-type male mice there were some treatment-related effects, but sometimes no clear dose–response relationship, perhaps because of the onset of cytotoxicity. CYP2b10 mRNA and PROD/BROD activities were increased at 0.3 and 1 µM, demonstrating Car activation. DNA synthesis was slightly increased (1.45-fold over control) at 1 µM. Because the test item had very little impact on CYP3a11 both at the mRNA level and with regard to enzyme activity, it is unlikely that there was no interaction at all with the Pxr receptor.

Similar observations were made in hepatocytes from wild-type female mice, but sometimes at higher concentrations (3 or 10 µM). In the hepatocytes from knockout mice, by contrast, no effects or much weaker ones were seen. The positive controls gave the expected responses but the effect of phenobarbital on knockout mouse hepatocytes was only very weak (Elcombe, 2016a).

In a related study by Elcombe (2016b), commercially available human (male and female) hepatocytes were cultured and exposed to concentrations of 0.1, 0.3, 1.0 and 3 µM mefentrifluconazole to investigate its potential to interact with nuclear Car and/or Pxr receptors and to stimulate cell proliferation. The first aim was achieved by measuring induction of CYP2B6 and CYP3A4 liver enzyme activites and the second by determination of hepatocytes in S phase. Higher concentrations could not be tested because of cytotoxicity. Again, phenobarbital and epidermal growth factor were used as positive control substances.

Mefentrifluconazole did not induce the CYP enzymes, neither on an mRNA nor a protein level, in contrast to phenobarbital. There a small increase in BROD activity in male human hepatocytes but this findings was not confirmed by increased CYP2B6 mRNA levels. Furthermore the test item (as well as phenobarbital) had no impact on DNA synthesis under the conditions of this in vitro study. It was concluded that no proliferation of human liver cells occurred and that human nuclear receptors Car and PxR were not activated (Elcombe, 2016b).

Endocrine disrupting properties

The only possibly hormone-mediated effects observed in mammals were confined to the highest doses in the respective studies and comprised higher placental weights (13% above control and still within the historical control range) in the developmental toxicity study in rats (Schneider et al., 2015) and thyroid effects, including a positive trend for adenoma, in the long-term mouse stud of Schoenmakers (2015b). With regard to the latter findings, it must be acknowledged that thyroid hormones had not been measured. As regards the perinatal litter losses in the two-generation study in rats by Schneider et al. (2015), an endocrine MOA is not likely but, cannot be completely excluded. A lower uterus weight and a higher weight of the testes as observed in 28- and 90-day studies in dogs, were not accompanied by histological changes and more importantly were not reproduced in the one-year study of Keller et al. (2016). So on balance, the evidence for a specific effect of mefentrifluconazole on the endocrine system is weak, in contrast to what is known from the experience with many other triazole fungicides. This lower potency was confirmed in a limited number of in vitro experiments on aromatase inhibition as described in the following paragraphs. No further mechanistic studies on endocrine disruption by mefentrifluconazole are available.

In a human recombinant aromatase assay, recemic mefentrifluconazole, its *R*- and *S*-enantiomers and the metabolite M750F022 were examined for their abilities to inhibit aromatase (CYP19) activity by interaction with the substrate binding site on the enzyme. In this non-GLP study, a modified method was followed in which the fluorometric substrate *O*-benzyl fluorescein benzyl ester was used instead of radiolabelled androstenedione. This method was validated in the same test facility by testing of a number of known aromatase inhibitors and substances with no or only weak inhibitory potency. Aromatase was expressed in insect cells where it had been transferred by means of a baculovirus vector.

A half-maximal inhibitory concentration (IC_{50}) of aromatase was determined for the racemate as 0.92 µM, for the *S*-enantiomer at 0.58 µM and for the *R*-enantiomer at 2.97 µM. In contrast, the IC_{50} of the metabolite was much higher at 715 µM. Based on this data, mefentrifluconazole, but not its metabolite M750F022, might be considered as an aromatase inhibitor in vitro. The toxicological relevance of these findings is equivocal since no related findings were observed in the apical studies. When the results of the validation study are taken into account, the aromatase inhibiting activity of mefentrifluconazole and its enantiomers was much less pronounced than that of other triazole compounds such as epoxiconazole or econazole nitrate (Mentzel, 2016a, b).

As a follow up to these studies and presumably in order to allow a better comparison to in vivo data obtained in rats, inhibition of rat aromatase was investigated in a similar study that was also accompanied or preceeded by a validation study. Racemic mefentrifluconazole, its *S*- and *R*-enantionmers and the (presumably more effective) fungicide epoxiconazole were tested in vitro for their effects on rat CYP19 (aromatase) activity. Rat CYP19 supersomes (aromatase plus reductase) were exposed to the test substances (or to the positive reference substance 4-hydroxyandrostenedione) at concentrations ranging from 3.16×10^{-11} M to 1×10^{-4} M as well as to the solvent DMSO. Enzyme activity was determined fluorometrically using dibenzylfluorescein as a substrate. Resulting activity values were fitted using a four-parameter regression model, which yielded sigmoidal inhibition curves and allowed for calculation of IC_{50} values.

Similarly as found with the human recombinant aromatase assay, mefentrifluconazole and its enantiomers had a measurable effect on aromatase (CYP19) activity. Under the study conditions, the *S*-enantiomer was found to have the lowest IC_{50} at 0.294 µM, followed by the racemate with an IC_{50} of 0.402 µM and then the *R*-enantiomer at 9.19 µM. This graduated response of aromatase inhibition by mefentrifluconazole and its enantiomers was reproducibly found in all individual test runs. However, epoxiconazole had a considerably stronger inhibitory effect on rat aromatase activity, with a calculated

IC_{50} value of 0.0082 µM. Epoxiconazole was even more active in this assay than the the reference compound 4-hydroxyandrostenedione for which an IC_{50} value of about 0.0417 µM was calculated.

On balance, the inhibitory effect of mefentrifluconazole (that is the racemate) on rat aromatase appeared a bit stronger than in the human recombinant aromatase assay, but by orders of magnitude weaker than that for epoxiconazole which is known (EFSA, 2008) to exhibit severe effects on reproduction and to cause tumours of hormone-producing and hormone-dependent organs (adrenals and ovaries) in the rat (Mentzel, 2017).

3. Observations in humans

No adverse health effects in manufacturing plant personnel were noted. At the time when this monograph was prepared there were no reports on poisoning incidents available. It must be acknowledged, however, that mefentrifluconazole is a new compound and therefore the number of people exposed to this substance or its metabolites has hitherto been very limited.

Comments

Biochemical aspects

Mefentrifluconazole was subject to extensive absorption, distribution, metabolism, excretion (ADME) investigations. Most of the experiments had been performed with test substance that was radiolabelled with ^{14}C in the chlorophenyl ring or in the triazole moiety, but some data were also available for trifluoromethyl ring-labelled material. The metabolic profile observed was not significantly affected by sex, repeated administration nor the position of the radiolabel.

Following oral administration of a single dose, absorption was rapid (T_{max} of radioactivity 0.5–1.2 h) and extensive (80% or higher) at 5 mg/kg bw, and somewhat less extensive (50–70%) at 180 mg/kg bw, suggesting some saturation of absorption. The applied radioactivity was widely distributed throughout the body. Total organ and tissue residues after seven days accounted for less than 1% of the applied dose, with highest concentrations found in the gut, in stomach and gut contents, in liver and kidneys.

In all experimental groups, the major part of excretion took place within 72 hours. Elimination was complete seven days after a single low dose and accounted for more than 90% after a single high dose given alone or after repeated (15 days) low dose administration of unlabelled compound. Most of the radioactivity was excreted via the faeces (60–90%, depending on dose and radiolabel) whereas urinary elimination was less important (5–15%). In bile duct-cannulated rats, 40–60% of radioactivity was found in the bile, demonstrating that this was in fact the major excretion route. There was limited evidence of enterohepatic circulation (Wenker, 2015; Fabian & Landsiedel, 2016; Birk, Bogen & Doebbe, 2016).

Plasma kinetics of mefentrifluconazole in the mouse were similar to those in the rat (Fabian & Landsiedel, 2014).

Metabolism in the rat was extensive and complex with a total of 68 identified metabolites resulting from phase I and phase II reactions. Main metabolic pathways comprised hydroxylation, methylation and cleavage of the ether group or of the triazole ring from the parent molecule, often followed by conjugations. Most metabolites occurred at comparatively low concentrations and only very few of them, like M750F015, M750F016 plus M750F017 in the faeces, accounted for more than 30% of the dose. In urine, the most abundant metabolite was 1,2,4-triazole (M750F001) with a maximum abundance of 10.5%. In bile, the five main metabolites were all glucuronides which had been formed subsequent to hydroxylation. Amongst individual experiments and groups the percentage of the remaining parent compound varied very greatly. In faeces, there was a 1:1 ratio of the two isomers of mefentrifluconazole, whereas a shift towards the *R*-enantiomer was observed in methanolic liver and kidney extracts (Birk, Bogen & Doebbe, 2016).

A comparative study in human, rat and mouse hepatocytes revealed a completely different metabolic pattern in vitro. In rat hepatocytes, biotransformation of the parent compound was complete

after three hours of incubation but there was only one metabolite that was most likely identical to one of those found in rat bile. The same metabolite was detected in the experiment with human hepatocytes, but accounted for only 19–21% of the applied radioactivity, suggesting more delayed metabolism. In mouse hepatocytes, no metabolism at all was observed since parent mefentrifluconazole remained unchanged. Based on this limited information, no reliable conclusions regarding similarities and differences in human and animal metabolism could be drawn (Funk, Wotske & Glaessgen, 2016).

Toxicological data

The acute oral median lethal dose (LD_{50}) in rats was >2000 mg/kg bw (Hoeger et al., 2013a) and the dermal LD_{50} > 5000 mg/kg bw (Hoeger et al., 2013b). The inhalation median lethal concentration (LC_{50}) was above 5.3 mg/L (Landsiedel & Wittmer, 2014). The compound was not irritating to the skin (Hoeger et al., 2013c) or to the eyes of rabbits (Hoeger & Lammer, 2013). The test substance was devoid of a phototoxic potential in vitro (Cetto & Landsiedel, 2015). However, mefentrifluconazole was identified a skin sensitizer in a Guinea pig maximization test (Vaeth, 2013).

Oral (feeding) short-term toxicity studies were performed in mice (28-day and 90-day) and rats (28-day and 90-day. Dogs were administered mefentrifluconazole in capsules for ca 30 or for 90 days, as well as in a one-year study. In these studies, the liver was the common target organ in all the species. In the dog and in the rat, body weight and its gain were also affected. Clinical signs of toxicity were observed, but only at higher dose levels in dogs. In the mouse, there was some evidence of higher mortality in high-dose animals under isoflurane anaesthesia during blood sampling. In the short-term studies, the rat and the dog appeared less sensitive than the mouse when NOAELs and LOAELs were compared.

In a 28-day range-finding study in mice, the dietary dose levels were 0, 30, 100, 300 or 1000 ppm (equal to 0, 4.8. 15.5, 47.9 and 128 mg/kg bw per day in males, 0, 5.8, 18.5, 51 and 145 mg/kg bw per day in females). The NOAEL was 30 ppm (equal to 5.8 mg/kg bw per day) based on a marked increase in liver weight with associated alterations in clinical chemistry parameters in females at 100 ppm (equal to 18.5 mg/kg bw per day) (Stark et al., 2014).

In a 90-day study in mice, dietary doses of 0, 10, 50, 250 or 750 ppm (equal to mean daily intakes of 0, 2, 11, 58 and 174 mg/kg bw in males, 0, 3, 15, 67 and 211 mg/kg bw in females) were administered. The NOAEL was 50 ppm (equal to 11 mg/kg bw per day), based on increased liver weight with associated histopathology such as cytoplasmic alterations (hyaline vacuoles) and single cell necrosis at 250 ppm (equal to 58 mg/kg bw per day) (Schoenmakers, 2015a).

In a 28-day study in rats, dietary dose levels of 0, 500, 1500 or 4000 ppm (equal to 0, 47, 135, and 388 mg/kg bw per day in males, 0, 47, 138, and 334 mg/kg bw per day in females) were fed to the animals. The NOAEL of 1500 ppm (135 mg/kg bw per day) was based on an increase in relative liver weight in females, and associated clinical chemistry findings and reductions in body weight and its gain at 4000 ppm (equal to 334 mg/kg bw per day) in both sexes (Buesen, 2015a).

In a 90-day study in rats, dietary doses of 0, 400, 1200 or 3600 ppm (equal to 0, 27, 76, and 256 mg/kg bw per day in males, 0, 30, 91, and 314 mg/kg bw per day in females) were administered. The NOAEL was 1200 ppm (equal to 76 mg/kg bw per day), based on weak evidence of liver toxicity (increased liver weight with associated clinical chemistry findings) and reductions in terminal body weight and body weight gain at 3600 ppm (equal to 256 mg/kg bw per day) (Buesen, 2015b).

In a four-week range-finding study in dogs, the LOAEL was 125 mg/kg bw per day, the lowest dose tested, based on clinical signs, reduced body weight and indications of liver toxicity (Keller, 2015a).

In a 90-day oral toxicity study in dogs, the dose levels were 0, 15, 90 or 180 mg/kg bw per day. The NOAEL was 180 mg/kg bw per day, the highest dose tested. Minor treatment-related effects on body and liver weight and liver function at lower dose levels were not considered adverse (Keller, 2015b).

In a one-year oral toxicity study in dogs, doses of 0, 10, 30 or 150 mg/kg bw per day were administered. The NOAEL was 30 mg/kg bw per day, based on a lower body weight and body weight gain at 150 mg/kg bw per day, supported by limited evidence of liver toxicity in both sexes (Keller, 2016).

In the 18-month feeding study in mice, mefentrifluconazole was administered at dietary doses

of 0, 20, 50 or 200 ppm (equal to 0, 3.5, 9.1 and 36 mg/kg bw per day) to males and of 0, 20, 50, or 250 ppm (equal to 0, 4.9, 12.6, and 61.5 mg/kg bw per day) to females. The NOAEL was 20 ppm (equal to 3.5 mg/kg bw per day) based on liver weight increase in both sexes, a higher frequency of macrovesicular fatty change in males and an increase in severity of fatty degeneration in male and female mice at 50 ppm (equal to 9.1 mg/kg bw per day). Increased follicular cell hyperplasia of thyroids was noted in high-dose animals which apparently progressed to thyroid follicular cell adenomas in a few animals in both sexes. Mechanistic studies conducted in liver in vivo and in vitro (see below) indicated that mefentrifluconazole, like other conazoles, could activate the CAR receptor. However, the evidence was against any induction of thyroxine–glucuronosyl transferase. In addition, there were a number of species and tissue differences that were not consistent with a MOA mediated by CAR alone for either the liver or the thyroid effects. Hence, the Meeting was unable to dismiss the potential human relevance of the thyroid tumours observed in the mouse and the NOAEL for carcinogenicity was 50 ppm (equal to 9.1 mg/kg bw per day) (Schoenmakers, 2015b).

In the two-year study in rats, mefentrifluconazole was administered at dietary concentrations of 0, 100, 600 or 3600 ppm (equal to 0, 4, 25, and 163 mg/kg bw per day in males, 0, 6, 38, and 302 mg/kg bw per day in females). The NOAEL for chronic toxicity was 600 ppm (equal to 25 mg/kg bw per day) based on lower body weight, higher relative liver weights and histopathological liver findings at 3600 ppm (equal to 163 mg/kg bw per day). There was no evidence of substance-related carcinogenicity in the rat. Accordingly, the NOAEL for carcinogenicity was 3600 ppm (equal to 163 mg/kg bw per day), the highest dose tested (Buesen, 2016a).

The Meeting concluded that there was limited evidence that mefentrifluconazole is carcinogenic in mice but no evidence for its carcinogenicity in rats.

Mefentrifluconazole was tested for genotoxicity in an adequate range of studies in vitro (Woitkowiak, 2014, 2015a; Schulz & Landsiedel, 2014; Wollny, 2015a, b; Sokolowski, 2015a; Naumann, 2019) and in vivo (Schulz, Becker & Landsiedel, 2014), all of which were negative.

The Meeting concluded that mefentrifluconazole is unlikely to be genotoxic.

In view of the lack of genotoxicity, the absence of carcinogenicity in the rat and since the higher incidence of benign thyroid tumours in the mouse was confined to the highest dose levels, the Meeting concluded that mefentrifluconazole is unlikely to pose a carcinogenic risk to humans via exposure from the diet.

In a two-generation study, mefentrifluconazole was administered to rats at dietary concentrations that were weekly adjusted to keep constant dose levels of 0, 25, 75 or 200 mg/kg bw per day over the whole course of the study. The NOAEL for parental effects was 25 mg/kg bw per day, based on increased liver weight and associated clinical chemistry findings at 75 mg/kg bw per day. A (conservative) reproductive toxicity NOAEL of 75 mg/kg bw per day was based on lower fertility amongst the high-dose (200 mg/kg bw per day) F1 females, and perinatal litter losses in both generations, suggesting a possible adverse effect on certain reproductive parameters that may be related to parental toxicity. The offspring NOAEL was 75 mg/kg bw per day, based on a lower pup body weight gain and a slight developmental delay at 200 mg/kg bw per day (Schneider et al., 2015).

In a developmental study in rats, mefentrifluconazole was administered by oral gavage at dose levels of 0, 50, 150 or 400 mg/kg bw per day. The maternal NOAEL was 150 mg/kg bw per day based on reduced body weight gain and food consumption at 400 mg/kg bw per day. In addition, mean placental weight was higher in this group. The highest dose level of 400 mg/kg bw per day was considered the developmental NOAEL. There was no increase in malformations (Schneider, 2015).

In a range-finding study in female rabbits (non-pregnant), mefentrifluconazole appeared more toxic than in other species since all animals receiving doses of 50 mg/kg bw per day or greater died or had to be killed for humane reasons. A single death was also noted after repeated administration of 25 mg/kg bw per day. Even though these fatalities cannot be considered acute effects, clinical signs were observed after only very few doses. These signs included a marked reduction in food intake, missing defaecation and steady weight loss. Based on the mainly gastrointestinal nature of these signs and the pathological findings in the decedents, this apparently treatment-related effect was described as "mucoid enteropathy". On the other hand, pregnant does in a subsequent range-finding study tolerated

administration of up to 20 mg/kg bw per day without any adverse effects, which tends to suggest a steep dose–response curve. In the main developmental study in rabbits, mefentrifluconazole was administered by oral gavage at doses of 0, 5, 15 or 25 mg/kg bw per day. Since no adverse effects on the does or the fetuses were observed, 25 mg/kg bw per day, the highest dose tested, was both the maternal and the developmental NOAEL (Schneider, 2016).

The Meeting concluded that mefentrifluconazole is not teratogenic.

In an acute neurotoxicity study in rats in which gavage doses of 0, 200, 600 or 2000 mg/kg bw were administered, a transient reduction in body weight gain, unsteady gait or reduced forelimb grip strength were observed at 2000 mg/kg bw . Accordingly, the overall NOAEL was 600 mg/kg bw. Since these effects had disappeared within seven days after dosing and since there were no neuropathological findings, they were not considered specific neurotoxic effects (Buesen et al., 2015a). A separate neurotoxicity study with repeated administration was not submitted, but no concern for neurotoxicity was identified from the available studies.

The Meeting concluded that mefentrifluconazole is unlikely to be neurotoxic.

An immunotoxicity study was not submitted but no concern was identified from the available studies.

The Meeting concluded that mefentrifluconzole is unlikely to be immunotoxic.

A number of mechanistic studies were conducted to further investigate the effects on the endocrine system and on the liver.

The few in vitro studies on the presumed endocrine disrupting potential of mefentrifluconazole were all on inhibition of human recombinant aromatase. They revealed a very weak inhibition of this enzyme by the parent compound (IC_{50} = 0.6–0.9 µM) whereas the metabolite M750F022 was inactive. The aromatase-inhibiting potential of mefentrifluconazole was by orders of magnitudes smaller than that of other compounds from the triazole group (Mentzel, 2016a, b).

A number of studies on liver enzyme induction and cell proliferation were conducted in vivo in rats and in both wild-type and PXR/CAR knock-out strains of mice, as well as in vitro. Some induction of a variety of CYP enzymes was observed. It seems that the liver effects of mefentrifluconazole were mainly CAR receptor-mediated, though PXR might be also involved, in contrast to the AH and PPAR receptors. It could also be shown that liver effects in rodents develop very early after the commencement of treatment. A final conclusion on the relevance of this to humans could not be drawn (Buesen et al., 2015b; Elcombe, 2015a, b, c, 2016a, b; Marxfield & van Ravenzwaay, 2016a, b).

Toxicological data on metabolites and/or degradates

M750F022

Toxicological studies are only available for the metabolite M750F022 that was detected in livestock matrices but not in rat metabolism. This metabolite was devoid of genotoxic potential in an adequate range of in vitro tests (Woitkowiak, 2015b; Schulz & Landsiedel, 2015; Sokolowski, 2015b). It is of low acute oral toxicity with an LD_{50} in the rat > 2000 mg/kg bw (Hoeger & Lammer, 2013).

In a 28-day feeding study in mice, the metabolite caused similar liver effects to the parent compound, but only at higher dose levels (Buesen et al., 2016b).

The Meeting concluded that M750F022 is of equal or lower toxicity than the parent compound and that the reference doses as established for mefentrifluconazole are applicable.

Other metabolites

For the metabolites M750F015, M750F016, M750F017, M750F038, and M750F039, negative in silico predictions for Ames mutagenicity and chromosome aberrations (clastogenicity and aneugenicity) were made in two independent, but complimentary, QSAR models. The same negative predictions were made for parent mefentrifluconazole and the metabolite M750F022, this being in line with the negative results obtained with both in experimental studies (Ehnes & Urbisch, 2021).

M750F015, M750F016 and M750F017 were considered structurally similar to the parent, and were present in rat metabolism (> 10%, taking into account downstream metabolites). Metabolite M750F019 was considered structurally similar to M750F016 and M750F017, and several other metabolites (M750F038, M750F043, M750F068) were considered structurally similar to metabolite M750F022.

Therefore, the Meeting concluded that the reference doses for mefentrifluconazole are applicable also to M750F015, M750F016, M750F017, M750F019, M750F038, M750F043, and M750F068, in case such a risk assessment should prove to be needed.

No genotoxicity concern was raised in the case of the metabolites M750F023, M750F024, and M750F025 due to their similarity to M750F022. However, it was noted that for these two metabolites the additional fatty acid side chain may cause differences in kinetics compared to M750F022, and therefore the Meeting concluded to use the TTC approach for non-genotoxic compounds of Cramer class III, that is 1.5 µg/kg bw per day. The same reasoning was adopted towards M750F072 which may be cleaved in the gastrointestinal tract to give the (presumably non-genotoxic) metabolite M750F039, but for which no further data was available. No conclusion on metabolite M750F034 could be drawn.

Microbiological data

No data for the impact of mefentrifluconazole on the human gut microbiome was available.

Human data

From observations in manufacturing personnel, no adverse health effects have been reported. As expected for a new compound, no information on poisoning incidents or other human exposure is available.

The Meeting concluded that the existing database on mefentrifluconazole 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 on the basis of the NOAEL of 3.5 mg/kg bw per day in the long-term study in mice. A safety factor of 100 was applied. The margin from this ADI to the dose level at which an increased incidence of thyroid tumours in mice was observed, was 900.

The Meeting established an ARfD of 0.3 mg/kg bw, based on the NOAEL of 25 mg/kg bw per day for maternal and developmental toxicity in the main developmental toxicity study in the rabbit. A safety factor of 100 was applied.

Levels relevant to risk assessment of mefentrifluconazole

Species	Study	Effect	NOAEL	LOAEL
Mouse	78-week study of toxicity and carcinogenicity [a]	Toxicity	20 ppm, equal to 3.5 mg/kg bw per day	50 ppm, equal to 9.1 mg/kg bw per day
		Carcinogenicity	50 ppm, equal to 9.1 mg/kg bw per day	200 ppm, equal to 36 mg/kg bw per day
Rat	Acute neurotoxicity study [b]	Neurotoxicity	600 mg/kg bw	2000 mg/kg bw
	Two-year studies of toxicity and carcinogenicity [a]	Toxicity	600 ppm, equal to 25 mg/kg bw per day	3600 ppm, equal to 163 mg/kg bw per day
		Carcinogenicity	3600 ppm, equal to 163 mg/kg bw per day [c]	-
	Two-generation study of reproductive toxicity [a]	Reproductive toxicity	75 mg/kg bw per day	200 mg/kg bw per day
		Parental toxicity	25 mg/kg bw per day	75 mg/kg bw per day
		Offspring toxicity	75 mg/kg bw per day	200 mg/kg bw per day
	Developmental toxicity study [b]	Maternal toxicity	150 mg/kg bw per day	400 mg/kg bw per day
		Embryo and fetal toxicity	400 mg/kg bw per day [c]	-
Rabbit	Developmental toxicity study [b]	Maternal toxicity	25 mg/kg bw per day [c]	-
		Embryo and fetal toxicity	25 mg/kg bw per day [c]	-
Dog	One-year study [d]	Toxicity	30 mg/kg bw per day	150 mg/kg bw per day
Metabolite M750F022				
Mouse	Four-week study of toxicity [a]	Toxicity	87 ppm, equal to 20 mg/kg bw per day	872 ppm, equal to 180 mg/kg bw per day

[a] Dietary administration

[b] Gavage administration

[c] Highest dose tested

[d] Capsule administration

Acceptable daily intake (ADI) applies to mefentrifluconazole and its metabolites M750F015, M750F016, M750F017, M750F019, M750F022, M750F038, M750F043, and M750F068, expressed as mefentrifluconazole:

0–0.04 mg/kg bw

Acute reference dose (ARfD) applies to mefentrifluconazole and its metabolites M750F015, M750F016, M750F017, M750F019, M750F022, M750F038, M750F043, and M750F068, expressed as mefentrifluconazole:

0.3 mg/kg bw

Information that would be useful for the continued evaluati on of the compound

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

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

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid (T_{max} = 0.5–1.2 h at low dose of 5 mg/kg bw); ≥ 80% at low dose, 50–70% at high dose of 180 mg/kg bw
Dermal absorption	No data
Distribution	Widely distributed; highest residues in liver, gut and stomach and their contents
Potential for accumulation	Not observed
Rate and extent of excretion	Nearly complete within 3 days, mainly via faeces (≥ 80%); biliary excretion accounting for elimination of most of absorbed dose (40–60%), urine less important (5–15%)
Metabolism in animals	Extensive with up to 68 metabolites occurring in the rat in the different matrices; mainly by hydroxylation, methylation and cleavage of ether bond or triazole ring, followed by conjugations
Toxicologically significant compounds in animals and plants	Parent compound and livestock metabolite M750F022 (others may be identified by FAO evaluation in 2022)
Acute toxicity	
Rat, LD_{50}, oral	> 2000 mg/kg bw
Rat, LD_{50}, dermal	> 5000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.3 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Not irritating
Dermal sensitization	Sensitizing (Magnusson & Kligman test)
Short-term studies of toxicity	
Target/critical effect	Liver (organ weight increase, histopathological lesions, clinical chemistry alterations) in all species; body weight loss in rats and dogs
Lowest relevant oral NOAEL	11 mg/kg bw per day (mouse)
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	Liver (histopathology, clinical chemistry organ weight) and reduced body weight in rodents; in addition thyroid (histopathology) only in mice
Lowest relevant NOAEL	3.5 mg/kg bw per day (mouse)
Carcinogenicity	Not carcinogenic in rats, carcinogenic in mice (thyroid adenoma) [a]
Genotoxicity	No evidence of genotoxicity in vitro or in vivo
Reproductive toxicity	
Target/critical effect	Reproductive toxicity: possible impact on female fertility and increase in perinatal losses Offspring toxicity: low pup weight and developmental delay Parental toxicity: increased liver weight, clinical chemistry
Lowest relevant parental NOAEL	25 mg/kg bw per day
Lowest relevant offspring NOAEL	75 mg/kg bw per day
Lowest relevant reproductive NOAEL	75 mg/kg bw per day

Developmental toxicity	
Target/critical effect	Maternal: decreased food intake and body weight loss, mortality and gastrointestinal signs in range-finding studies in rabbits Developmental: none
Lowest relevant maternal NOAEL	25 mg/kg bw per day, highest dose tested in main study (rabbit)
Lowest relevant embryo/fetal NOAEL	25 mg/kg bw per day, highest dose tested in main study (rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	No evidence of a specific neurotoxic potential up to 2000 mg/kg bw
Subchronic neurotoxicity NOAEL	No data, but no evidence from routine studies
Developmental neurotoxicity NOAEL	No data
Phototoxicity	No data, no concern from routine studies
Mechanistic studies	Negative in vitro; Early-occurring liver effects (more pronounced in mice) most likely CAR-mediated High cytotoxicity in vitro confirmed; low potential for aromatase inhibition in vitro (in particular as compared to other triazole fungicides)
Studies on toxicologically relevant metabolites	***M750F022*** Acute oral toxicity: LD_{50} > 2000 mg/kg bw (rat); 28-day feeding study in mice: NOAEL 20 mg/kg bw per day, liver and possible kidney identified as target organs; Genotoxicity: negative in vitro (experiment and QSARs); Aromatase inhibition: much lower effect than with parent ***M750F015, M750F016, M750F017, M750F038, M750F039*** Negative in silico predictions for genotoxicity
Human data	Not available for this new compound

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

Summary

	Value	Study	Safety factor
ADI	0.04 mg/kg bw [a]	18-month carcinogenicity study (mouse)	100
ARfD	0.3 mg/kg bw [a]	Developmental toxicity (rabbit)	100

[a] Applies to mefentrifluconazole and its metabolites M750F015, M750F016, M750F017, M750F019, M750F022, M750F038, M750F043 and M750F068, expressed as mefentrifluconazole

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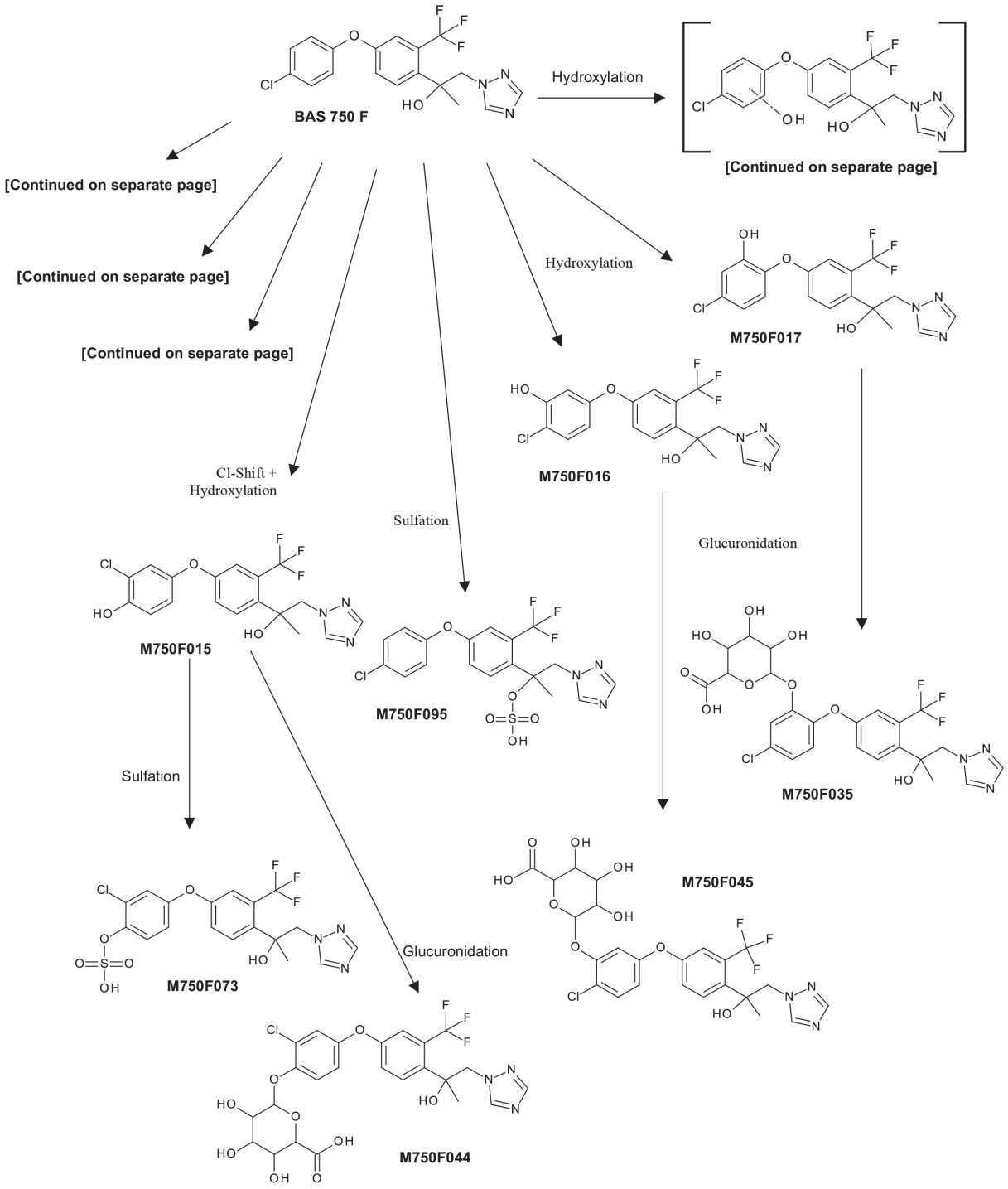
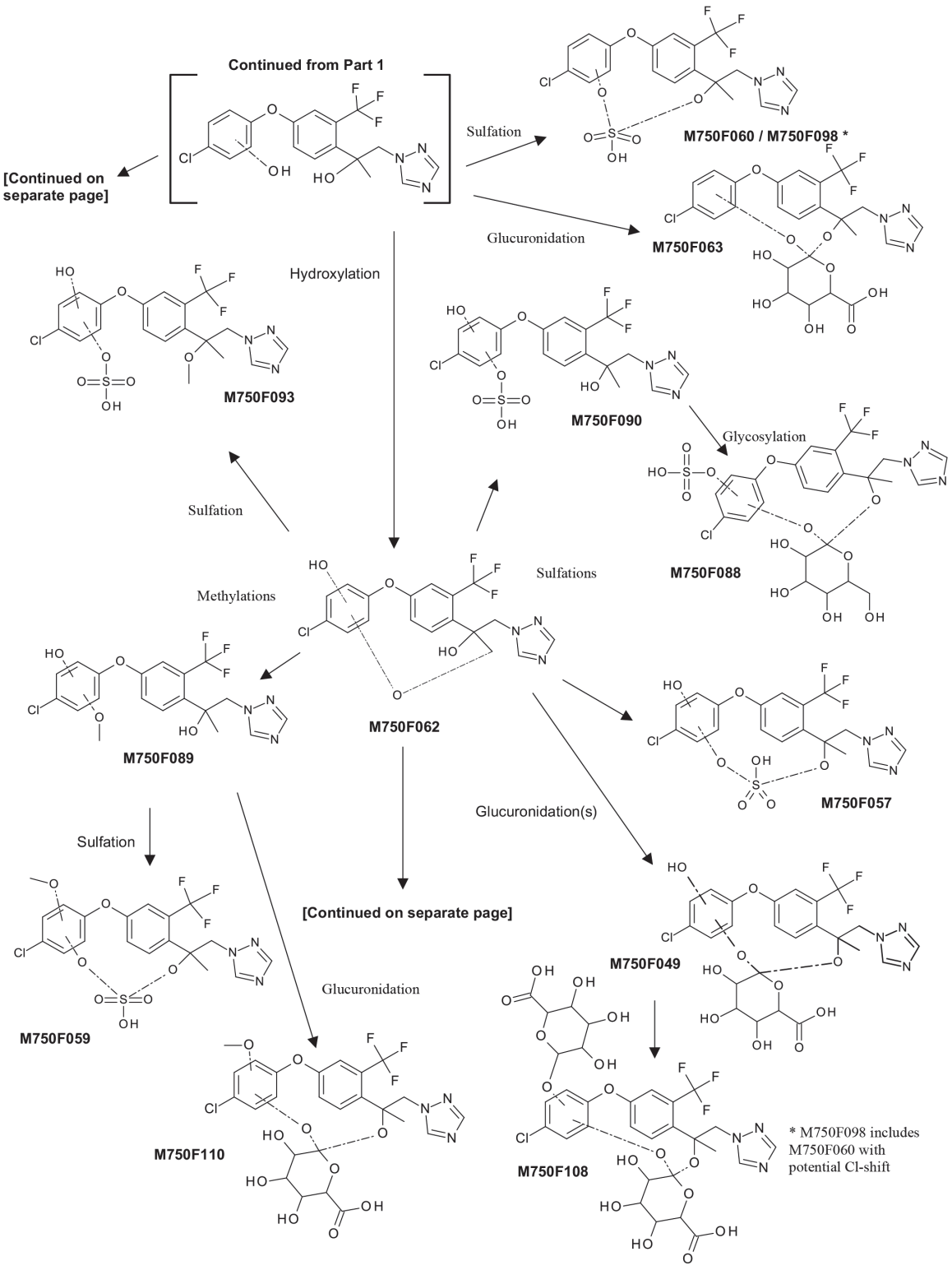
Appendix 1. Metabolic pathways for mefentrifluconazole in the rat as proposed by the Sponsor

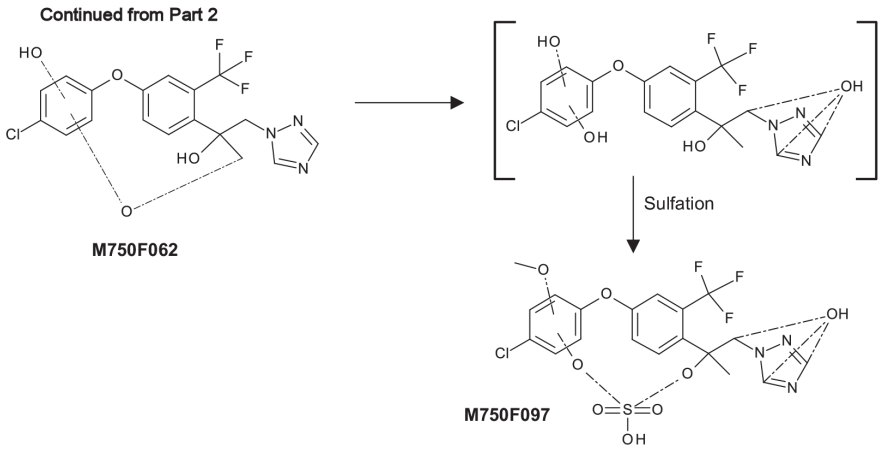
Part 1: General overview



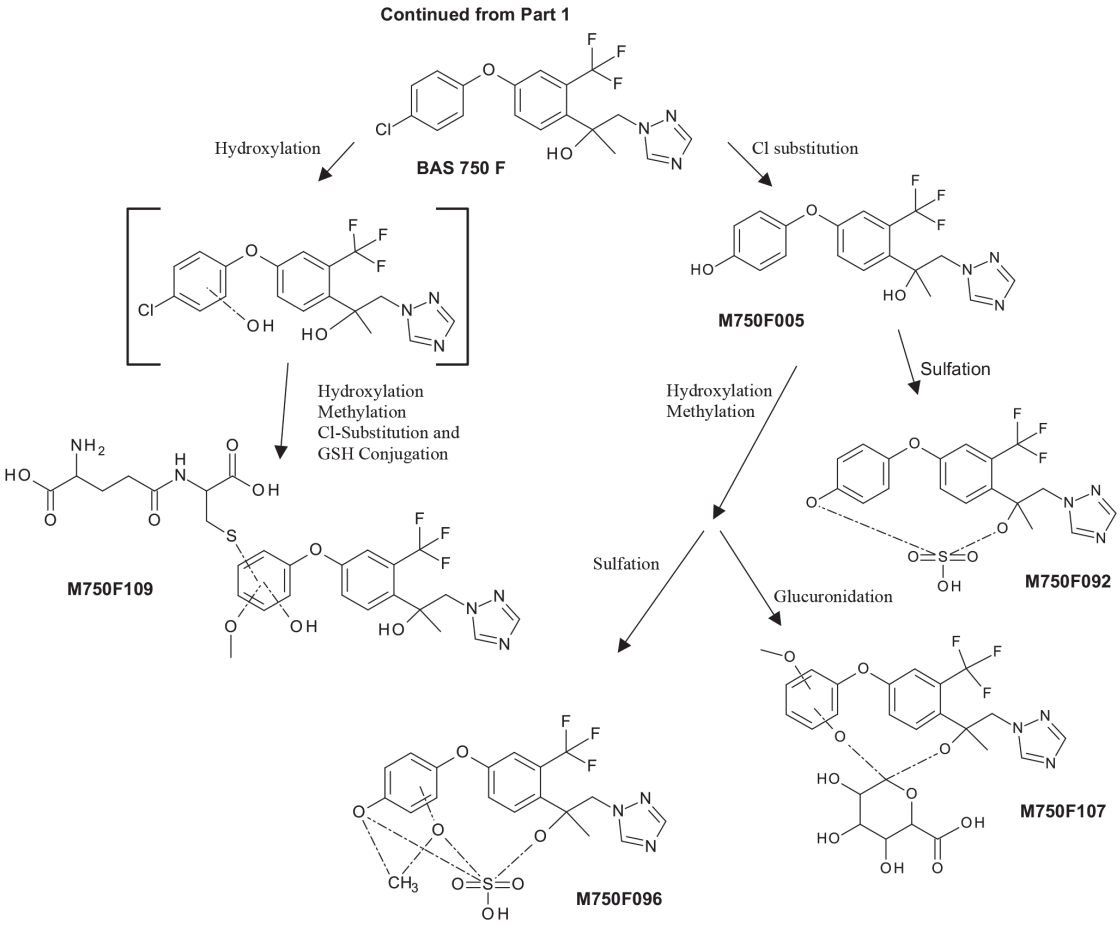
Part 2: First and second hydroxylation, multiple Phase I and Phase II



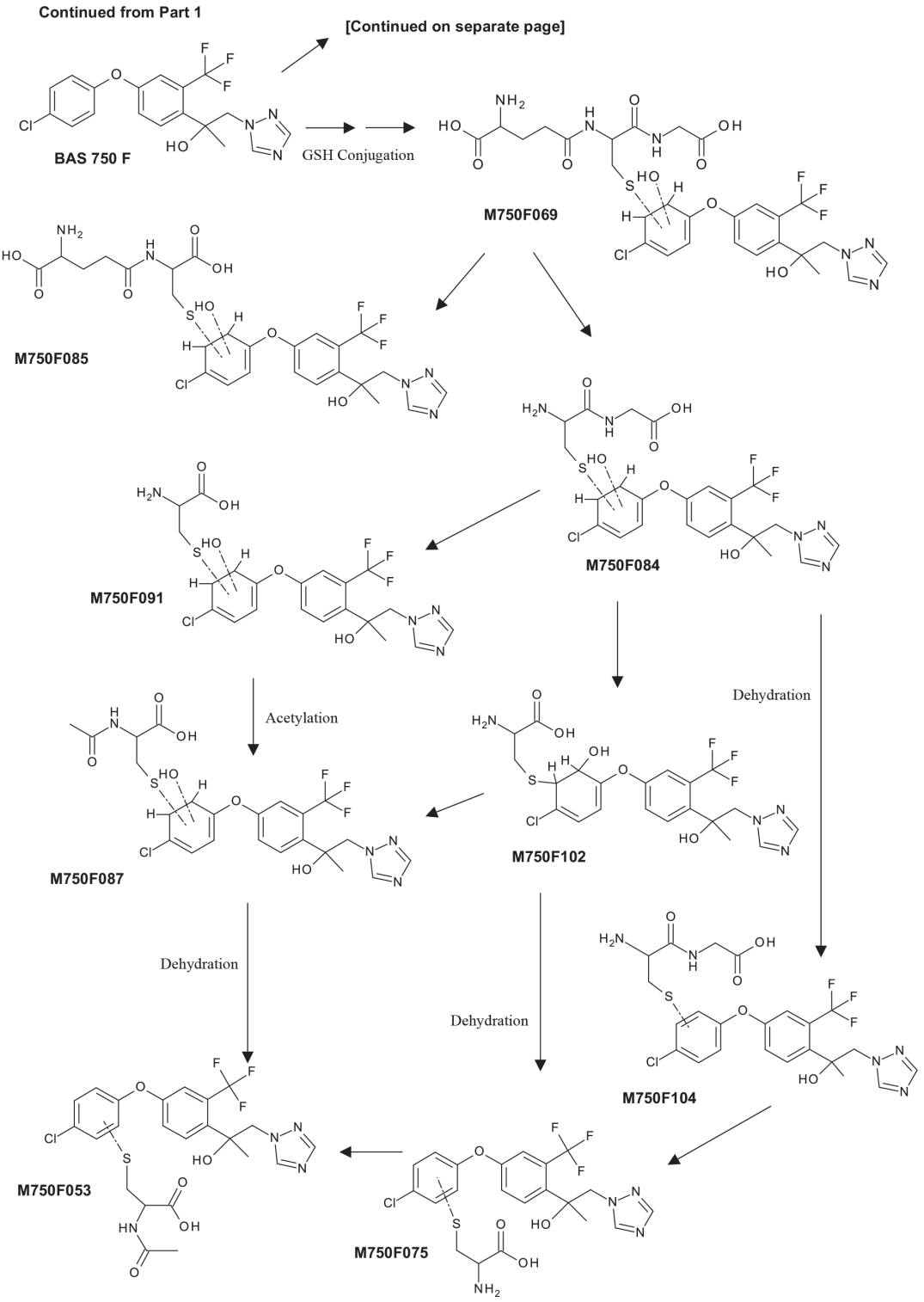
Part 3: Third hydroxylation, multiple Phase I and Phase II



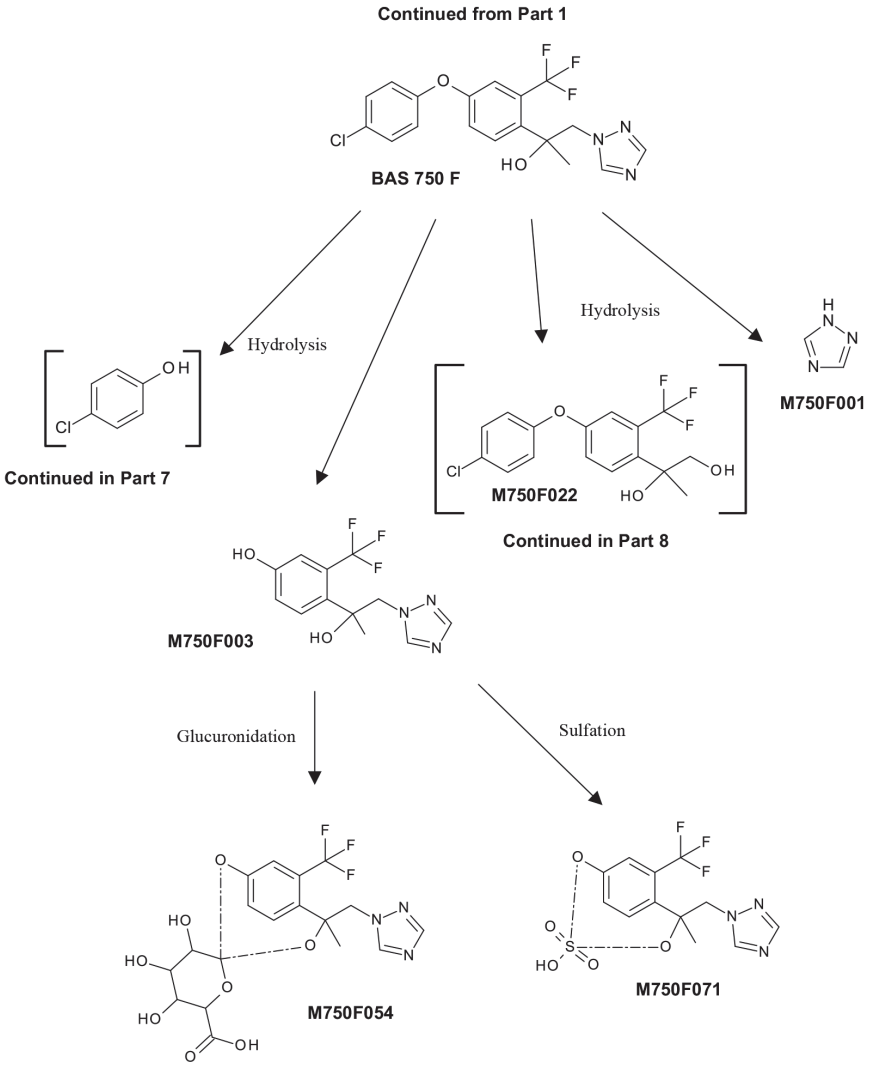
Part 4: Cl substitution, multiple Phase I and Phase II



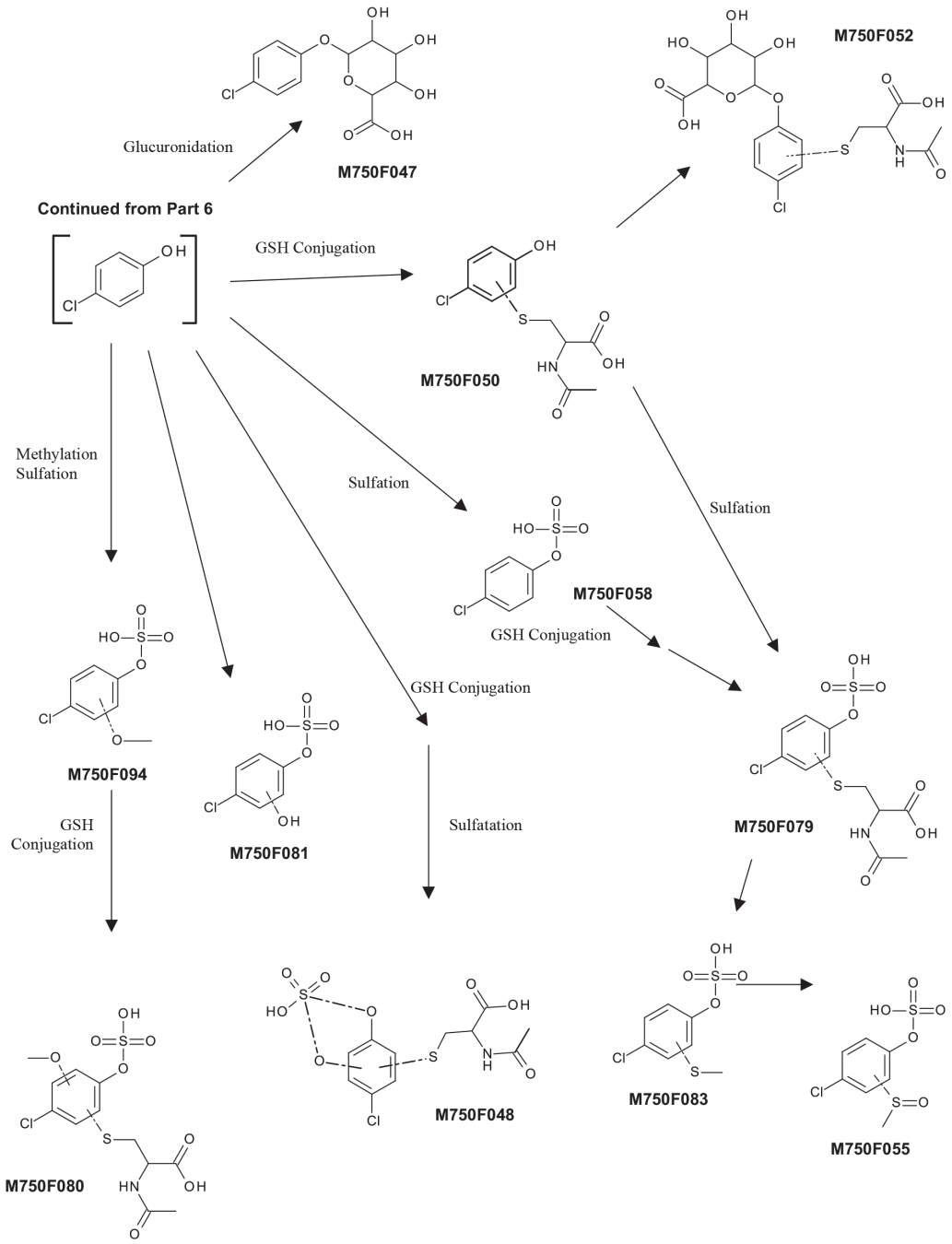
Part 5: GSH conjugation



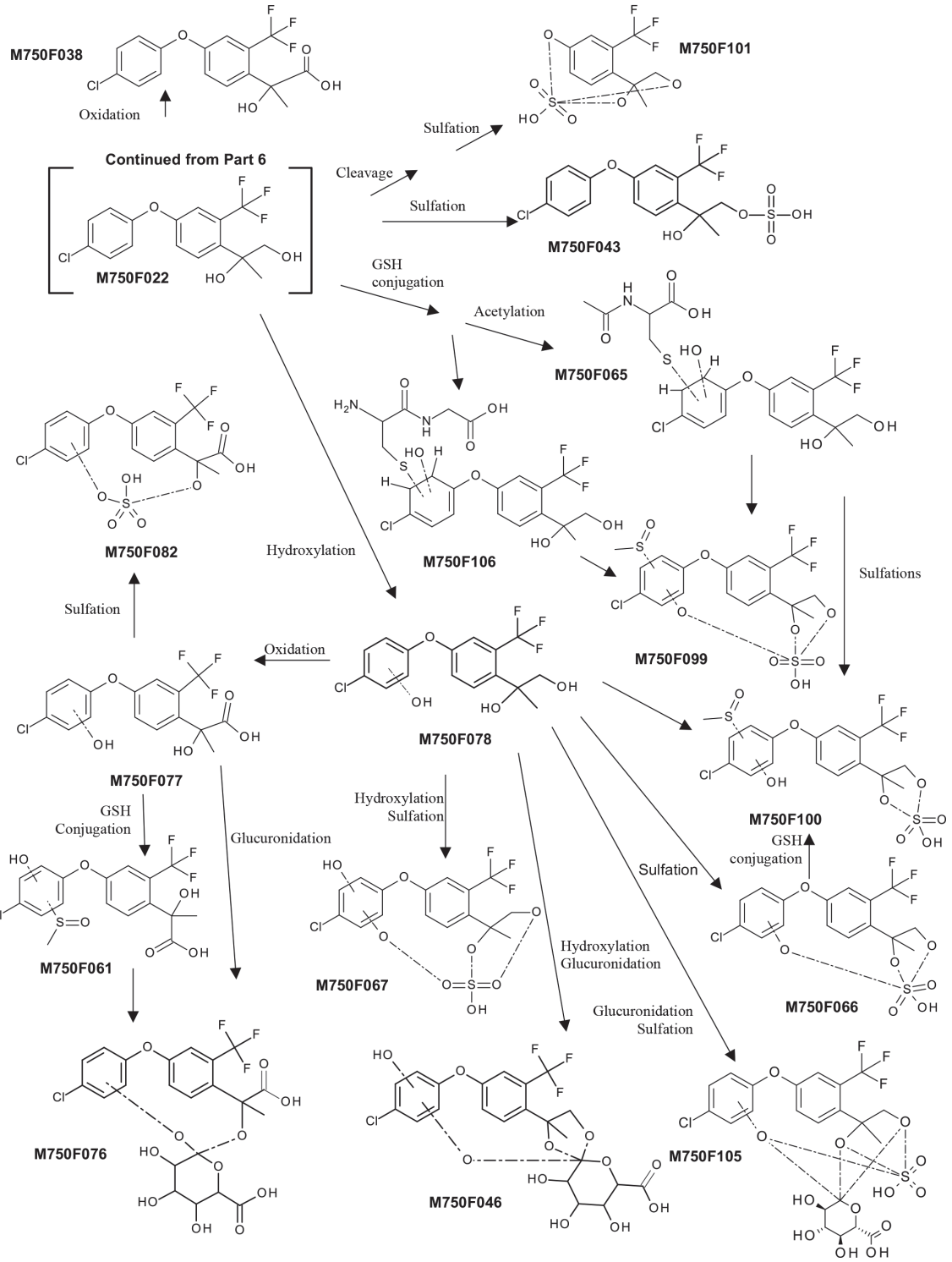
Part 6: Ether cleavage and hydrolysis



Part 7: Ether cleavage with subsequent conjugations



Part 8: Multiple Phase I and Phase II after hydrolysis



METALAXYL

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Explanation

Metalaxyl is a 1:1 mixture of (*R*)-2-[(2,6-dimethylphenyl)methoxyacetylamino]propionic acid methyl ester (*R*-enantiomer) and (*S*)-2-[(2,6-dimethylphenyl)methoxyacetylamino]propionic acid methyl ester (*S*-enantiomer). Metalaxyl-M is the ISO common name for methyl *N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)alaninate (IUPAC). Metalaxyl-M is a single enantiomer (*R* enantiomer) of the racemic mixture metalaxyl. The two compounds are fungicides used in agriculture, horticulture, and forestry, and they act by inhibiting mycelial growth and spore formation. They are of the phenylamide class of pesticides and are thought to act by inhibition of RNA polymerase I.

The toxicity of metalaxyl and of metalaxyl-M was evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1982. Investigations with metalaxyl-M were confined to studies of its absorption, distribution, metabolism and excretion (ADME), acute and short-term toxicity, mutagenicity and developmental toxicity, and were designed to establish whether there are qualitative or quantitative differences between the toxicological properties of metalaxyl-M and metalaxyl. None of the studies revealed any unexpected effects of metalaxyl-M, and the quantitative dose–effect relationships found with the racemate and the *R*-enantiomer were similar. Therefore, the 2002 Meeting concluded that the database on metalaxyl could be used for the toxicological evaluation of metalaxyl-M. The JMPR Meeting in 2002 established a group acceptable daily intake (ADI) for metalaxyl and metalaxyl-M (alone or in combination) of 0–0.08 mg/kg body weight (bw) per day on the basis of a no-observed-adverse-effect level (NOAEL) of 8 mg/kg bw per day in a two-year toxicity study in dogs with metalaxyl, and applying a safety factor of 100. The 2002 Meeting concluded that it was not necessary to establish an acute reference dose (ARfD).

Metalaxyl and metalaxyl-M were reviewed by the 2021 Meeting under the periodic review programme of the Codex Committee on Pesticide Residues (CCPR). The only study submitted since the previous JMPR evaluation of metalaxyl and metalaxyl-M was a study of the absorption and distribution of metalaxyl-M following 14-day, repeat-dose administration.

The evaluation of the biochemical and toxicological aspects of metalaxyl and metalaxyl-M was based on previous JMPR evaluations; they have been re-evaluated and updated in this report to meet current scientific standards.

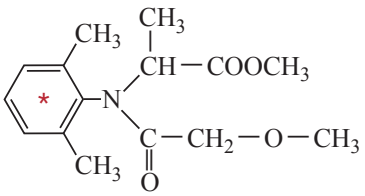
All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with relevant national or international test guidelines, unless otherwise specified. A search of the open literature did not reveal any relevant publications that would have an impact on the evaluation.

Evaluation for acceptable intake

1 Biochemical aspects

1.1 Absorption, distribution and excretion

Figure 1. Radiolabel position of metalaxyl or metalaxyl-M



* Indicates position of radiolabel

(a) Oral route

In a rat metabolism study, [^{14}C]metalaxyl (radiochemical purity > 99%) was followed in four male and four female RAI rats given a single oral gavage dose of 0.5 or 25 mg/kg bw. The animals were kept in individual metabolism cages where urine, faeces and expired carbon dioxide were collected separately for analysis at 24-hour intervals. When the rats were killed at 144 hours after dosing, the liver, fat, kidney, muscle, blood, heart, brain, lungs, spleen, ovary, testis and remaining carcass were examined for residual radioactivity.

In both sexes, irrespective of dose, more than 60% of the administered radioactivity was excreted within 24 hours, and the compound was almost completely eliminated within 144 hours (Table 1). While renal elimination was the predominant route in female rats, males excreted greater amounts in faeces. Males and females at 0.5 mg/kg bw excreted 37% and 55% of the administered dose (AD) in urine and 66% and 45% in faeces, respectively. At 25 mg/kg bw, by contrast, males and females excreted 38% and 63% in urine and 63% and 35% in faeces, respectively. Less than 0.02% of AD appeared in expired air.

Table 1. Excretion of radioactivity after a single oral dose of [^{14}C]metalaxyl in rats (% of AD)

		Dose level (mg/kg bw)			
		0.49 (low)	**0.54 (low)**	**24.1 (high)**	**26.7 (high)**
		Male	**Female**	**Male**	**Female**
Urine	0–24 hours	26.92	37.56	28.34	45.74
	24–48 hours	7.49	11.28	6.72	9.90
	48–72 hours	1.90	3.88	1.70	4.53
	72–144 hours	0.85	1.98	0.82	2.46
	Subtotal	37.16	54.70	37.58	62.63
Faeces	0–24 hours	37.79	26.26	34.00	17.62
	24–48 hours	20.41	14.10	24.37	12.87
	48–72 hours	4.02	2.67	3.30	3.28
	72–144 hours	3.48	1.94	1.37	1.34
	Subtotal	65.70	44.97	63.04	35.11
Expired air	0–48 hours	0.46	0.01	< 0.01	0.01
	48–144 hours	0.00	0.00	< 0.01	< 0.01
Total excretion		103.31	99.68	100.63	97.75
Tissue residues[a]		0.08	0.12	0.12	0.20
Cage wash		0.67	0.37	0.32	1.66
Total recovery		104.06	100.17	101.07	99.61

AD: Administered dose; Source: Hamboeck, 1977

[a] Calculated from residual radioactivity values, assuming that fat, blood and muscle represent 11%, 6.4% and 45% of body weight respectively, but without using carcass values

In animals fed a nominal dose of 0.5 mg/kg bw, residues were found at levels above the limit of quantitative determination (LOQ) only in liver, blood and carcass, still accounting for < 0.005 ppm of metalaxyl equivalent (Table 2). Animals at 25 mg/kg bw showed higher concentrations, with 0.1–0.23 ppm in liver, carcass, fat and blood and < 0.1 ppm of metalaxyl equivalent in all other tissues. The concentration of residual radioactivity in tissues was generally higher in females than in males.

Table 2. Residual radioactivity (ppm of metalaxyl equivalent) in selected tissues after a single oral gavage dose of [^{14}C]metalaxyl in rats 144 hours after dosing

	Dose level (mg/kg bw)			
Tissue	**Male: 0.49 (low)**	**Female : 0.54 (low)**	**Male: 24.1 (high)**	**Female: 26.7 (high)**
Spleen	< LOQ, > LD	< LOQ, > LD	0.025	0.046

Tissue	Dose level (mg/kg bw)			
	Male: 0.49 (low)	Female : 0.54 (low)	Male: 24.1 (high)	Female: 26.7 (high)
Carcass	LOQ	0.003	0.093	0.172
Liver	0.002	0.004	0.146	0.225
Fat	<LOQ, >LD	<LOQ, >LD	0.056	0.194
Kidney	<LOQ, >LD	LOQ	0.032	0.063
Muscle	LOQ	<LOQ, >LD	0.009	0.016
Blood	<LOQ, >LD	0.002	0.068	0.117
Brain	<LOQ, >LD	<LOQ, >LD	0.009	0.019
Heart	<LOQ, >LD	<LOQ, >LD	0.018	0.034
Lungs	<LOQ, >LD	LOQ	0.032	0.074
Testis	<LOQ, >LD	-	0.005	-
Ovary	-	<LOQ, >LD	-	0.046

<LOQ: Less than the limit of determination/quantification; From: Hamboeck (1977)

>LD: Greater than the limit of detection

Two-dimensional thin-layer chromatography (TLC) of the urine in various solvent systems demonstrated the presence of four to six major metabolite fractions and about 10 minor ones, most of the metabolites being relatively polar. The pattern of metabolites was not significantly influenced by dose or by the sex of the animals. No unchanged metalaxyl was detected in urine. The results of this study show that orally administered metalaxyl is readily absorbed from the gastrointestinal (GI) tract into the general circulation and rapidly excreted in rats. The predominant route of excretion is via the urine for females and the faeces for males. Because of the rapid elimination of the compound, the residual radioactivity in tissues was generally low (Hamboeck, 1977, 1981a).

In a series of metabolism studies, the absorption, distribution and excretion of [^{14}C]metalaxyl (radiochemical purity >98%) were studied in groups of five male and five female Sprague Dawley rats given metalaxyl as a single oral gavage dose of 2 or 80 mg/kg bw, or 2 mg/kg bw by intravenous injection. The animals were kept in individual metabolism cages, and urine, faeces and expired carbon dioxide were collected separately for analysis at 24 hour intervals for three days after dosing. Biliary excretion was investigated in male rats given metalaxyl at 80 mg/kg bw via oral gavage. Biliary excretion was monitored by taking bile samples every six hours up to 24 hours after administration. Blood samples were taken (0.5, 1, 2, 3, 4, 5, 7.5, 10, 15, 20, 25 and 30 minutes after dosing) from the caudal vein (after oral administration) or jugular vein (after intravenous injection), and portions of the samples were radioassayed. To prevent enterohepatic circulation during determination of the rate of disappearance of radioactivity from blood in animals given 2 mg/kg bw intravenously, their bile ducts were cannulated under anaesthesia, and the concentration of metalaxyl in whole blood and plasma measured. The distribution of radioactivity in plasma, blood, brain, thyroid, lung, heart, thymus, liver, kidney, adrenal, spleen, pancreas, duodenum, testis, uterus, ovary, abdominal fat and hypogastrium, abdominal and dorsal skin, femoral muscle and bone marrow was measured 1, 24 and 72 hours after administration.

After gavage, the compound was taken up readily into the general circulation. At 2 mg/kg bw, the maximum concentration (C_{max}) of radioactivity in blood was reached after 20 min in males (T_{max} = 0.48 µg/mL) and 40 minutes in females (T_{max} = 0.93 µg/mL) (Table 3). The decline in radioactivity showed a biphasic relationship, with a half-life ($t_{½}$) of 1.1 and 72 hours in males and 2 and 22 hours in females for the first and second phases, respectively. In the group given [^{14}C]metalaxyl at 80 mg/kg bw, the concentration in blood reached a maximum more slowly than with 2 mg/kg bw: T_{max} reached 40 minutes after administration in males and by 100 minutes in females The C_{max} was once again higher in the females (38 µg/mL) than in the males (19 µg/mL). The half-lives were l.5 hours and 125 hours in males and 3 hours and 96 hours in females for the first and second phases, respectively. The decreasing blood concentrations after six hours suggested enterohepatic circulation of metalaxyl or its metabolites, and this hypothesis is supported by rapid excretion in the bile, in bile duct-cannulated rats (described below).

Table 3. Concentration of radioactivity in blood following a single oral dose of [^{14}C]metalaxyl – selected times after dosing (µg equivalents of metalaxyl/mL)

	Dose level			
	2 mg/kg bw		80 mg/kg bw	
	Male	Female	Male	Female
20 min	0.48	0.87	19.0	23.2
40 min	0.37	0.93	19.3	31.9
60 min	0.25	0.85	16.3	34.5
4 hour	0.07	0.26	4.2	27.9
5 hour	0.06	ND	3.6	20.7
8 hour	0.09	0.18	2.4	8.4
12 hour	0.10	0.13	2.7	4.1
24 hour	0.09	0.09	2.3	3.1

ND: Not determined; Source: Uesugi, 1988

The rate of disappearance of radioactivity from blood of animals treated intravenously with metalaxyl at 2 mg/kg bw fitted a two-compartment model. The half-life in whole blood was 0.42 h in males and 0.64 h in females, and the half-life in plasma was 0.41 h in males and 0.56 h in females (Table 4).

Table 4. Concentration of radioactivity in blood following a single oral dose of [^{14}C]metalaxyl – selected times after dosing (µg equivalents of metalaxyl/mL)

	Dose level			
	2 mg/kg bw		80 mg/kg bw	
	Male	Female	Male	Female
0.5 min	2.62	2.65	2.84	2.99
1 min	2.64	2.68	2.90	3.07
2 min	2.16	2.61	2.38	2.82
4 min	1.80	2.28	1.96	2.49
10 min	1.28	1.80	1.47	2.27
20 min	0.95	1.52	1.04	1.86
30 min	0.78	1.35	0.85	1.46

Source: Uesugi, 1988

The distribution of radioactivity in tissues after oral administration of metalaxyl is shown in Table 5. The concentration of radioactivity in all organs (except brain) and in tissues of all treated animals reached a maximum one hour after administration and was higher than that in plasma. At 2 mg/kg bw, high concentrations of radioactivity were observed after one hour in liver, kidney and duodenum in males and in thyroid, liver, kidney, duodenum and abdominal fat in females. The concentrations in these organs and in plasma in females was higher than that in males. Thereafter, the concentrations in most organs declined gradually, and by 72 hours after administration the concentrations in liver and kidney had decreased to one-sixth to one-tenth of the values one hour after administration.

At 80 mg/kg bw, high concentrations of radioactivity were observed in thyroid, liver, kidney, duodenum and abdominal fat in males and in thyroid, liver, kidney, adrenal, spleen, duodenum and fat in females. After one hour the concentrations in most organs declined gradually and the values 72 hours after administration were one-half to one-tenth of those at one hour after dosing. It is notable that in a number of tissues in females, levels of radioactivity increased in the period 24–72 hours after administration, and this at both doses.

Table 5. Concentration of radioactivity in tissues following a single gavage oral dose of [^{14}C]metalaxyl (µg equivalents of metalaxyl/mL)

	Dose level											
	2 mg/kg bw						80 mg/kg bw					
	Male			Female			Male			Female		
Tissue	1 h	24 h	72 h	1 h	24 h	72 h	1 h	24 h	72 h	1 h	24 h	72 h
Plasma	0.08	0.05	0.01	0.40	0.03	0.01	21.84	5.04	0.32	24.60	2.36	0.56
Blood	0.09	0.08	0.05	0.36	0.05	0.08	33.00	8.00	1.64	36.88	3.20	1.56
Brain	0.06	0.06	0.03	0.31	0.03	0.04	12.28	3.27	1.48	27.84	2.20	2.03
Thyroid	0.17	0.33	0.23	1.06	0.03	0.25	79.41	58.66	14.68	52.39	5.78	27.15
Lung	0.12	0.14	0.10	0.57	0.08	0.15	27.03	11.12	6.01	21.17	3.43	5.57
Heart	0.12	0.23	0.12	0.44	0.05	0.12	18.83	6.77	5.68	31.13	3.56	6.48
Thymus	0.11	0.11	0.06	0.40	0.04	0.09	23.52	7.76	2.74	24.83	3.34	3.26
Liver	0.48	0.28	0.07	1.41	0.22	0.10	49.20	10.87	5.59	56.25	8.76	3.92
Kidney	0.45	0.27	0.08	1.51	0.25	0.10	71.50	26.34	5.86	58.04	13.92	7.90
Adrenal	0.11	0.26	0.15	0.74	0.06	0.20	35.48	11.43	6.64	59.24	4.29	11.57
Spleen	0.21	0.16	0.08	0.43	0.05	0.13	28.75	15.43	5.73	64.65	5.81	7.34
Pancreas	0.21	0.12	0.06	0.52	0.07	0.08	27.05	8.51	3.18	44.91	5.84	10.70
Duodenum	0.73	0.21	0.10	1.32	0.30	0.14	59.95	24.13	2.63	81.34	16.40	7.26
Testis	0.06	0.06	0.02	-	-	-	17.04	3.22	0.99	-	-	-
Uterus	-	-	-	0.42	0.20	0.07	-	-	-	28.50	30.77	4.45
Ovary	-	-	-	0.77	0.09	0.08	-	-	-	44.00	6.53	3.93
Fat, abdomen	0.07	0.11	0.03	1.70	0.03	0.07	36.23	9.11	2.26	83.96	5.33	3.58
Fat, hypogastrium	0.06	0.06	0.05	1.47	0.09	0.07	91.67	5.96	4.00	84.83	44.76	3.65
Skin, abdomen	0.14	0.13	0.14	0.72	0.06	0.23	24.07	9.89	9.74	32.01	4.95	10.67
Skin, dorsum	0.11	0.16	0.16	0.55	0.06	0.10	32.58	5.68	6.42	26.47	4.12	8.02
Muscle, femur	0.12	0.10	0.04	0.38	0.06	0.07	22.50	6.66	3.33	25.10	5.53	5.21
Bone marrow	0.13	0.19	0.17	0.49	0.02	0.27	29.40	20.28	8.59	32.05	3.16	10.90

Source: Uesugi, 1988

The amounts of radioactivity excreted in urine and faeces and in expired air are shown in Table 6. Urinary and faecal excretion was rapid, both males and females excreting 67–84% of the administered dose within 24 hours and 92–100% within 72 hours. The amounts excreted in expired air by rats at 2mg/kg bw was below the level of detection at all times, whilst at 80 mg/kg bw the mount excreted was 0.001–0.006% of AD.

Table 6. Cumulative excretion of radioactivity in rat urine and faeces following a single oral dose of [^{14}C]metalaxyl (% of dose)

	Dose level											
	2 mg/kg bw						80 mg/kg bw					
Time	Male			Female			Male			Female		
(hours)	Urine	Faeces	Total	Urine	Faeces	Total	Urine	Faeces	Total	Urine	Faeces	Total
0–24	31.3	42.6	73.8	49.0	22.2	71.2	45.6	38.7	84.2	53.5	13.0	66.5
48	36.8	52.7	89.6	57.0	30.8	87.8	49.7	48.1	97.7	64.6	27.4	92.0
72	38.9	56.5	95.4	59.5	32.7	92.2	50.7	49.9	100.7	67.2	29.7	96.9

Source: Uesugi, 1988

When cannulated rats were given a 2 mg/kg bw oral dose of [^{14}C]metalaxyl, males excreted 31.4% of the radioactivity into the bile within one hour, 49.1% within two hours and 71.0% within 24 hours (Table 7). Females excreted 10.6% within one hour, 33.1% within two hours and 65.8% within 24 hours. Sex differences were seen when treated with [^{14}C]metalaxyl at the higher dose level of 80 mg/kg bw. Males excreted 15.0% in bile within one hour, 29.3% within two hours and 69.4% within 24 hours, whereas females excreted 1.2% in bile within one hour, 4.4% within two hours and 54.5% within 24 hours. When bile duct-cannulated rats were given a 2 mg/kg bw intravenous dose of [^{14}C]metalaxyl, males excreted 30.2%, 66.8% and 90.7% of the radioactivity into the bile duct within 10 minutes, 30 minutes and five hours of administration, respectively (Table 8). Females excreted 9.1%, 35.8% and 91.2% of the radioactivity into the bile duct within 10 minutes, 30 minutes and five hours, respectively. These results show that for metalaxyl, transportation and metabolism involving the liver and bile duct differ between males and females.

Table 7. Biliary excretion of radioactivity following a single oral dose of [^{14}C]metalaxyl in bile duct-cannulated rats (cumulative excretion of radioactivity as % of AD)

Time (hours)	Dose level							
	2 mg/kg bw				80 mg/kg bw			
	Male		Female		Male		Female	
	Bile	Urine	Bile	Urine	Bile	Urine	Bile	Urine
0–1	31.4	-	10.6	-	15.0	-	1.2	-
2	49.1	-	33.1	-	29.3	-	4.4	-
3	58.4	-	49.0	-	37.1	-	9.9	-
4	63.0	-	56.1	-	40.3	-	16.6	-
5	64.9	-	58.8	-	42.9	-	22.8	-
6	66.3	-	60.5	-	45.6	-	28.2	-
8	68.3	-	62.4	-	50.6	-	36.3	-
10	69.1	-	63.7	-	54.8	-	40.3	-
12	69.5	-	64.3	-	58.1	-	42.6	-
24	71.0	24.4	65.8	29.1	69.4	22.3	54.5	14.3

AD: Administered dose

Source: Uesugi, 1988

Table 8. Biliary excretion of radioactivity following a single intravenous dose of [^{14}C]metalaxyl in bile-cannulated rats (cumulative excretion of radioactivity (% of dose)

Sampling time	Dose level 2 mg/kg bw			
	Male		Female	
	Bile	Urine	Bile	Urine
0-10 min	30.2	ND	9.1	ND
20	54.8	ND	24.3	ND
30	66.8	ND	35.8	ND
40	73.8	ND	44.5	ND
50	78.2	ND	51.3	ND
1 h	81.4	ND	57.0	ND
2	88.5	ND	76.8	ND
3	90.0	ND	85.1	ND
4	90.5	ND	89.2	ND
5	90.7	7.7	91.2	3.7

ND: Not determined;

Source: Uesugi, 1988

The results of this study show that orally administered metalaxyl is rapidly absorbed in rats through the digestive tract. The two-fold higher C_{max} in females than in males may have been due to different excretory rates into bile, and the subsequent biphasic pattern of disappearance showed enterohepatic circulation. Metalaxyl and its metabolites were excreted rapidly in urine and faeces, the differences between the sexes being due to the differences in biliary excretion. The higher excretion rate in urine by females suggests qualitative and quantitative differences in the metabolites in bile, a difference in the reabsorption rate from the digestive tract and a different flow to enterohepatic circulation. The rates of excretion in bile were high in both sexes. No difference was found in the total amounts excreted by males and females over 24 hours, but males showed a higher excretion rate in the early stages, as confirmed in experiments with intravenous administration. The average absorption of metalaxyl was thus about 90%. Metalaxyl translocated readily to all tissues except brain, with maximal amounts one hour after oral administration, decreasing to relatively low concentrations in all tissues by 72 hours after administration (Uesugi, 1988).

The absorption, distribution and excretion of [^{14}C]metalaxyl (radiochemical purity 97.2–97.3%) were studied after intravenous and oral administration to groups of five male and five female Sprague Dawley rats. Group I was dosed by intravenous injection at low dose, Groups II and III were dosed by oral gavage at low dose and Group IV was dosed by oral gavage at high dose. The actual mean dose levels received were 1.08, 1.13, 1.12 or 203.0 mg/kg bw for Groups I, II, III and IV respectively. Groups II and IV received a single oral dose at the low and high dose level respectively. Group III was preconditioned by treatment with a series of fourteen daily oral doses at the low dose level, of unlabelled metalaxyl followed by a single oral low dose of [^{14}C]metalaxyl. Faeces and urine were collected at various time points over a seven-day period. Rats were killed at the completion of the study and specified tissues collected.

The mean total recovery of ^{14}C administered to the forty rats was 102.0%. Total mean balance recoveries were102.8%, 99.7%, 103.0% and 101.6% for Groups I, II, III, and IV respectively (Table 9).

Table 9. Recovery of radioactivity (% of AD) in rats after oral or intravenous administration of [^{14}C]metalaxyl

	Dose level [^{14}C]metalaxyl (mg/kg bw)							
	Males				Females			
Sample type	1.08 Group I	1.13 Group II	1.11 Group III	203.0 Group IV	1.08 Group I	1.13 Group II	1.11 Group III	203.0 Group IV
Urine	44.09	35.00	32.00	46.68	65.63	66.79	74.12	70.43
Faeces	59.38	62.13	63.60	54.19	35.68	34.66	35.44	31.32
Tissues	0.37	0.31	0.29	0.30	0.42	0.56	0.44	0.31
Red blood cells	0.012	0.009	0.008	0.007	0.013	0.009	0.007	0.009
Plasma	0.003	0.002	0.001	0.001	0.004	0.004	0.002	0.001
Total	103.86	97.45	95.90	101.18	101.75	102.02	110.01	102.07

Group I: Single intravenous low dose; AD: Administered dose Source: Jamerson, 1990

Groups II, III and IV: Oral gavage dose

Excretion of radioactivity was rapid and complete in all groups (Table 10), the amount eliminated in excreta ranging from 95.5% to 109.4%. More than 89% was eliminated within the first 48 hours after dosing. The pattern of elimination was different in males and females, elimination via faeces predominating in males (60%) and elimination in urine predominating in females (70%). The similar That the patterns of excretion from oral and intravenous administration were similar indicates that the compound was well absorbed. The high recovery of radioactivity in the faeces of intravenously-dosed rats (59% in males, 36% in females) suggests the involvement of biliary secretion, which was more extensive in males.

Table 10. Cumulative urinary and faecal excretion in male rats of radioactivity (as % of AD)

	Dose level [^{14}C]metalaxyl (mg/kg bw)											
	1.08 (Group I)			1.13 (Group II)			1.11 (Group III)			203.0 (Group IV)		
Time	Urine	Faeces	Total	Urine	Faeces	Total	Urine	Faeces	Total	Urine	Faeces	Total
12 h	20.21	NS	20.21	15.33	NS	15.33	13.47	NS	13.47	30.80	NS	30.80
24 h	30.90	40.27	71.17	25.84	44.08	69.92	25.02	46.79	71.81	37.88	38.91	76.79
48 h	39.23	53.52	92.75	32.11	57.26	89.37	30.08	59.75	89.83	43.68	50.48	94.16
72 h	41.37	57.10	98.47	33.66	59.93	93.59	31.13	62.25	93.38	45.15	52.79	97.94
7 days	43.83	59.38	102.91	34.77	62.13	96.90	31.89	63.60	95.49	46.41	54.19	100.60
Cage wash	44.09		102.91	35.00		96.90	32.00		95.49	46.68		100.60

Group I: Single intravenous low dose; AD: Administered dose Source: Jamerson, 1990

Groups II, III and IV: Oral gavage dose; NS: Not sampled

At the low dose, the concentrations of [^{14}C]metalaxyl equivalent were low in all tissues (Table 11). After seven days, the highest concentrations of residue (average for males and females) were found in intestine (0.03 ppm) and liver (0.007 ppm). The tissue levels were not affected by the route of administration. At the high dose, residues were measurable in all tissues, the highest concentrations at seven days being observed in the intestine (3.1 ppm) and liver (0.81 ppm). No significant difference between the sexes was seen at either dose. In all groups, <1% of the dose was recovered in tissues after seven days. Low concentrations were found in blood fractions; the highest average values at the high dose (males and females) were 0.62 ppm for erythrocytes and 0.057 ppm for plasma.

Table 11. Residual radioactivity (ppm metalaxyl equivalents) in rats after oral and intravenous administration of [^{14}C]metalaxyl

	Dose level [^{14}C]metalaxyl (mg/kg bw)							
	Males				Females			
Sample	1.08 Group I	1.13 Group II	1.11 Group III	203.0 Group IV	1.08 Group I	1.13 Group II	1.11 Group III	203.0 Group IV
Bone	0.00029	0.00027	0.00048	0.045	0.00041	0.00038	0.00035	0.094
Brain	<LOQ	0.0013	0.0002	0.055	<LOQ	<LOQ	0.00028	0.10
Fat	0.00036	<LOQ	<LOQ	0.38	<LOQ	0.0021	<LOQ	0.67
Testes	<LOQ	<LOQ	<LOQ	0.028	-	-	-	-
Ovaries	-	-	-	-	0.0011	0.0012	0.00099	0.25
Heart	<LOQ	<LOQ	<LOQ	0.098	<LOQ	0.00094	0.00047	0.14
Kidneys	0.0016	0.0012	0.0017	0.16	0.0028	0.0023	0.0021	0.28
Liver	0.0054	0.0037	0.004	0.64	0.010	0.0090	0.0082	0.98
Lungs	0.0024	0.00044	<LOQ	0.12	0.0068	0.0056	0.0037	0.20
Muscle	<LOQ	<LOQ	<LOQ	0.066	<LOQ	0.00034	<LOQ	0.11
Spleen	<LOQ	<LOQ	0.0012	0.086	0.0029	0.0012	0.00095	0.17
Stomach	0.0008	<LOQ	0.0013	0.071	0.00032	<LOQ	0.0020	0.15
Intestine	0.021	0.029	0.018	3.53	0.030	0.045	0.045	2.67
Carcass	0.0013	0.00028	0.0023	0.18	0.0018	0.0030	0.0026	1.13
RBC	0.004	0.003	0.003	0.51	0.006	0.004	0.004	0.72
Plasma	0.0007	0.0005	0.0003	0.050	0.001	0.001	0.0005	0.063

RBC: Red blood cells; <LOQ: Below the limit of quantification; Source: Jamerson, 1990

This study confirmed the previous finding that metalaxyl is rapidly and well absorbed and eliminated, with differences in excretion between males and females. Pretreatment with 14 daily doses of 1 mg/kg bw of unlabelled metalaxyl or intravenous administration did not affect the rate or route of excretion. Reflecting the rapid elimination of the compound, the residual radioactivity in tissues was generally low; even after an oral dose of 200 mg/kg bw the concentration was < 1 ppm in all tissues (Jameson, 1990).

The absorption, distribution, metabolism and excretion of metalaxyl-M and metalaxyl were compared at two doses of [*phenyl-U-*^{14}C]-labelled test substance (radiochemical purity 98.5% and 97.3% respectively) in groups of four male and four female Tif:RAif (SPF) rats. Groups received metalaxyl or metalaxyl-M at a single oral dose of 1 or 100 mg/kg bw. Urine and faeces were collected at 24 hour intervals up to 168 hours after treatment, and urine was collected after eight hours (12 hours for the group given the higher dose of metalaxyl-M). Blood samples were taken 0.25, 0.5, l, 2, 4, 8, 24 and 48 hours after dosing from all animals. Seven days after dosing, bone, brain, fat, gonads, heart, kidneys, liver, lungs, plasma, skeletal muscle, spleen, uterus, whole blood and residual carcass were taken for analysis.

The concentrations of radioactivity from both compounds reached a maximum in blood in the period 0.5–1 hour after administration, irrespective of the dose: the only exception was for females in the higher dose group where the maximum occurred at four hours after dosing (Table 12). The short half-lives of 9–14 hours, which indicate a rapid depletion from blood, were also independent of test substance, dose or sex. The total areas under the blood concentration–time curve (AUC) for metalaxyl-M and metalaxyl were similar at the lower dose, but increased proportionally at the higher dose, except for females at the higher dose of metalaxyl, for which the AUC was 179-fold higher than at the lower dose. Generally, the bioavailability of both compounds was higher in females than in males.

The urinary excretion and tissue residues indicated that the extent of absorption was similar for metalaxyl-M (37–62%) and metalaxyl (48–61%) (Table 13). As shown previously, most of an oral dose of metalaxyl was eliminated with the bile. As the absorption process is generally not influenced by chirality, it can be assumed that both test compounds were completely absorbed. Distribution occurred rapidly: seven days after the low dose of either substance the concentrations of residues in all tissues were very low, not exceeding 0.01 ppm of parent equivalents. The pattern of distribution was similar at the higher dose but approximately 100-fold greater than at low dose. The total residues remaining after seven days at the low dose were 0.23%, 0.27%, 0.16% and 0.55% of AD in males and females for metalaxyl-M and metalaxyl, respectively. At the high dose the total residues remaining after seven days were 0.17%, 0.24%, 0.17% and 0.43% of AD in male and female for metalaxyl-M and metalaxyl, respectively. Depletion of the racemate metalaxyl from adipose tissue was markedly slower than that of the *R*-enantiomer metalaxyl-M, owing to a slightly greater tendency of the racemate to form lipophilic metabolites than the *R*-enantiomer alone and subsequent deposition of the resulting metabolites in adipose tissue. However, this metabolic difference applied only to 0.01% and 0.03% of the dose of metalaxyl-M and metalaxyl, respectively.

Table 12. Blood kinetics of metalaxyl-M and metalaxyl after oral administration to rats

	[*phenyl-U-*^{14}C] metalaxyl-M				**[*phenyl-U-*^{14}C] metalaxyl**			
	1 mg/kg bw[a]		**100 mg/kg bw[a]**		**1 mg/kg bw[a]**		**100 mg/kg bw[a]**	
Kinetics	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
C_{max} (µg parent equivalent/g)	0.07	0.21	26	17	0.08	0.23	18	28
T_{max} (h)	0.5	0.5	0.5	1.0	0.5	1.0	0.5	4.0
Half-life (h)	14	12	11	10	12	9.4	11	8.5
$AUC_{0–48\,h}$ (µg × h/g)	0.9	1.4	120	130	0.9	1.5	83	270

[a] Actual doses: metalaxyl-M: 1 and 110 mg/kg bw for males and fem ales; Source: Muller, 1997
metalaxyl: 1.2 and 100 mg/kg bw for males, 1.1 and 120 mg/kg bw for females

Table 13. Absorption (percent of dose) of metalaxyl-M and metalaxyl after oral administration to rats

	[*phenyl-U-*^{14}C] metalaxyl-M				[*phenyl-U-*^{14}C] metalaxyl			
	1 mg/kg bw[a]		100 mg/kg bw[a]		1 mg/kg bw[a]		100 mg/kg bw[a]	
Tissue	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Urine	50	62	37	46	47	20	49	59
Tissue	0.23	0.27	0.17	0.24	0.16	0.55	0.17	0.43
Absorption	50	62	37	47	48	61	49	60

[a] Actual doses: metalaxyl-M: 1 and 110 mg/kg bw for males and females; Source: Muller, 1997
metalaxyl: 1.2 and 100 mg/kg bw for males, 1.1 and 120 mg/kg bw for females

The excretion pattern was essentially the same for metalaxyl-M and metalaxyl. In all groups, females showed slightly greater renal elimination than males. With both compounds, the administered dose was rapidly and almost completely eliminated, independently of dose or sex. The blood kinetics, absorption, distribution and rate and route of excretion were not influenced by chirality (Muller, 1997)

In a repeat dose study, thirty six female Wistar rats were given up to 14 consecutive daily oral doses of 2 mg/kg bw of [*phenyl-U-14C*]metalaxyl-M (radiochemical purity 99.4%). At predetermined intervals, animal were killed and selected tissues/organs removed, then these and remaining the carcasses were removed and analysed for radioactivity.

Following repeated daily oral administration of 2 mg/kg bw of [^{14}C]metalaxyl-M to female rats for 14 days, [^{14}C]metalaxyl-M related residues were well distributed in the range of tissues collected. Mean concentrations of total radioactivity in each tissue generally increased with each sampling time during the period of dosing and were detectable in all tissues analysed 24 hours after the third dose. Mean tissue concentrations were generally at the highest observed concentration 24 hours after the tenth dose, with the concentration of ^{14}C reaching steady state in all tissues. Following the cessation of dosing, all tissue concentrations declined steadily.

Mean tissue concentrations of radioactivity were highest in the liver and kidney (Table 14), consistent with both biliary and urinary elimination of [^{14}C]metalaxyl-M and its products. The lungs displayed the next highest mean concentrations of radioactivity. Until 56 days after the final dose, residues in all other tissues were generally below blood concentrations. Thereafter, blood concentrations were below the limit of detection. This indicates that material related to [^{14}C]metalaxyl-M related can freely distribute into tissues and that a small proportion of the radioactive residues remained for a period of time before being eliminated. By the final sampling time (77 days after dose 14), mean concentrations were detectable only in the lungs and kidneys. The total tissue and carcass residues at the final sampling time accounted for less than 0.02% of AD.

Circulating concentrations of radioactivity were higher in blood than in plasma during the entire sampling period. This suggests an association of total radioactivity with the cellular component of whole blood. Mean concentrations in plasma fell below a reliably detectable level by seven days after dose 14.

Table 14. Mean residues of radioactivity in tissues during and following repeated (up to 14 days) daily oral dosing of 2 mg/kg bw [^{14}C]metalaxyl-M to female rats (expressed as % of AD)

Tissue	**24 h PD 3**	**24 h PD 7**	**24 h PD 10**	**24 h PD 14**	**2 d PD 14**	**4 d PD 14**	**7 d PD 14**	**10 d PD 14**	**14 d PD 14**	**21 d PD 14**	**56 d PD 14**	**77 d PD 14**
Adrenals	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***
Brain	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	***<0.01***	***<0.01***
GI tract	4.50	2.16	0.94	1.30	0.45	0.11	0.02	<0.01	<0.01	<0.01	***<0.01***	***<0.01***
GIT contents	10.38	3.86	2.87	2.83	0.83	0.24	0.06	0.01	<0.01	***<0.01***	***<0.01***	***<0.01***
Heart	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	***<0.01***	***<0.01***

Tissue	24 h PD 3	24 h PD 7	24 h PD 10	24 h PD 14	2 d PD 14	4 d PD 14	7 d PD 14	10 d PD 14	14 d PD 14	21 d PD 14	56 d PD 14	77 d PD 14
Kidneys	0.02	0.01	0.02	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Liver	0.30	0.17	0.14	0.09	0.05	0.03	0.02	0.01	0.01	<0.01	***<0.01***	***<0.01***
Lungs	0.02	0.01	0.01	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Ovaries	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***
Pancreas	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***
Residual carcass	1.31	0.97	0.66	0.47	0.32	0.29	0.22	0.13	0.07	0.04	<0.03	<0.02
Spleen	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	***<0.01***	***<0.01***
Thymus	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***
Thyroid	<0.01	<0.01	<0.01	<0.01	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***
Uterus	<0.01	<0.01	<0.01	<0.01	<0.01	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***	***<0.01***
Tissues + carcass	16.55	7.18	4.64	4.71	1.66	0.68	0.33	0.17	0.08	0.05	0.03	0.02

PD: Post dose; h: Hours; d: Days Source: Shaw & Montgomery, 2011

<0.01: In bold italics indicates that the mean includes results calculated from data less than 30 d.p.m above background

The terminal half-life for tissue depletion was variable with the shortest estimate in the plasma and longest in the lungs, 1.0 day and 37.4 days respectively. The terminal half-life for brain, heart, kidneys, liver, muscle, spleen and whole blood were 17.11, 24.11, 18.44, 18.35, 10.06, 24.12 and 7.41 days, respectively (Shaw & Montgomery, 2011).

1.2 Biotransformation

The degradation of [^{14}C]metalaxyl (purity > 99%) was investigated in a preliminary study in 16 female Tif:RAI (SPF) rats after a single oral dose of 28 mg/kg bw. Urine and faeces were collected for 48 hours. Within that time, 64% of the radioactivity was excreted in urine and 33% in faeces. The identified metabolites accounted for about 30% of the radioactivity in urine, equivalent to 19% of the dose. The following urinary metabolites were identified chromatographically and spectroscopically:

- *N*-(2-hydroxymethyl-6-methylphenyl)-*N*-(methoxyacetyl) alanine methyl ester (14% of the dose; designated Metabolite 8, B isomer)
- *N*-(2,6-dimethylphenyl)hydroxy acetamide or *N*-hydroxyacetyl-2,6-dimethylaniline (3%; designated Metabolite 5)
- *N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)alanine (2%; designated Metabolite 1)
- *N*-(2,6-dimethylphenyl)methoxyacetamide (only in free form) 0.3% designated Metabolite 10).

The structures identified show that metabolism of metalaxyl proceeds primarily via oxidative and hydrolytic processes: (i) methyl ester hydrolysis, (ii) *N*-dealkylation, (iii) methyl ether cleavage and (iv) benzylic methyl oxidation. Most of the metabolites formed were subsequently conjugated by glucuronic acid and excreted via the kidney. They were therefore found in urine in both free and conjugated forms. Products formed by ring hydroxylation in the aniline moiety, as reported for lidocaine, mepivacaine and bupivacaine, which contain the same aniline moiety, were not found in this study (Hamboeck, 1978).

In a follow-up to the previous studies, the metabolic fate of [^{14}C]metalaxyl (radiochemical purity > 98%) was investigated in 24 female Tif:RAI (SPF) rats after administration of a single oral dose of 28 mg/kg bw. Urine and faeces were collected for 48 hours. Within this time 58% of the radioactivity was excreted in urine and 32% in faeces. The metabolites present in urine and faeces that were identified chromatographically and/or spectroscopically were:

- *N*-(2,6-dimethylphenyl)-*N*-(hydroxyacetyl)alanine
  (39% of the dose; designated Metabolite 6)
- *N*-(2-hydroxymethyl-6-methylphenyl)-*N*-(methoxyacetyl)alanine methyl ester
  (14%; designated Metabolite 8, A isomer)
- *N*-(2-6-dimethylphenyl)-*N*-(methoxyacetyl)alanine (4.1%; designated Metabolite 1),
- *N*-hydroxyacetyl-2,6-dimethyl aniline (2.9%; designated Metabolite 5)
- *N*-(2-carboxy-6-methylphenyl)-*N*-(methoxyacetyl)alanine methyl ester
  (1.2%; designated Metabolite 9)
- N-(2,6-dimethyphenyl)-N-(hydroxyacetyl)alanine methyl ester
  (0.9%; designated Metabolite3)
- 4-(2,6-dimethylphenyl)-3-methylmorpholine-2,5-dione
  (0.6%; the isomeric lactone form of Metabolite 6),
- metalaxyl; unmetabolized parent (0.4%)
- *N*-(2,6-dimethyl-5-hydroxyphenyl)-*N*-(methoxyacetyl)alanine methyl ester
  (0.3%; designated Metabolite 7).
- The urinary metabolites were partially conjugated with glucuronic acid.

At least four independent pathways of biotransformation degrade metalaxyl in rats: (i) hydrolytic cleavage of the carboxyl methyl ester group, (ii) hydrolytic (or oxidative) cleavage of the methyl ether moiety, (iii) oxidation of the toluene methyl side-chain to the benzylic alcohol derivative and (iv) oxidation of the phenyl moiety to form phenols. Secondary biotransformation pathways are *N*-dealkylation at the 2-aniline propionic acid bond, oxidation of benzylic alcohol to the benzoic acid derivative and conjugation of metabolites with glucuronic acid.

Metalaxyl was effectively metabolized by rats, preferentially by hydrolytic and oxidative reactions. The products formed were readily excreted in urine and faeces, and also as conjugates with glucuronic acid in urine. Urine and faeces generally contained the same metabolite structures, indicating their common origin from the general circulation (Hamboeck, 1981b).

Four groups of five male and five female Sprague Dawley rats, were given an intravenous or oral dose of [^{14}C]metalaxyl. Group I (intravenous dose), and group II (low oral dose), received a single administration of 1.08 and 1.13 mg/kg bw of [^{14}C]metalaxyl, respectively. Group III (preconditioned low oral dose), received 14 daily oral administrations of 1.36 mg/kg bw metalaxyl prior to receiving, on day 15, a single oral dose of 1.12 mg/kg bw [^{14}C]metalaxyl. Group IV (high oral dose), received a single oral administration of 203 mg/kg bw of [^{14}C]metalaxyl. Urine and faeces were collected over a seven-day period, and [^{14}C] residue quantitated. Rats were sacrificed at 168 hours post dose and tissues taken for residue determination (Jameson, 1990). Samples were subjected to metabolite identification.

Metalaxyl was almost completely eliminated (93–98%) in the urine and faeces of rats within 72 hours of intravenous or oral administration of [^{14}C]-labelled metalaxyl. Metabolites in the urine were characterized by TLC and sequential enzymatic hydrolysis. Metabolite patterns were qualitatively similar regardless of sex or dose. Metabolism was extensive with a possible 33 metabolites localized. Ten metabolites were identified with the aid of cochromatography against known standards (Table 15). Nine metabolites (including metalaxyl) were purified and identified from mass spectrometry (MS) and nuclearmagnetic resonance (NMR) spectral data. CGA107955 (Met. 6; 3.2–20.3%), CGA62826 (Met. 1; 0.1–4.9%), CGA108905 (Met. 9; 1.2–2.6%) and the CGA94689 B isomer (Met. 8; 0.1–5.2%) were the major metabolites in rat urine. CGA37734 (Met. 5; 0.7–1.8%) and CGA79353 (Met. 4; 0.2–1.2%) were present in moderate amounts. CGA94689 A isomer (Met. 8; 0.1–0.5%), CGA67867 (Met. 10; 0.1–0.3%) and CGA100255 (Met. 7; 0.1%) were minor components in urine. Metalaxyl co-chromatographed with CGA67869 (Met. 3) as minor components (0.2–1.8%). A newly identified metabolite, CGA108905 (Met. 9), accounted for 0.1–2.3% of AD. Between 7.9% and 30.5% of the AD was identified as free metabolites which accounted for 19.6–51.4% of the total [^{14}C] residue in urine. A high percentage (49.9–96.8%) of the urinary radioactive recovery was conjugate in nature. Following enzymatic hydrolysis, most of the metabolites were partitionable into ethyl ethanoate and co-chromatographed

with free metabolites and standards. Urinary conjugation included glucuronides, sulfates and possibly peptide adducts.

Faeces were extracted with a methanol/water mix, 80:20 (v/v), which solubilised a minimum of 91% of the radioactive residue. In faeces, Met. 6 was the main metabolite in females. Metabolite patterns of extracts from faeces and urine were similar when analysed by TLC. CGA107955 (Met. 6) was quantitated as a major metabolite in the female rat ranging 7.1–10.4% of AD. CGA107955 (Met. 6) and CGA108905 (Met. 9) co-chromatographed as a major metabolite zone in the male rat (9.0–11.0%). CGA108905 in the female rat ranged 0.7–1.2% of dose. CGA62826 (Met. 1; 0.3–2.2%) and the CGA94689 B isomer (Met. 8; 0.8–3.3%) were present in moderate amounts. Metalaxyl and CGA67869 (Met. 3) co-chromatographed (0.2–0.8%) as minor components. CGA94689 A isomer (Met. 8; 0.2–1.6%), CGA37734 (Met. 5; 0.1–0.7%), CGA79353 (Met. 4; 0.2%), CGA67867 (Met. 11; 0.1–0.3%), CGA100255 (Met. 7; 0.1–0.2%) and a newly identified metabolite, CGA108905 (Met. 9; 0.1–0.4%) were all minor faecal metabolites. Unconjugated metabolites identified in faecal extracts ranged from 10.6–19.1% of dose and accounted for 46.4–75.9% of the total [^{14}C] residue in faeces.

Conjugated metabolites represented 28.7–50.9% of the faecal radioactivity, which had been hydrolysed predominately to aglycones with β-glucuronidase (18.4–41.6%). Sulfate conjugates accounted for another 3.8–11.6% of the matrix radioactivity.

Table 15. Major metabolites of metalaxyl in rats

Chemical Name	Met. Number	Company code	Percentage of dose			
			Urine		Faeces	
			Male	Female	Male	Female
N-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)alanine methyl ester	Metalaxyl (parent)	CGA48988	0.1[a]	0.2–1.8[a]	0.4–0.8[a]	0.2–0.4[a]
N-(2,6-dimethylphenyl)-*N*-(hydroxyacetyl)alanine methyl ester	3	CGA67869 A isomer				
N-(2-hydroxymethy1-6-methylphenyl)-*N*-(methoxyacetyl)alanine methyl ester	8	CGA94689 isomers A & B	0.6	0.9–5.7	1.0–4.9	1.1–2.9
N-(2,6-dimethyl-5-hydroxyphenyl)-*N*-(methoxyacetyl)alanine methyl ester	7	CGA100255	<0.1	0.1	0.1–0.2	<0.1
N-hydroxyacteyl-2,6-dimethyl aniline	5	CGA37734	1.0–1.8	0.7–1.3	0.1–0.7	0.1
N-(2,6-dimethy lphenyl)-*N*-(methoxyacetyl) alanine	1	CGA62826	0.1	0.6 - 4.9	1.7–2.2	0.3–0.4
N-(2,6-dimethylphenyl)-*N*-(hydroxyacetyl) alanine	6	CGA107955	3.2–3.61	10–20	7.1–10.4	9.0–11
N-(2-carboxy-6-methylphenyl)-*N*-(methoxyacetyl) alanine methyl ester	9	CGA108905	1.2–2.1	1.5–2.6		0.7–1.2
N-(carboxycarbonyl)-*N*-(2,6-dimethylphenyl) alanine methyl ester	4	CGA795353	0.2–0.4	0.5–1.2	0.2	0.2
N-(2-hydroxymethyl)-6-methylphenyl)-*N*-(methoxyacetyl)alanine	2	CGA100255	0.1–0.4	0.6–2.3	0.1–0.3	0.1–0.4

[a] Metalaxyl and its A isomer (Met.3) were cochromatographed so results apply to combination Source: Itterly, 1990

Metalaxyl was extensively metabolized in all groups investigated, with little or no unchanged substance being eliminated in urine or faeces. Based on TLC, biotransformation products were similar in all groups and sex with approximately the same relative proportions being observed in urine and faeces. In urine, 7.9–30.5% of dose was identified, accounting for 19.6–51.4% of the total [^{14}C] residue in urine. For faeces, 10.6–19.1% of dose was identified and this accounted for 46.4–75.9% of the total [^{14}C] residue in faeces.

A sizable proportion of the metabolites were conjugated in both urine and faeces, especially in the low-dose groups. The metabolic route appears to be subdivided into three major and one minor pathway (see Fig. 2). Demethylation of the ether giving the alcohol, CGA67869 (Met. 3), with stepwise demethylation of the ester forming the alcohol acid, CGA107955 (Met. 6). CGA107955 (Met. 6) is the major metabolite in urine and feces. Further oxidation of the alcohol CGA67869 (Met. 3) forms the acid CGA79353 (Met. 4). *N*-dealkylation of CGA67869 (Met. 3) gives CGA37734 (Met. 5), the hydroxyacetamide.

Oxidation of the aromatic methyl of metalaxyl forms the benzylic alcohol isomers, A and B, of CGA94689 (Met. 8). The methyl ester of isomer A is demethylated forming an acid, CGA108905 (Met. 9), the benzylic alcohol of CGA62826 (Met. 1). The B isomer is stepwise oxidized to the benzoic acid CGA108905 (Met. 9). Demethylation of the ester of metalaxyl forms the acid ether, CGA62826 (Met. 1), which is a major urinary (high-dose females) and faecal (male) metabolite.

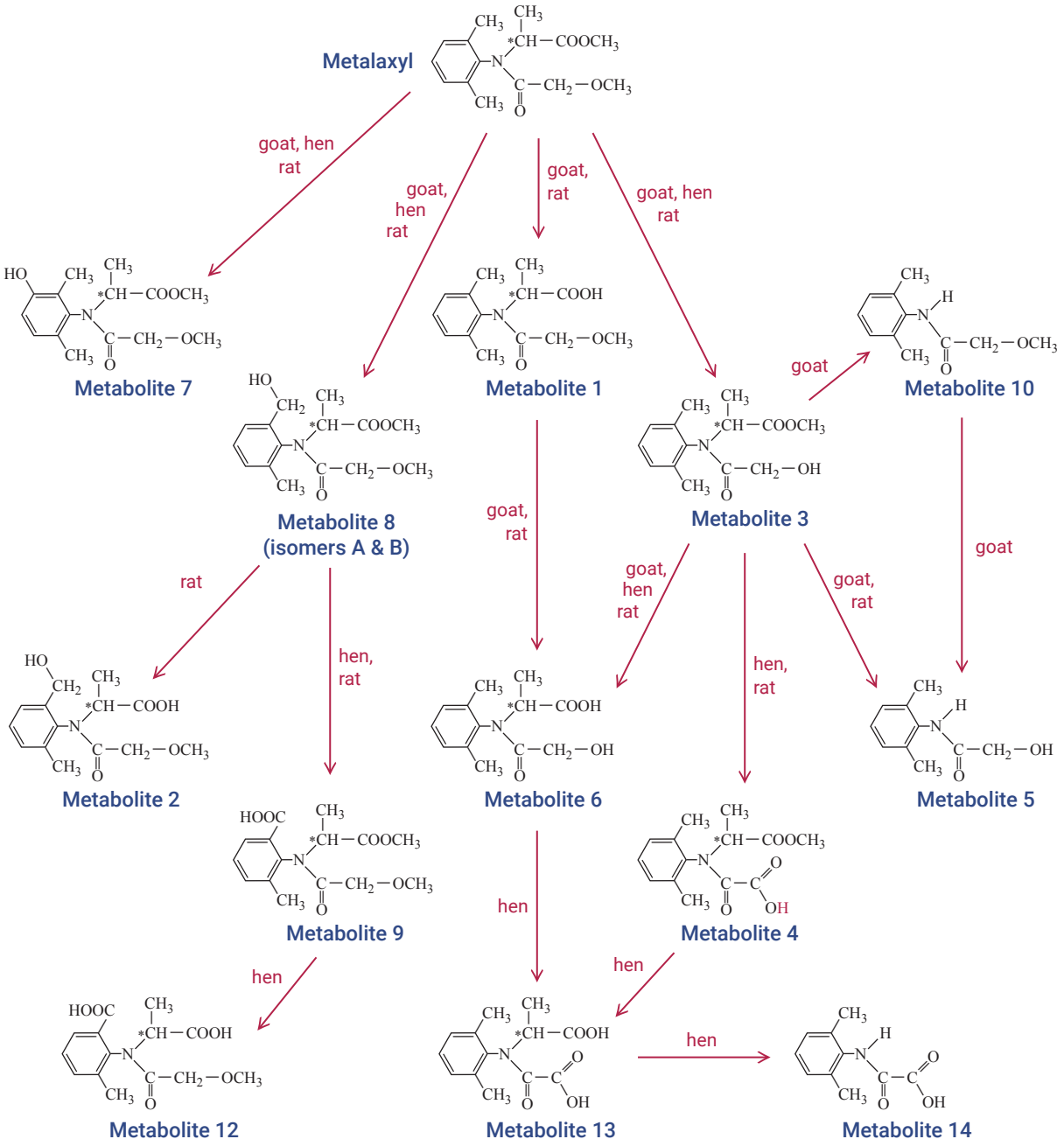
In the minor pathway, metalaxyl undergoes hydroxylation at the *meta* position on the phenyl ring forming a mixture of CGA100255 (Met. 7) isomers. All metabolites isolated undergo phase II conjugation reactions and are present as glucuronide and sulfate conjugates (Itterly, 1990).

The pattern of metabolites of the *R*-enantiomer, metalaxyl-M, and the racemate, metalaxyl, in rats was also investigated in the study of Miller (1997), the protocol of which is described above. Faeces were extracted with acetonitrile and an acetonitrile/water mix, such that 91–95% of the radioactivity present was extracted, and the composite urines from each group and the faecal extracts were analysed quantitatively by two-dimentional TLC.

Apart from stereochemistry, the metabolic patterns of metalaxyl-M and metalaxl were similar. Both were extensively metabolized, yielding 17 and 13 metabolite fractions in urine and faeces, respectively. In both cases this occurred independently of dose and sex. The dose- and sex-related differences in the concentrations of metabolites of metalaxyl found by Itterly (1990) were also seen for metalaxyl-M. Only the concentration of metabolite fraction U4 in male rats at the higher dose depended markedly on stereochemistry. Therefore, the metabolic pathways deduced for metalaxyl are also valid for metalaxyl-M (Fig. 2).

The study of ltterly (1990) showed that metalaxyl undergoes extensive phase II reactions, namely conjugation with sulfuric and glucuronic acids. Sulfonation and glucuronidation are competing pathways for hydroxylated metalaxyl metabolites, and sulfonation can be superseded by glucuronidation at increasing concentrations of substrate, resulting in a quantitative shift of metabolite distribution. These reactions are catalysed by sulfontransferases and UDP-glucuronyltransferases, which are known to discriminate between enantiomers. In the case of metabolite U4, the preferred substrate for a sex- and dose-dependent shift of conjugation appeared to be the *S*-enantiomer, as the concentration of the *R*-enantiomer was not affected by dose. The pattern of metabolites in urine and faeces thus indicated that, aside from stereochemistry, the metabolic pathways of metalaxyl-M (the *R*-enantiomer) and metalaxyl (the racemate) are similar (Muller, 1997).

Figure 2: Proposed metabolic pathway for metalaxyl and metalaxyl-M in rats, goats and hens



Metabolite 1: *N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)alanine;
Metabolite 2: *N*-[(2-hydroxymethyl)-6-methylphenyl]-*N*-(methoxyacetyl)alanine;
Metabolite 3: *N*-(2,6-dimethyphenyl)-*N*-(hydroxyacetyl)alanine methyl ester;
Metabolite 4: *N*-(carboxycarbonyl)-*N*-(2,6-dimethylphenyl)alanine methyl ester;
Metabolite 5: *N*-hydroxyacetyl-2,6-dimethylaniline;
Metabolite 6: *N*-(2,6-dimethylphenyl)-*N*-(hydroxyacetyl)alanine;
Metabolite 7: *N*-(2,6-dimethyl-5-hydroxyphenyl)-*N*-(methoxyacetyl)alanine methyl ester;
Metabolite 8: *N*-(2-hydroxymethyl-6-methylphenyl)-*N*-(methoxyacetyl)alanine methyl ester;
Metabolite 9: *N*-(2-carboxy-6-methylphenyl)-*N*-(methoxyacetyl)alanine methyl ester;
Metabolite 10: *N*-(2,6-dimethylphenyl)methoxyacetamide;
Metabolite 11: *N*-(2,6-dimethylphenyl)alanine;
Metabolite 12: *N*-(2-carboxy-6-methylphenyl)-*N*-methoxyacetyl)alanine;
Metabolite 13: *N*-(carboxycarbonyl)-N-(2,6-dimethylphenyl)alanine;
Metabolite 14: [(2,6-dimethylphenyl)amino]oxoacetic acid **(No conjugates are shown)**

2. Toxicological studies

2.1 Acute toxicity of metalaxyl-M

Results of studies on the acute toxicity of metalaxyl-M in rats and rabbits are summarized below in Table 16.

Table 16. Acute toxicity of metalaxyl-M

Route	Species	Strain	Sex	Vehicle	Purity	LD_{50}/LC_{50} (mg/kg bw)	Reference
Oral	Mouse	Tif:MAG (SPF)	M & F	0.5% CMC in 0.1% aqueous polysorbate 80	97.1%	>1000 (male) >500 and <1000 (female)	Winkler, 1996a
Oral	Rat	TifRAIf (SPF)	M & F	0.5% CMC in 0.1% aqueous polysorbate 80	97.9%	953 (male) 375 (female) 667 (combined)	Schoch, 1994a
Dermal	Rat	TifRAIf (SPF)	M & F	Undiluted	97.3%	>2000	Schoch, 1994b
Inhalation	Rat	Wistar (Crl:WI WU)BR)	M & F	Undiluted	97.1%	>2.3 mg/L	Arts, 1995
Skin Irritation	Rabbit	NZW	M	Undiluted	97.3%	Non-irritant	Marty, 1994a
Eye Irritation	Rabbit	NZW	M & F	Undiluted	97.3%	Severe irritant	Marty, 1994b
Skin sensitization (Magnusson-Kligman)	Guinea pig	Pirbright white	M & F	Undiluted	97.3%	Non-sensitizer	Marty, 1994c
Skin sensitization (Buehler)	Guinea pig	Crl:(HA) BR	M	Undiluted	96.6%	Non-sensitizer	Glaza, 1995

M: Male; F: Female; NZW: New Zealand White

(a) Oral administration

Mouse

In an acute oral toxicity study, groups of five male and five female fasted Tif:MAG (SPF) mice were given metalaxyl-M (purity 97.1%) orally at a single gavage dose of 500 or 1000 mg/kg bw for males and 200, 500 or 1000 mg/kg bw for females, and were observed for 14 days before sacrifice.

At 1000 mg/kg bw, two of the five males were found dead one day after dosing. Three of the five females were found dead within 10 minutes to one hour of dosing, the remaining two female mice were killed for animal welfare reasons after two hours. At 500 mg/kg bw, one of the five females was found dead 10 minutes after administration of the test article, whereas all males survived. At 200 mg/kg bw all females survived. At l000 mg/kg bw, male and female animals showed the following treatment-related effects: ventral or lateral recumbency, severe dyspnoea, reduced locomotor activity, convulsions, tremor and tonic spasms. Ataxia, hunched posture and piloerection were found in surviving males only, all of which recovered fully by day 7. At 500 mg/kg bw, ventral or lateral recumbency were seen in two males and two females, and dyspnoea, reduced locomotor activity, hunched posture and piloerection were seen in all animals, whereas tonic spasms, convulsions and ataxia were seen in some animals of both sexes. All males had recovered by day 4 and all surviving females by day 2. At 200 mg/kg bw, all females presented with hunched posture and piloerection but fully recovered within one day. At autopsy no deviations from normal morphology were found. The body weights of surviving animals were not affected by the treatment.

The acute oral lethal median dose (LD_{50}) of metalaxyl-M in male mice was >1000 mg/kg bw and in female mice >500 and <1000 mg/kg bw (Winkler, 1996a).

Rat

In an acute oral toxicity study, groups of fasted Tif:RAIf(SPF) rats were given a single oral dose of metalaxyl-M (purity 97.9%), in 0.5% carboxymethyl cellulose (CMC) in 0.1% aqueous polysorbate 80, at doses of 200 mg/kg bw (females), 500 mg/kg bw (males and females), 1000 mg/kg bw (males) or 2000 mg/kg bw (males). All animals were observed for 14 days before sacrifice.

Four of five females at 500 mg/kg bw, three of five males at 1000 mg/kg bw and all males at 2000 mg/kg bw died on the day of administration after observation of clinical signs such as piloerection, abnormal body position, dyspnoea and reduced locomotor activity. Convulsions and/or tonic spasms occurred in two males and four females given 500 mg/kg bw and all males at 1000 and 2000 mg/kg bw. Ataxia was seen in two females at 200 mg/kg bw and tremor in one male at 500 mg/kg bw. Four females at 500 mg/kg showed increased irritability. Males at 2000 mg/kg bw showed vocalization, respiratory sounds and cyanosis. The surviving animals recovered within 3–6 days. At autopsy, one male at 2000 mg/kg bw (which died shortly after treatment) had a spotted thymus. No deviations from normal morphology were found in the other animals.

The acute oral LD_{50} of metalaxyl-M in male rats was 953 mg/kg bw and in female rats 375 mg/kg bw; for male and female rats combined the LD_{50} was 667 mg/kg bw (Schoch, 1994a).

(b) Dermal application

Rat

In an acute dermal toxicity study, a group of five male and five female, Tif:RAIf(SPF) rats were dermally exposed to undiluted metalaxyl-M (purity 97.3%) for a single 24 hour application at a dose of 2000 mg/kg bw. A dose volume of 2 mL/kg bw was applied to an area of shaved skin on the back, at least 10% of the body surface, under a semi-occlusive dressing. After 24 hours, the dressing was removed and the skin cleaned with lukewarm water. The animals were observed daily for 14 days. Necropsies were perfomed at termination.

There were no mortalities and no clinical signs of systemic toxicity or skin irritation. Slight body weight loss was recorded in two females during the first week after treatment but both animals gained weight during the second week. All other animals gained weight steadily during the observation phase. At autopsy, no deviations from normal morphology were found.

The demal LD_{50} of metalaxyl-M in rats was > 2000 mg/kg bw (Schoch, 1994b).

(c) Exposure by inhalation

Rat

In an acute inhalation study, five male and five female Wistar-derived (Crl:WI(WU)BR) rats were exposed in a nose-only system to metalaxyl-M (purity 97.1%) for four hours at a mean aerial concentration of 2.3 mg/L (the highest attainable concentration) measured in the breathing zone. The test atmosphere was generated by nebulizing the material into small droplets with a compressed air-driven machine. The mass median aerodynamic diameter (MMAD) of the particles was 2.1 µm with a geometric standard deviation (GSD) of 1.4 µm, which ensured exposure of the bronchioles and alveoli of the animals. The animals were observed for 14 days before sacrifice.

No deaths occurred. Slight shallow breathing was observed in all rats during the first three hours of exposure, and clear restlessness was seen from the second hour of exposure onwards. Two female rats also showed slightly decreased breathing frequency during the last hour of exposure. Shortly after exposure four females had a hunched appearance, and one female had slightly decreased breathing frequency and incoordination. The only abnormalities seen during the 14-day observation period were fatty, yellow, discoloured fur and a small alopecic area in one female. No abnormalities in body weight gain were observed. At autopsy, no abnormalities were found.

The acute inhalation median lethal concentration (LC_{50}) of metalaxyl-M in rats was > 2.3 mg/L (Arts, 1995).

(d) Dermal irritation

In an acute primary skin irritation study, three male New Zealand White rabbits were exposed for four hours to 0.5 mL of undiluted metalaxyl-M (purity 97.3%) under an occlusive dressing, applied to an area of approximately 6 cm^2 on the shaved right flank. Skin reactions were evaluated 1, 24, 48 and 72 hours after removal of the patch. Slight erythema (score 1 for two rabbits) was only observed one hour after removal of the bandages, but no skin reactions were seen subsequently in any animals, and the study was terminated.

Metalaxyl-M was not irritating to the skin of rabbits (Marty, 1994a).

(e) Ocular irritation

In a primary eye irritation study, 0.1 mL of undiluted metalaxyl-M (purity 97.3%) was instilled into the conjunctival sac of the left eye of one male and two female New Zealand White rabbits. The other eye was left untreated to serve as a control. The eyes were examined for irritation with a slit lamp, and any ocular reactions recorded at 1, 24, 48 and 72 hours after instillation, and thereafter at 7, 10, 14, 17 and 21 days post treatment.

Clear signs of ocular irritation were seen in all animals, comprising corneal opacity grades 1–2, grade l iridal lesions and conjunctival redness and chemosis (grades 2 and l, respectively). Vascularization of the cornea was observed in two animals on days 7 and 10, but all symptoms were reversed within 14 days. In the third animal, vascularization was observed on day 14, and corneal opacity was still present at the end of the observation period.

Metalaxyl-M was considered as severe ocular irritant in rabbits (Marty, 1994b).

(f) Dermal sensitization

The skin sensitization potential of metalaxyl-M (purity, 97.3%) was assessed in 10 male and 10 female Pirbright white Guinea pigs using the Magnusson-Kligman maximization test. The animals received one intradermal injection of 0.1 mL of a 5% solution of metalaxyl-M in peanut oil with Freund's adjuvant, followed after one week by one epidermal application of 0.4 g of undiluted metalaxyl-M. The animals were challenged on day 21 with a 30% emulsion of metalaxyl-M in Vaseline®. After the dressing had been removed, on day 10, irritation of the application site was observed in all treated animals. One animal showed grade 1 skin reactions 24 and 48 hours after the challenge application, but all the others remained unaffected.

Under the study conditions utilized, metalaxyl-M was considered not to be sensitizing to Guinea pigs (Marty, 1994c).

In a dermal sensitization study with metalaxyl-M (purity 96.6%), male albino Crl:(HA)BR Guinea pigs (10 per group in the test, rather than the 20 required by current guidelines) and naïve control groups were tested using the method of Buehler. On the basis of the results of a preliminary test, Guinea-pigs were given undiluted material for both the induction phase and the challenge. In the induction phase, each animal received metalaxyl-M once a week for three weeks, at a dose of 0.4 mL on an adhesive patch, which was semi-occluded and left in place for six hours. Two weeks after application of the third induction dose a challenge dose of 0.4 mL was administered in the same manner.

No dermal reactions were observed in the animals in either the induction or the challenge phase of the study.

Under the study conditions utilized, metalaxyl-M was non-sensitizing to Guinea pigs (Glaza, 1995).

2.2 Acute toxicity of metalaxyl

Table 17. Acute toxicity of metalaxyl

Route	Species	Strain	Sex	Vehicle	Purity	LD_{50}/LC_{50} [95% CL]	Reference
Oral	Mouse	Tif:MAG (SPF)	M & F	2% CMC	99.4%	790 mg/kg bw [630–990]	Sachsse & Bathe (1976a)
Oral	Rat	Tif RAIf (SPF); fasted	M & F	2% CMC	99.4%	669 mg/kg bw [515–868]	Sachsse & Bathe (1976b)
Oral	Rabbit	Himalayan	M & F	2% CMC	99.4%	700 mg/kg bw [500–960]	Sachsse & Ullmann (1976a)
Dermal	Rat	Tif RAIf (SPF)	M & F	2% CMC	99.4%	> 3100 mg/kg bw	Sachsse & Bathe (1976c)
Dermal	Rabbit	Himalayan	M & F	50% PEG 400 and physiological saline (70:30 ratio)	99.4%	> 6000 mg/kg bw	Sachsse & Ullmann (1978)
Inhalation	Rat	Tif RAIf (SPF)	M & F	Undiluted	96.1%	> 3.6 mg/L	Hartmann (1992)
Intraperitoneal	Rat			2% CMC	99.4%	310 mg/kg bw	Sachsse & Bathe (1976d)
Skin Irritation	Rabbit	Russian breed	3M and 3F	Undiluted	99.4%	Non-irritating	Sachsse & Ullmann, (1976b)
Eye Irritation	Rabbit	Russian breed	3M and 3F	Undiluted	99.4%	Minimally irritating	Sachsse & Ullmann, (1976c)
Skin Sensitization (Magnusson-Kligman)	Guinea pig	Pirbright white	M & F	PEG and saline (70 : 30)	99.4%	Non-sensitizing	Sachsse & Ullmann, (1976d)
Skin sensitization (Buehler)	Guinea pig	Albino	F	25% ethylene glycol acetate	96.1%	Non-sensitizing	Arcelin, (1991)

M: Male; F: Female; CMC: Carboxymethyl cellulose; PEG: Polyethylene glycol CL: Confidence limits

(a) Oral administration

Mouse

In an acute oral toxicity study, groups of five male and five female fasted Tif:MAG (SPF) mice were given metalaxyl (purity 99.4%) orally as a single gavage dose (in 2% CMC as vehicle) of 320, 460, 600, 1000 or 2200 mg/kg bw for males and females. Mice were observed for 14 days before termination.

Within two hours of dosing, the treated mice in all dose groups showed sedation, dyspnoea, exophthalmos, curved or ventral position and ruffled fur. Additionally, in the three highest dose groups, tonic-clonic muscle spasms were also observed. Sedation became more accentuated with increasing doses. One female died within 24 hours of treatment at 464 mg/kg bw. Four males died within 24 hours of treatment at 600 mg/kg bw. Two males and four females died within 24 hours at 1000 mg/kg bw and all males and females died within 24 hours at 2150 mg/kg bw. The surviving animals recovered within 7–12 days. At necropsy, no treatment-related macroscopic changes were observed.

The acute oral LD_{50} of metalaxyl in male and female mice was 788 mg/kg bw (95% confidence limits given as 626–991 mg/kg bw) (Sachsse & Bathe, 1976a).

Rat

In an acute oral toxicity study, groups of five male and five female fasted Tif:MAG (SPF) rats were given metalaxyl (purity 99.4%) orally in 2% CMC vehicle, as a single gavage dose 215, 464, 775, 1000, 1290 or 2150 mg/kg bw for males and females. The rats were observed for 14 days before termination.

Within two hours of dosing, the treated rats in all dose groups showed sedation, dyspnoea, exophthalmos, curved, or ventral position, tonic-clonic muscle spasms and ruffled fur. The surviving animals recovered within 9–11 days. At necropsy no treatment-related macroscopic changes were observed.

The acute oral LD_{50} of metalaxyl in both male and female rats was 669 mg/kg bw (95% confidence limits given as 515–868 mg/kg bw) (Sachsse & Bathe, 1976b).

Rabbit

In an acute oral toxicity study, groups of two male and two female Himalayan rabbits were given metalaxyl (purity 99.4%) orally in 2% CMC as vehicle, as a single gavage dose 215, 464, 1000 or 3590 mg/kg bw for males and females. The rabbits were observed for 14 days before termination.

In all groups the animals showed sedation, curved position and ataxy. In addition to these symptoms, the rabbits in both groups where mortalities occurred showed tremor and tonic-clonic muscle spasms. All treated rabbits died within 24 hours of treatment at 1000 and 3590 mg/kg bw. The surviving animals recovered within two days. At necropsy no treatment-related macroscopic changes were observed.

The acute oral LD_{50} of metalaxyl in male and female rabbits was 697 mg/kg bw (95% confidence limits given as 505–961 mg/kg bw) (Sachsse & Ullmann, 1976a).

(b) Dermal application

Rat

In an acute dermal toxicity study, a group of three male and three female, Tif:RAIf (SPF) rats were dermally exposed to metalaxyl (purity 99.4%) via a single 24 hour application (in 2% CMC) of 2150 or 3170 mg/kg bw. After 24 hours the dressing was removed and the skin cleaned with lukewarm water. The animals were observed daily for 14 days. Necropsies were perfomed at termination.

There were no mortalities and no clinical signs of systemic toxicity or skin irritation. At autopsy, no deviations from normal morphology were found.

The demal LD_{50} of metalaxyl in rats was > 3170 mg/kg bw (Sachsse & Bathe, 1976c).

Rabbit

In an acute dermal toxicity study, a group of three male and three female Himalayan rabbits were dermally exposed to metalaxyl (purity 99.4%) via a single 24 hour application (in 2% CMC) of 1000 or 6000 mg/kg bw. After 24 hours the dressing was removed and the skin cleaned with lukewarm water. The animals were observed daily for 14 days. Necropsies were perfomed at termination.

There were no mortalities and no clinical signs of systemic toxicity. The animals inthe 6000 mg/kg bw dose group showed a slight erythema on the application site just after removing the dressing. At autopsy, no deviations from normal morphology were found.

The demal LD_{50} of metalaxyl in rabbits was > 6000 mg/kg bw (Sachsee & Ullmann, 1978).

(c) Exposure by inhalation

Rat

In an acute inhalation study (GLP compliant), five male and five female Tif: RAI f (SPF) rats were exposed to metalaxyl (purity 96.1%) for 4 h in a nose-only system to a mean aerial concentration of 3.6 mg/l (3638 mg/m3). The mass median aerodynamic diameter (MMAD) of the particles was between 1.3 and 1.5 µm with a geometric standard deviation (GSD) of 2.3 to 2.7 µm, which ensured exposure of

the bronchioles and alveoli of the animals. The animals were observed for 14 days before termination.

No deaths occurred. The animals of both sexes exposed to the test article experienced the symptoms piloerection, hunched posture, dyspnea and reduced locomotor activity. No abnormalities in body weight gain were observed. At autopsy, no abnormalities were found.

The acute inhalation LC_{50} of metalaxyl in rats was > 3.6 mg/L (Hartmann, 1992).

(d) Dermal irritation

In an acute primary skin irritation study, three male and three female Russian breed rabbits were exposed to 0.5 g of undiluted metalaxyl (purity 99.4%) for four hours under an occlusive dressing. The skin reactions were evaluated immediately after removal of dressing and at 24, 48 and 72 hours. Slight erythema (score 1) was observed in rabbits 24 hours after application.

Metalaxyl was not irritating to the skin of rabbits (Sachsse & Ullmann, 1976b).

(e) Ocular irritation

In a primary eye irritation study, 0.1 g of metalaxyl (purity 97.3%) was instilled into the conjunctival sac of the left eye of three males and three females Russian breed rabbits. The other eye was left untreated to serve as a control. The eyes were examined for irritation with a slit-lamp, and any ocular reactions were recorded 1, 2,3, 4 and 7 days after instillation.

The irritation index was recorded as 2 for cornea, 0 for the iris and 2.2 for the conjuctivae.

Metalaxyl was considered as minimally irritating to the eyes of rabbits (Sachsse & Ullmann, 1976c).

(f) Dermal sensitization

The skin sensitization potential of metalaxyl (purity, 99.4%) was assessed in 10 male and 10 female Pirbright white Guinea pigs in the Magnusson-Kligman maximization test. Ten males and 10 female Guinea pigs were used as a vehicle control group and the same number as positive control group (dinitrochloro benzene, DNCB). The animals each received one intradermal injection of 0.1 mL of a 0.1% suspension of metalaxyl in polyethylene glycol and saline (70 : 30) every second day (except weekends) to a total of 10 intradermal injections. A challenge dose of 0.1% suspension was injected after 14 days.

Seven of 20 treated animals and one of 20 vehicle control animals showed positive reactions after challenge, while all 20 animals in the positive control (dinitrochlorobenzene) group gave positive reactions. The authors considered that metalaxyl did not have skin sensitizing potential in Guinea pigs. In the metalaxyl-treated group, the average increase in skin reactions after challenge over that seen after induction was only marginal, increasing by a factor of 1.2. By contrast in the positive control group the average increase in skin reactions at challenge was 18-fold. The reactions in the vehicle control group during induction were more marked than in the metalaxyl-treated group at induction and at challenge, by factors of 1.7 and 1.3 respectively. Use of an irritating vehicle and irritating concentrations of metalaxyl at challenge made it difficult to differentiate sensitization reactions from irritation. The results were below the threshold for significance set by the laboratory ($p = 0.02$). The Meeting in 2002 considered the results to have been equivocal but that they did not represent a positive effect (Sachsse & Ullmann,1976d).

In a GLP compliant dermal sensitization study with metalaxyl (purity 96.1%), female Ibm:GDHI (SPF) Guinea pigs (20 per group in the test and 10 in the control group) were tested using the method of Buehler. Metalaxyl was administered in 25% ethylene glycol acetate.

No deaths occurred during the study. Neither local nor systemic symptoms were observed during the study.

No dermal reactions were observed in the animals in either the induction or the challenge phase of the study.

Under the study conditions utilized, metalaxyl was not sensitizing to Guinea pigs (Arcelin, 1991).

(g) Intraperitoneal administration

In an acute intraperitoneal toxicity study, groups of five male and five female fasted Tif:MAG (SPF) rats were administered metalaxyl (purity 99.4%) intraperitoneally (in 2% CMC as vehicle) of 100, 215, 278 , 359 or 464 mg/kg bw for males and females. Rats were observed for 14 days before termination.

Within two hours of dosing the treated rats in all dose groups showed sedation, dyspnoea, exophthalmus, curved or ventral position, tonic-clonic muscle spasms and ruffled fur. The surviving animals recovered within 10–12 days. At necropsy, no treatment-related macroscopic changes were observed.

The acute intraperitoneal LD_{50} of metalaxyl in male and female rats was 312 mg/kg bw (95% confidence limits given as 282–345 mg/kg bw) (Sachsee & Bathe, 1976d).

2.3 Short-term studies of toxicity

(a) Oral administration

Rat

Study 1

A study was conducted to detect possible differences in the toxicological properties of metalaxyl-M and the racemate metalaxyl. Metalaxyl-M (purity 97.3%) and metalaxyl (purity 96.1%) suspended in water containing 0.5% CMC and 0.1% Tween 80 were administered by gavage to groups of five male and five female Sprague Dawley-derived Tif:RAif (SPF) rats at a dose of 0, 10, 50, 150 or 300 mg/kg bw per day, for seven days per week over a four week period.

No deaths occurred. Animals given doses of 150 or 300 mg/kg bw per day showed transient hypoactivity after the first treatment with metalaxyl-M (one female each at 150 and 300 mg/kg bw per day), and two females at 300 mg/kg bw per day were prostrate. These signs were present for two hours post dosing on day 1 of treatment only No further clinical signs were observed. Body weight gain and food consumption were similar in treated and untreated rats. The mean water consumption of males at the highest dose was reduced by 10% throughout treatment with metalaxyl-M, whereas treatment with metalaxyl increased the mean water consumption of animals at this dose by 10% in males and 22% in females.

The haematological examinations revealed no changes. Minimally lower plasma sodium levels, minimally higher plasma chloride levels and a tendency to decreased urea levels were recorded among males at the highest dose of both metalaxyl-M and metalaxyl. In addition, males treated with metalaxyl-M at this dose had a minimally lower plasma bilirubin level, and females showed increased plasma albumin and globulin and reduced plasma bilirubin. The differences in these parameters compared with untreated controls were statistically significant for animals receiving metalaxyl-M at 300 mg/kg bw per day and for those given metalaxyl at 150 and 300 mg/kg bw per day.

No treatment-related gross changes in organs were seen at necropsy. The absolute and relative weights of the liver were increased by 9–12% in males and females at the highest dose of metalaxyl and females at the highest dose of metalaxyl-M.

Histopathological examination revealed minimal to moderate hypertrophy of centrilobular hepatocytes in females given metalaxyl-M at 150 mg/kg bw per day and in both sexes at 300 mg/kg bw per day. Minimal hepatocellular hypertrophy was also seen in the females given metalaxyl at 300 mg/kg bw per day. A minimally increased incidence of extramedullary haematopoiesis in the spleen was found in females given the two higher doses of metalaxyl.

In summary, treatment of rats with metalaxyl and metalaxyl-M resulted in similar effects. The main target organ of both substances was the liver, which reacted to treatment with hypertrophy. Metalaxyl-M had no unexpected toxicological properties that would differentiate the *R*-enantiomer from the racemic form of metalaxyl when viewed qualitatively. Quantitative differences in the effects of the two substances were generally minimal and remained within the range of biological variation.

It was therefore justified to conclude that the two compounds are toxicologically equivalent.

The NOAEL for both substances was 300 mg/kg bw per day, the highest dose tested, as the effects on the liver observed at doses of 150 mg/kg bw per day were considered not to be adverse. The study author established no-observable-effect level (NOEL) for both compounds at 50 mg/kg bw per day (Gerspach, 1994).

Study2
In a 28-day toxicity study in rats (non-GLP), metalaxyl (purity 94.6%) in 2% CMC was administered by gastric intubation to groups of 10 male and 10 female Tif:RAif (SPF) rats at an initial daily dose of 0, 10, 30 or 100 mg/kg bw day. As the treatment produced no overt toxic reaction, the doses were raised to 0, 30, l00 and 300 mg/kg bw per day from day 15 to 21 and finally to 0, 60, 200 and 600 mg/kg bw per day from day 22 to 28. During the study, animals were observed daily for mortality and clinical signs; body weights and food consumption were recorded weekly. All animals were given an eye examination and a hearing test prior to the start of dosing and at the end of treatment. On day 21 blood was collected from five rats/sex per group for haematology analysis. Blood was also collected from a different five rats/sex per group for clinical chemistry analysis on days 21 and 29. Urinalysis determinations were carried out on the same five animals/sex per group as used for clinical chemistry analysis on day 21. After 28 days of dosing the rats were killed and given a macroscopic examination and selected organs were weighed. A range of tissues was collected and preserved but not examined microscopically.

No deaths occurred. After the dose had been increased to 600 mg/kg bw per day (that is, to near the acute oral LD_{50} for rats) tremors were observed within two hours of treatment. On subsequent days the animals adapted to the treatment and no clinical symptoms were seen on days 26, 27 and 28.

No treatment-related effects were noted on body weight gain, food consumption, ophthalmic examination, haematology, blood biochemistry or urine analysis. The mean absolute and relative weights of the liver were increased in all treated groups, with a positive trend from control to the highest dose. This effect was more pronounced in females than in males. In males at the highest dose, the absolute weight of the testes was significantly increased. The relative weight of the adrenals was significantly increased in females at the highest dose. The only gross pathological change observed was atrophy of the left testis in one male at the highest dose. This study indicated that the liver responded to treatment with hypertrophy, as shown by the dose-related increases in absolute and relative liver weights (Sachsse, 1979).

Study 3
In a 90-day toxicity study, groups of 10 male and 10 female Sprague-Dawley-derived rats (Tif:RAif (SPF) were given diets containing technical-grade metalaxyl-M (purity, 97.l%) at a concentration of 25, 50, 250, 625 or 1250 ppm. A group of 10 rats of each sex receiving the vehicle (acetone) served as controls. An additional group of 10 rats of each sex from the control and highest-dose groups were kept for a 4-week recovery period before termination. The doses were equal to mean intakes of l.7, 3.5, 17, 45 and 91 mg/kg bw per day for males and 1.9, 3.7, 18, 49 and 95 mg/kg bw per day for females. Overt signs of toxicity were recorded daily, and body weight, food consumption and water consumption were recorded weekly throughout the study. Ophthalmoscopy was performed on animals from the control and highest-dose groups before treatment, towards the end (day 87) of treatment and towards the end of the recovery period (day 115). Haematological, blood chemistry and urine analyses were carried out on all surviving treated animals at the end of treatment and at the end of the recovery period. All animals killed after treatment underwent a detailed necropsy and comprehensive microscopic evaluation of tissues; the livers of animals allowed to recover for four weeks were examined.

There was no mortality or treatment-related clinical signs. No treatment-related effects were observed on body weights, body weight gains, food or water consumption, haematological or clinical chemistry parameters, nor from ophthalmological examination or urinalysis. No treatment-related ophthalmological changes were noted, similarly changes in haematology, clinical chemistry and urine analysis revealed nothing that could be attributed to treatment.

At termination, no treatment-related gross changes were found in organs. The absolute and relative weights of organs were unaffected by treatment, although a slight (maximum 7%) increase in the mean liver : body weight ratio was seen in females at concentrations of 625 ppm. Histopathological examination revealed a dose-related occurrence of hepatocellular inclusion bodies (consisting of ring- or whorl-shaped eosinophilic particles located within the cytoplasm of perilobular hepatocytes) in males

at 625 and 1250 ppm. The occurrence of these inclusions was sometimes associated with enlargement of perilobular hepatocytes. Females at these concentrations showed increased incidences of minimal hepatocellular hypertrophy, located centrilobularly. Both findings were completely reversed within the four-week recovery period.

The NOAEL was 1250 ppm (equal to 91 mg/kg bw per day, the highest dose tested) as the effects on the liver observed at 625 ppm were considered not to be adverse (Gerspach, 1995). The study author determined the NOAEL to be 250 ppm, equal to 16.8 mg/kg bw per day in males and 17.9 mg/kg bw per day in females (Gerspach, 2006).

Study 4

In a 90-day toxicity study (non-GLP), groups of 20 male and 20 female Sprague Dawley-derived Tif:RAif (SPF) rats received diets containing metalaxyl (purity 93.5%) at a concentration of 0, 10, 50, 250 or 1250 ppm (equal to mean intakes of 0, 0.66, 3.5, 15 and 72 mg/kg bw per day for males, 0, 0.67, 3.6, 16 and 74 mg/kg bw per day for females). Overt signs of toxicity were recorded daily, and body weight and food consumption were recorded weekly throughout the study. Ocular examinations and an auditory test were performed before and after treatment. Animals allowed to recover were examined during week 17. Haematological, blood chemistry and urine analyses were carried out on 10 rats of each sex per group in weeks 4, 8 and 12. At the end of treatment, all animals were subjected to a detailed necropsy and a comprehensive microscopic evaluation of tissues.

No deaths or clinical signs were seen. Body weight gain and food consumption remained unaffected by treatment. Ophthalmic inspections and hearing examinations before and after the application period revealed no evidence of a reaction to treatment. No treatment-related findings were observed in urine parameters. The laboratory investigations revealed a slight decrease in leukocyte count for males at the highest concentration at week 12. Slightly higher values in the blood urea concentration were observed in females of the 250 and 1250 ppm groups at 8 and 12 weeks of treatment when compared to the controls. At necropsy slightly increased adrenal weights were seen in males at concentrations of 50 ppm, 250 ppm and 1250 ppm (160%, 180% and 170% of control values, respectively). There were no treatment-related effects on macroscopic findings. Histopathological examination of the organs and tissues revealed no changes related to administration of metalaxyl.

The maximum tolerated dose was not reached in this study. The concentration of 50 ppm (equal to 3.5 mg/kg bw per day) and above caused changes in the adrenal weights in males. However, the effect was not observed in females and was not confirmed by blood biochemistry or histopathology. Moreover, this effect has not been observed in other studies. This study was not taken into account in the final evaluation (Gfeller, 1980).

Study 5

In a 90-day toxicity study (non-GLP), groups of 20 male and 20 female Sprague Dawley rats received diets containing metalaxyl (purity 99%) at a concentration of 0, 50, 250 or 1250 ppm (equal to mean intakes of 3.2, 16 and 79 mg/kg bw per day for males, 3.5, 18 and 86 mg/kg bw per day for females). Five males and five females from the control and highest-dose groups were kept for a four-week recovery period before they were killed. Overt signs of toxicity were recorded daily, and body weight and food consumption recorded weekly throughout the study. Ophthalmoscopy was performed on all animals before treatment and on 10 males and females from the control and highest-dose groups during weeks 5, 9 and 13. Animals allowed to recover were examined during week 17. Haematological, blood chemistry and urine analyses were carried out on all surviving treated animals at weeks 5, 9 and 13, and also at week 17 for rats that were allowed to recover. Animals that died or were killed during the study and all animals that survived until scheduled sacrifice were autopsied. A comprehensive microscopic evaluation of tissues from all treated animals was carried out, and the livers and ovaries from the five female rats killed after the four-week recovery period were examined.

No deaths and no overt signs of toxicity were reported. Ophthalmic examination revealed no treatment-related changes. Males at the highest dose had minimally decreased body weight gain (terminal weight 97% of controls) and food consumption (at week 13, 94% of controls). Haematology, blood biochemistry and urine analysis revealed no treatment-related changes. At autopsy, no macroscopic changes were seen, and there were no relevant changes in organ weights. Histopathological examination revealed minimal hypertrophy in a few hepatocytes in the females at the two higher concentrations, with

incidences of 0/20, 0/20, 2/20 and 7/20. Large ovarian cysts were reported in 5/20 females at 1250 ppm, but these were regarded as of doubtful significance. In the group allowed to recover, no changes were noted in the liver, and the ovaries were similar to those of controls.

As the liver-cell hypertrophy at 1250 ppm was considered not to be adverse, this concentration, equal to 79 mg/kg bw per day, the highest dose tested, was the NOAEL (Drake, 1977).

Dog

The toxicity of metalaxyl-M in dogs was investigated in a 13-week study of dietary administration. Metalaxyl was tested in a six-month study with administration in the diet and in a two-year study with administration in capsules.

Study 1

In a 90-day toxicity study, groups of four male and four female beagle dogs were given diets containing technical grade metalaxyl-M (purity 97.1%) in acetone at a concentration of 0, 50, 125, 250 or 1250 ppm. The doses were selected to allow direct comparison with the previous short-term study conducted with metalaxyl, and were equal to a mean daily intake of 1.6, 4.1, 7.3 and 39 mg/kg bw per day for males and 1.6, 4.3, 7.9 and 40 mg/kg bw per day for females. Overt signs of toxicity, body weight and food consumption were recorded daily throughout the study. Ophthalmoscopy was performed before and at the end of treatment. Haematological, blood chemistry and urine analyses were conducted before treatment and at weeks 7 and 13. At 13 weeks all animals were killed and necropsied and a comprehensive histological examination was carried out.

Treatment caused no deaths or any clinical signs of toxicological significance, and the ophthalmic examination revealed no changes. All dogs ate similar quantities of food, and the body weight development was similar in treated and control groups. No treatment-related changes in haematological parameters were observed. Increased alkaline phosphatase (ALP) activity was seen in males and females at the highest concentration after seven weeks of treatment (190% increase over controls for males and 220% increase for females) and 13 weeks of treatment (210% for males and 260% for females). Treatment had no effect on the urine parameters investigated. At termination, the mean absolute weights (25% in males, 28% in females) and relative weights (25% in males, 33% in females) of the liver were increased in animals at the highest concentration. Mean thyroid : body weight ratio was increased in 1250 ppm males. Individual absolute thyroid weights were, however, within the expected biological range and in the absence of any histopathological findings this was considered not to be toxicologically relevant. Other statistically significant organ weight changes were considered not to be substance-related, either because they represented normal physiological variation or because of a lack of dose–response relationship.

Metalaxyl-M was thus well tolerated at concentrations up to 1250 ppm. The liver was the target organ, as indicated by increases in relative and absolute weights and ALP activity.

As in the case of increased liver weights and hepatocellular hypertrophy in dogs, recent publications (Yokoyama et al., 2019, 2021) have indicated that ALP is not a good marker of toxicity in dogs. As such, in the absence of other markers of hepatotoxicity, the changes in ALP values were not considered adverse.

In conclusion, the NOAEL was 1250 ppm (equal to 39 mg/kg bw per day) the highest dose tested (Altmann, 1995).

Study 2

In a six-month toxicity study, groups of six male and six female beagle dogs were given diets containing technical grade metalaxyl (purity 92%) in acetone at a concentration of 0, 50, 250 or 1000 ppm. Two additional dogs of each sex from the control group and the 1000 ppm group were kept for a four-week recovery period before they were killed. The doses were equal to mean daily intakes of 0, 1.6, 7.8 and 31 mg/kg bw per day for males and 0, 1.7, 7.4 and 32 mg/kg bw per day for females. Overt signs of toxicity, body weight and food consumption were recorded daily throughout the study. Ophthalmoscopy was performed before and at the end of treatment. Haematological and blood chemical analyses were conducted before treatment and at 30-day intervals until termination of dosing. Urine was analysed before treatment and at 60-day intervals until termination of dosing. Blood samples were collected from dogs allowed to recover four weeks after termination of dosing, and urine was collected before

necropsy. At six months all animals were killed and subjected to a detailed necropsy and a comprehensive histological examination.

No deaths occurred and no treatment-related changes were found in clinical signs, body weight gain, food consumption or ophthalmic parameters. In comparison with the control animals, the erythrocyte (RBC) count, erythrocyte volume fraction and haemoglobin concentration were significantly lower in males at the highest concentration from day 60 of treatment onwards, and these haematological changes were still present in males allowed to recover, though they were not statistically significant (Table 18). Blood biochemical analyses revealed a slight increase in plasma ALP activity in animals of both sex at the highest concentration from day 30 onwards, which became statistically significant during the second half of the treatment period. After 180 days of treatment ALP activity was 170% of the control value in males and 190% in females. In the group allowed to recover, ALP activity was only minimally above the control values (120% of control value in males and 110% in females). Urinary parameters were all within normal limits, and no trends by dietary concentration were detected.

Table 18. Intergroup comparison of haematology – selected parameters, selected days

	Dietary concentration (ppm)							
	Males				Females			
Days	**0**	**50**	**250**	**1000**	**0**	**50**	**250**	**1000**
				Red blood cell count ($\times 10^6$)				
60	7.84	7.30	7.32	7.08*	7.60	7.83	7.80	7.80
90	7.96	7.47	7.75	7.16*	7.80	7.87	8.28	7.65
180	8.20	7.95	7.85	7.39*	7.58	8.02	8.00	7.58
				Haematocrit (%)				
60	48.8	46.0	46.3	44.1*	49.5	49.5	48.5	49.3
90	48.8	46.7	48.0	44.3*	49.6	49.2	50.8	48.6
180	51.5	49.5	48.5	46.1*	49.4	50.2	50.3	47.4
				Haemoglobin (g/dL)				
60	16.94	15.80	15.85	15.09*	16.94	17.03	16.93	16.94
90	16.95	16.27	16.60	15.38*	17.26	17.02	17.97	16.70
180	17.78	17.32	16.87	16.15*	17.01	17.01	17.60	16.48

* Statistically significant difference from control group mean, $p < 0.05$ Source: Beck & deWard, 1981

Although not statistically significant, there appeared to be a dose-related increase in absolute liver weight, with increases of 8% at 50 ppm, 14% at 250 ppm and 16% at 1000 ppm, and in the liver : body weight ratios in males and especially in females, with increases of 2% at 50 ppm, 13% at 250 ppm and 20% at 1000 ppm. These changes were reversible in males but less so in females when allowed to recover. The difference in liver : brain weight ratio attained statistical significance (120% of control values) in females at 1000 ppm after 180 days of treatment; the value was 110% of the control in the group that was allowed to recover. Gross and histopathological examination showed no treatment-related changes.

As in the case of increased liver weights and hepatocellular hypertrophy in dogs, recent publications (Yokoyama et al., 2019, 2021) have indicated that ALP is not a good marker of toxicity in dogs. As such, in the absence of other markers of hepatotoxicity, the changes in ALP values was not considered adverse.

In conclusion, the NOAEL was 250 ppm (equal to 7.8 mg/kg bw per day) on the basis of decreased RBC counts, haematocrit and haemoglobin levels in male dogs (minor changes but consistent throughout the study and supported by the same effects observed in a two-year dog toxicity study) at 1000 ppm (equal to 31 mg/kg bw per day) the highest dose tested (Beck & deWard, 1981).

Study 3

In a two-year toxicity study, metalaxyl (purity 92.7–94.1%) was administered in gelatine capsules to groups of six male and six female beagle dogs at a dose of 0, 0.8, 8 or 80 mg/kg bw per day once a day, on seven days a week. All animals were observed daily for mortality and clinical signs. Before the first dose and on weeks 4, 13, 26, 52, 78 and 103 all dogs were given an ophthalmoscopic examination. Individual body weights were recorded weekly. Individual food consumption was measured daily. Water consumption was measured before the first dose and during weeks 12, 25, 51, 77 and 103. Likewise, before the first dose and after 4, 13, 26, 52, 78 and 103 weeks, haematology, blood biochemistry and urinalysis assessments were made. All surviving animals were killed at termination of the study at week 103.

Animals at the highest dose frequently showed spasms and/or salivation, especially during the first 52 weeks of treatment (first observed during the first week of treatment). The symptoms usually occurred 10–30 minutes after dosing and disappeared within 0.5–2 minutes of their onset. Two males and two females died during weeks 20–52. There were no treatment-related effects on body weight, although male dogs at 0.8 mg/kg bw per day weighed slightly more than controls during the study. There were no treatment-related effects on food consumption, water consumption or ophthalmic end-points.

A slight decrease or a decreasing trend in the specific gravity of the urine was observed in males at 80 mg/kg bw per day from 13 weeks of treatment. At 80.0 mg/kg bw per day group, males and females showed consistently lower (10–20% less) values of haematocrit, haemoglobin and RBC count than controls, this from 26 weeks of treatment (Table 19). In addition, male dogs exhibited a significant increase in mean corpuscular haemoglobin concentration (MCHC) at 13, 78 and 103 weeks of treatment and an increase in platelet count at 52 and 103 weeks of treatment. There were no abnormalities in reticulocyte count or morphology of erythrocytes. Statistically significant changes were also observed in some other parameters in this group during the study, but were considered not be treatment-related as they did not correlate with the treatment period.

Table 19. Intergroup comparison of blood haematology – selected parameters, selected weeks

	Dose level (mg/kg bw per day)							
	Males				Females			
Week	**0**	**0.8**	**8.0**	**80**	**0**	**0.8**	**8.0**	**80**
				Haematocrit (%)				
26	48.7	51.2	48.9	41.9*	50.5	49.9	55.4*	45.1*
52	48.0	51.6*	49.4	44.0	50.5	44.0*	51.4	41.7**
78	50.4	48.3	49.9	44.6*	50.1	46.9	52.2	44.5
103	50.3	47.7	51.9	39.3**	50.1	47.8	54.2	45.1
				Haemoglobin (g/dL)				
26	16.6	17.4	16.5	14.5*	17.4	17.1	19.0*	15.6*
52	16.7	18.1*	17.4	15.6	17.7	15.5*	18.1	14.6**
78	17.1	16.4	17.3	15.6	17.0	16.2	18.1	15.4
103	17.0	16.4	17.8	13.7**	17.0	16.3	18.6	15.5
				RBC count (cells/10^6 per mL)				
26	7.25	7.56	7.37	6.33*	7.56	7.47	8.27	6.88
52	7.22	7.72*	7.58	6.47	7.58	6.74	7.91	6.26**
78	7.50	7.23	7.58	6.55*	7.49	7.14	7.91	6.70
103	7.49	7.14	7.90	5.79**	7.57	7.31	8.27	6.79
				MCHC (g/dL)				
13	33.7	34.2	34.1	34.8*	33.4	34.7*	34.5*	33.7
78	34.0	34.0	34.7	35.0*	34.0	34.5	34.7	34.7
103	33.7	34.3	34.2	35.0**	34.1	34.2	34.3	34.5

	Dose level (mg/kg bw per day)							
	Males				Females			
Week	0	0.8	8.0	80	0	0.8	8.0	80
	Platelet count (/10^3 per cm^3)							
52	264	276	278	404*	291	289	278	515
103	277	288	268	371*	287	283	284	421

RBC: Red blood cell; MCHC: Mean corpuscular haemoglobin concentration; Source: Harada, 1984

* Statistically significant difference from control group mean, $p < 0.05$, ** $p < 0.01$

Animals at 80 mg/kg bw per day had significantly increased serum activities of ALP and alanine aminotransferase (ALT) compared to controls (Table 20). The values for ALP during the study were 1.5–2.5 times higher than those determined before initiation of treatment, and 2–5.6 times higher than those in the corresponding control groups. The ALP activity in males at 8 mg/kg bw per day and in controls decreased gradually during the study from that determined before initiation of treatment, and only a slight (maximum, two-fold) but statistically significant increase in ALP activity was found. The increase in ALT activity in males at the intermediate dose was slight (maximum 50%) and often not dose-related. Animals at the highest dose also had elevated concentrations of albumin (9–20%), total protein (6.5–13%) and calcium (11% males only). Males showed decreased aspartate aminotransferase (AST) activity and globulin and creatinine concentrations.

Table 20. Intergroup comparison of blood clinical chemistry – selected parameters, selected weeks

	Dose level (mg/kg bw per day)							
	Males[a]				Females[a]			
Week	0	0.8	8.0	80	0	0.8	8.0	80
	Alkaline phosphatase activity [SD] (IU/L)							
Before	227 [26]	200 [27]	224 [33]	196* [19]	217 [69]	211 [38]	225 [49]	208 [24]
4	218 [35]	190 [33]	216 [33]	296 [100]	190 [39]	182 [45]	218 [55]	408* [204]
13	148 [29]	149 [51]	203* [42]	395* [215]	148 [38]	140 [45]	157 [31]	390* [185]
26	107 [24]	122 [47]	169* [53]	443*[166]	132 [29]	109 [38]	132 [39]	292** [77]
52	88 [27]	125 [74]	176* [68]	367* [161]	112 [41]	94 [35]	118 [43]	421 [339]
78	78 [25]	108 [49]	160* [68]	451* [191]	110 [46]	93 [67]	93 [34]	442 [457]
103	75 [23]	137 [83]	186* [82]	423* [139]	110 [40]	76 [28]	94 [26]	460 [389]
	Alanine aminotransferase activity [SD] (IU/L)							
Before	22 [3]	25 [6]	27 [8]	33 [17]	25 [4]	30 [25]	23 [6]	31 [6]
4	20 [3]	24 [6]	27* [5]	25 [5]	27 [5]	27 [5]	29 [7]	27 [3]
13	22 [4]	25 [4]	29* [6]	28 [6]	25 [3]	25 [6]	26 [3]	33 [9]
26	21 [4]	25 [5]	28 [12]	39* [10]	23 [3]	24 [5]	25 [3]	49* [19]
52	22 [6]	29 [9]	28 [11]	65* [24]	24 [3]	25 [5]	26 [5]	56 [32]
78	22 [5]	28 [13]	30 [9]	66* [17]	25 [4]	25 [3]	27 [5]	96* [31]
103	22 [6]	27 [8]	33* [9]	86* [31]	27 [5]	23 [6]	27 [6]	86* [30]

SD: Standard deviation [in brackets] Source: Harada, 1984

[a] Six animals examined per data point, except males at 80 mg/kg bw per day, only five of which were examined after 26 weeks of treatment and only four thereafter and females at this dose, of which only four were examined from week 52 onwards

* Statistically significant difference from control group mean, $p < 0.05$, ** $p < 0.01$

At necropsy, animals at the highest dose frequently showed enlargement of the liver and increased liver weights, with increases in absolute weight of 58% in males and 16% in females and an increase in relative weight of 16% in both sexes. Males also had significantly increased kidney weights (by 34%) in comparison with controls. Histopathology revealed no specific changes attributable to treatment.

The incidences of hepatic lesions such as focal inflammation, fibrosis and brown pigment deposition tended to be higher in animals at 80 mg/kg bw per day than in controls, however the lesions could not definitively be related to treatment as they were relatively mild.

The changes in ALP and ALT activity observed in animals at the highest dose indicated hepatic effects of treatment and were considered to correlate with the increased liver weights. However, even though the difference in ALP activity between males at 8 mg/kg bw per day and concurrent controls was statistically significant, it was slight, and in the absence of other biologically significant changes in clinical chemistry or histopathology, it was considered not to be adverse. Furthermore, this conclusion is supported by recent publications (Yokoyama et al., 2019, 2021) which have indicated that ALP is not a good marker for toxicity in dogs. As such, in the absence of other markers of hepatotoxicity, the changes in ALP values were not considered adverse.

The liver was the target organ of metalaxyl, as indicated by the changes in blood chemistry and increased liver weight. Although a decrease in urine specific gravity and increased kidney weights were observed in males, there were no histopathological abnormalities in the kidney and it is not clear whether this effect was related to treatment. The haematological end-points indicating anaemia occurred only after long-term treatment and were not relevant to acute exposure.

The NOAEL was 8 mg/kg bw per day based on anemia (decreased haematocrit, haemoglobin, red blood cell count and mean corpuscular haemoglobin concentration) at the LOAEL of 80 mg/kg bw per day; the highest dose tested (Harada, 1984).

(b) Dermal application

Rat

In a dermal toxicity study, metalaxyl-M (purity, 97.1%) suspended in 1% (w/v) CMC in 0.1% (w/v) aqueous polysorbate 80 was applied to the clipped skin of groups of five Sprague Dawley-derived Tif:RAIf (SPF) rats of each sex at a dose of 0, 50, 250 or 1000 mg/kg bw per day under an occlusive dressing for six hours each day, five days per week, for four weeks. No application was made on the weekend days of weeks 1–3. Clinical signs, body weight, food consumption and deaths were monitored throughout the study. Haematological and blood chemistry analyses were performed at the end of treatment. At sacrifice, the animals were examined macroscopically and organ weights recorded. Organs and tissues were collected and prepared for histopathological examination.

Treatment with metalaxyl-M produced no clinical signs or behavioural changes and no signs of local irritation. Although the food intake of treated and control animals was similar, males at the highest dose gained less weight than controls. At the end of treatment the mean body weight of males at the highest dose was 6% lower and the mean body weight gain 21% less than that of controls. Haematological and blood chemistry analyses gave no indication of a treatment-related effect. Males at the highest dose had a 16% decreased mean spleen weight, and the liver : body weight ratios were increased by 8% in males and 16% in females compared to controls. Macroscopic and histopathological examination showed no treatment-related findings. Dermal treatment with metalaxyl-M was thus well tolerated, with no irritating effect.

The NOAEL for systemic effects was 1000 mg/kg bw per day, the highest dose tested, as the modifications in liver and spleen weight were not confirmed at necropsy or supported by histopathological findings (Gerspach, 1998).

Rabbit

In a dermal toxicity study, powdered metalaxyl (purity 92%) was applied under a semi occlusive dressing to the clipped skin of groups of 10 male and 10 female New Zealand White rabbits at a dose of 0, 10, 100 or 1000 mg/kg bw per day for six hours per day, five days per week, for three weeks. The skin of half of the animals was abraded once a week in order to enhance dermal absorption. Clinical signs, deaths and signs of dermal irritation were recorded daily throughout the study. Body weight and food consumption were monitored at the beginning of treatment and then twice a week until sacrifice or death. Haematological and blood chemistry analyses were performed before and at the end of treatment. After death or at sacrifice the animals were examined macroscopically and organ weights recorded. Organs and tissues were collected and prepared for histopathological examination.

The application was tolerated at all doses with no signs of systemic toxicity. Body weight and food consumption were similar in all groups. One male at 100 mg/kg bw per day was found dead on day 20, but the cause of death was not reported. Abnormal skin reactions were limited to lesions from the tape used to secure the dressing, and pimple-like eruptions were seen in all groups at a frequency that was not dose-related; none of these were considered to be related to treatment. Haematological and clinical chemistry analyses and postmortem examinations for organ weight, gross and histopathological lesions in brain, pituitary, heart, thyroid, adrenals, genital organs, liver, kidney and skin revealed no treatment-related changes. The experimental procedure induced multifocal dermatitis in treated and untreated areas of the skin in both treated and control rabbits. Dermal treatment with metalaxyl was thus well tolerated.

The NOAEL for systemic toxicity was 1000 mg/kg bw per day, the highest dose tested (Calkins, 1980).

(c) Exposure by inhalation

Rat

In a study of the toxicological effects of repeated inhalation of the pyrolysis products of cigarette tobacco treated with metalaxyl, groups of 10 male and 10 female Fischer 344 rats were exposed for five days per week, for 13 weeks, to the smoke from 18 cigarettes that had been spiked with 0, 130, 3900 or 13 000 ppm of metalaxyl (technical grade; purity not specified). In addition, five groups of two rats of each sex were exposed once to the smoke of each concentration of spiked tobacco and to ambient air, before initiation of the 90-day study, for analyses of plasma nicotine. The criteria used to evaluate treatment-related effects included death, moribundity, appearance, behaviour, body weight, clinical pathology, absolute and relative organ weights, gross pathology and histopathology. No distinct clinical changes were noted in any of the treated animals during the study. No treatment-related effects or trends were observed in body weight gain, food consumption, haematological or clinical chemistry parameters, gross or histological appearance. This study was not taken into consideration in the final evaluation (Coate, 1982).

2.4 Long-term studies of toxicity and carcinogenicity

Mouse

In a carcinogenicity study, groups of 60 male and 60 female ICI-derived Swiss mice were given diets containing metalaxyl (purity 93–94.6%) at a concentration of 0, 50, 250 or 1250 ppm for 104 weeks, (equal to mean daily intakes of 4.5, 22.7 and 119 mg/kg bw per day for males, 5.0, 21.5 and 132 mg/kg bw per dayfor females). These values were recalculated from individual values for food consumption and body weight, taking into account the content of the test compound in the food batches used. In earlier evaluations of this study, standard conversion factors were used to calculate the intake in mg/kg bw per day. The mice were inspected twice daily for mortality or evidence of reaction to treatment or ill-health during the first two weeks of treatment, once daily until week 29 and thereafter twice daily. In addition all mice were submitted to a physical examination and palpation once weekly. Body weights were measured, at a minimum, weekly for the first 14 weeks, monthly for the next 40 weeks and weekly thereafter. Food consumption was measured weekly and food conversion ratios and achieved doses calculated. Water consumption was assessed daily. All mice killed in extremis and those killed at scheduled termination were killed by carbon dioxide inhalation, followed by a detailed necropsy; a blood smear and a bone marrow smear were also prepared. Animals found dead were also given a detailed necropsy. A comprehensive range of tissues were taken from all animals and preserved. A comprehensive range of tissues taken from controls and 1250 ppm animals were processed and examined microscopically. In addition a microscopical examination from the 50 and 250 ppm groups was made of all tissue masses, suspected tumours, and lymph nodes draining the region of the mass or tumour, and all other tissues which had shown macroscopic change, also taking samples from all animals for examination.

The animals showed no clinical signs of toxicity, and the mortality rate was similar in all groups. Liver changes, consistent with Tyzzer's disease (unrelated to treatment as it was also seen in controls), were observed in a total of 116 mice (51 males and 65 females) which died or were killed in extremis. At 78 weeks, the survival rates were 53%, 55%, 57% and 50% for males and 67%, 53%, 53% and 52%

for females in the control group and at the low, intermdiate and high dietary concentrations, respectively. At 104 weeks the mortality rates were increased, with survival rates of 8%, 10%, 10% and 17% for males and 13%, 13%, 8% and 10% for females, respectively. During weeks 11–30 of treatment, males at 1250 ppm gained less weight (11 g) than control males (17 g), corresponding to a reduction of 31% and leading to a difference of 9% at week 30. The animals recovered slowly thereafter, but the cumulative body weight gain was still reduced by 12% at week 30 and 10% at week 56 for males at 1250 ppm. A slight, 8% reduction in body weight gain in comparison with controls was observed in weeks 0–10 among females at 1250 ppm (Table 21). Terminal body weights of treated and untreated groups were similar. Food consumption and water intake of treated and control animals were essentially identical throughout the treatment period. Food conversion efficiency was reduced in males at 1250 ppm during weeks 11–30. Water consumption was not affected by treatment.

Table 21. Intergroup comparison of body weight gains (g) at selected time points in a 24 month carcinogenicity study in mice [% change compared with control]

	Dietary concentration (ppm)							
	Males				Females			
	0	50	250	1250	0	50	250	1250
Weeks 1–10	28	28	28	28	19	19	19	18
Body weight; week 10	50	50	50	50	41	41	40	39
Weeks11–30	17	15	17	11*	14	14	15	16
Body weight; week 30	67	65	67	61* [↓9%]	55	55	55	55
Weeks 31–56	7	8	9	7	9	9	7	6
Body weight; week 56	74	73	76	68 [↓9%]	63	64	62	61
Weeks 1–104	32	30	35	37	38	38	33	36
Body weight; week 104	54	52	57	59	59	60	54	57

* Significantly different from controls, $p < 0.01$ Source: McSheehy, Macrae & Whitney, 1980a, b

Macroscopic and histopathological examination of a wide range of tissues, including blood and bone marrow smears, revealed no significant treatment-related changes. The histopathological results for the main target organ, the liver, are outlined in Table 22. There was no substance-related increase in the incidence of any tumour type, although there was a high incidence of hepatic adenomas in all dose groups including controls. A re-evaluation of the liver slides (by the study authors) did not alter this conclusion and the liver tumours in male mice were considered not to be treatment-related. This conclusion is supported by the following findings:

1. The liver tumours were age-related, occurring predominantly after 18 months of the study.
2. The survival of the animals was not affected by administration of metalaxyl.
3. Neither general health, body weight nor food consumption was compromised by treatment.
4. The incidence of Tyzzer's disease was 15, 14, 14 and 8 at 0, 50, 250 or 1250 ppm, respectively. The presence of Tyzzer's disease did not obscure the presence of liver tumours, and non-neoplastic lesions in the liver (hepatocytic fatty vacuolation and bile duct proliferation) were no different in control and treated groups and would therefore not have affected the distribution of liver tumours.
5. The liver tumour incidence was not statistically significantly different ($\chi^2 = 5.92$) between control and treated groups ($p = 0.12$).
6. The liver tumour incidence did not show a dose-related trend (Mantel trend test; $p = 0.15$).
7. The liver tumour incidence in the control group (23%) was within the range found in six other control groups in the same laboratory (12–37%).

Table 22. Incidences of histopathological liver changes in mice given diets containing metalaxyl for two years (incidence/animals in group)

	Dietary concentration (ppm)							
	0		50		250		1250	
	Males	Females	Males	Females	Males	Females	Males	Females
Fatty vacuolation (total incidence)								
Dead 0–52 weeks	1/10	3/7	1/8	1/10	0/9	0/5	2/7	10/14
Dead 53–104 weeks	20/46	25/44	19/46	14/42	20/45	16/49	18/43	26/40
Killed at 104 weeks	2/4	6/8	0/6	2/8	1/6	0/5	7/10	4/5
Total	23/60	34/59	20/60	17/60	21/60	16/59	27/60	40/59
Adenomas								
Dead 0–52 weeks	1/10	0/7	0/8	0/10	0/9	0/5	1/7	0/14
Dead 53–104 weeks	10/46	4/44	19/46	1/42	13/45	5/49	13/43	1/40
Killed at 104 weeks	2/4	0/8	2/6	2/8	1/6	1/5	6/10	1/5
Total	13/60	4/59	21/60	3/60	14/60	6/59	20/60	2/59
Carcinoma								
Dead 0–52 weeks	0/10	0/7	0/8	0/10	0/9	0/5	0/7	0/14
Dead 53–104 weeks	1/46	1/44	1/46	0/42	2/45	1/49	3/43	1/40
Killed at 104 weeks	1/4	0/8	0/6	0/8	1/6	0/5	0/10	0/5
Total	2/60	1/59	1/60	0/60	3/60	1/59	3/60	1/59
Adenomas plus carcinomas								
Dead 0–52 weeks	1/10	0/7	0/8	0/10	0/9	0/5	1/7	0/14
Dead 53–104 weeks	11/46	5/44	20/46	1/42	15/45	6/49	16/43	2/40
Killed at 104 weeks	3/4	0/8	2/6	2/8	2/6	1/5	6/10	1/5
Total	15/60	5/59	22/60	3/60	17/60	7/59	23/60	3/59

Source: McSheehy, Macrae & Whitney, 1980a, b

Treatment with metalaxyl did not increase the incidence of benign or malignant tumours in mice of either sex. The NOAEL was 1250 ppm (equal to 119 mg/kg bw per day) the highest dose tested (McSheehy, Macrae & Whitney, l980a, b).

Rat

In a combined chronic toxicity and carcinogenicity study, groups of 80 male and 80 female CD Sprague Dawley-derived rats were fed diets containing metalaxyl (purity 93.0–94.6%) at concentrations of 0, 50, 250 or 1250 ppm (equal to mean daily intakes of 0, 1.7, 8.7 and 43 mg/kg bw per day for males, 0, 2, 10 and 55 mg/kg bw per day for females). After 55 weeks of treatment ten male and ten female rats from each group were terminated. The remaining, surviving animals were killed after 105 weeks. The animals were observed twice daily for mortality and clinical signs and in addition, all rats were submitted to a physical examination and palpation once weekly. All rats were given an ophthalmoscopic examination pretest, and the control and high-dose groups were examined during weeks 13, 26, 51, 78 and 102. Body weight and food and water consumption were measured weekly for the first 13 weeks, at week 16 and monthly thereafter. Food conversion ratios and achieved doses were calculated. Blood from 10 rats/sex per group was analysed for haematology and blood chemistry after 13, 25, 51, 78 and 104 weeks. After 13, 26, 52, 78 and 103 weeks, overnight urine samples were collected from 10 rats/sex per group and urinalysis determinations made. All rats killed in extremis and those killed after 55 and 105 weeks of treatment were killed by carbon dioxide inhalation; a detailed necropsy followed. Animals found dead were also given a detailed necropsy. A selection of organs from all animals, was weighed. A comprehensive range of tissues were taken from all animals, preserved and processed for microscopic examination. Three coronal sections of the head (to include nasal cavity, paranasal sinuses, tongue, oral cavity, nasopharynx and middle ear) were prepared and examined from 10 rats/sex per group. Ovaries were not weighed, γ-glutamyl transpeptidase activity not measured nor were the ovaries weighed.

No deaths or clinical signs of toxicity resulted from treatment. While no differences between treated and control animals were seen in food consumption or absolute body weight, the body weight gain of females at 1250 ppm was reduced transiently in weeks 26–52 (by 10% compared to controls). Haematological, clinical chemistry and urine parameters remained within normal limits, and no remarkable intergroup differences were found. Ophthalmoscopy revealed no abnormalities attributable to the administration of metalaxyl.

At interim termination, the liver : body weight ratio was significantly increased (120% of control) in females at 1250 ppm. At final termination increased absolute and relative liver weights were found in animals of each sex at 1250 ppm. The increase in relative liver weight in males at 250 ppm was due at least partly to a decreased carcass weight in this group. The increased relative weights of the liver observed in females at 1250 ppm at week 55 and in males at 250 and 1250 ppm at weeks 105, were not accompanied by underlying hepatic damage, indicating that the changes in weight indicated a mild change in the liver that was not adverse.

An increased severity of eosinophilic vacuolation was seen in males that were killed at week 55 from the two higher concentration groups. This finding was considered to represent a transitory response that was not adverse because it was not observed in animals dying during the study or at final termination. Centriacinar, periacinar and panacinar hepatocytic vacuolation in the liver of various degrees of severity was observed, indicating fatty changes. The centriacinar and panacinar vacuolation was not related to treatment. In males, no significant, dose-dependent increase in the incidence of hepatocytic vacuolation was observed in any treated group. In animals that died up to week 52, no relationship with treatment was found for hepatocyte vacuolation in the few animals that were so affected. Increased incidences of periacinar hepatocyte vacuolation were observed in all treated females at 55 and 105 weeks, but the distribution among groups was uneven and there was no clear relationship to dose. The increase in severity was not dose-related at any time, except in females at 1250 ppm at final termination , in which the severity was increased compared with their concurrent controls and with females at this concentration at earlier times. Therefore, only a mostly slight-to-moderate increase in the incidence of periacinar hepatocytic vacuolation was observed with metalaxyl-treated females (considered to be a fatty change and occurring spontaneously also in control animals), with an increase in severity only for females at the highest concentration at week 105. Except in these animals, there was a slight decrease in the overall average severity of hepatic vacuolation between termination at week 55 and at week 105. A second histopathological evaluation (Faccini, 1985) confirmed the overall result of marginal changes in the liver. It also showed an increased incidence of centrilobular hepatocytomegaly in male and female rats at 1250 ppm and possibly a marginally higher incidence in males at 250 ppm. A higher incidence of foci of cellular alteration was seen in males at 250 ppm, with a possible trend towards the same effect in females at this concentration.

A statistically significant increase ($p < 0.05$) was found in the incidence of C-cell (parafollicular cell) adenomas in females at 250 ppm (Table 23). However, no tumours were detected in animals that died before 78 weeks of treatment, no dose–response relationship was established, and the incidence was within that of controls from 15 contemporary studies in the same laboratory (total adenomas, 0–15%; adenomas in females killed at termination of study: 0–30%). Therefore, the distribution was considered to be incidental and not related to treatment with metalaxyl.

Table 23. Incidence of C-cell adenomas of the thyroid in rats given diets containing metalaxyl for two years (incidence/animals in group)

	Dietary concentration (ppm)							
	0		50		250		1250	
	Males	Females	Males	Females	Males	Females	Males	Females
Dead 0–52 weeks	0/6	0/0	0/3	0/2	0/2	0/4	0/6	0/4
Killed after 55 weeks	0/10	0/10	1/10	0/10	0/10	0/10	0/10	0/10
Dead at 53–105 weeks	2/44	0/38	1/36	4/42	3/46	5/37	3/36	0/42
Killed after 105 weeks	2/20	2/32	0/31	3/26	1/22	5/28	1/28	5/24
[%]	[10]	[3.6]	[0]	[12]	[4.5]	[18]	[3.6]	[2.1]
Total	4/80	2/80	1/80	7/80	4/80	10/79	4/80	5/80
[%]	[5]	[2.5]	[1.3]	[8.8]	[5.0]	[13]*	[5]	[6.3]

* Statistically significant at $p < 0.05$ Source: Ashby & Whitney, 1980a, b

Thus, metalaxyl administered to rats at concentrations up to 1250 ppm in the diet for two years was well tolerated. The finding of hepatocytic vacuolation is age-related and was considered not to be adverse. The hepatocellular changes were not accompanied by degenerative, irreversible or cytotoxic lesions such as necroses, which can be observed in the cases of severe fatty change of the liver, and no accompanying inflammatory changes were found. Blood chemistry showed no altered liver parameters that pointed to hepatocellular damage. In general, most evidence suggests that moderate hepatic fatty change alone does not impair hepatic function (Popp & Cattley, 1991). Centrilobular hepatocytomegaly was considered to be the main effect, occurring in rats of each sex at the highest concentration. This change might have been a consequence of a mild enzyme-inducing effect due to the test compound (Uesugi, 1988). The treatment had no effect on the incidence or distribution of neoplastic lesions. A re-evaluation of C-cell (parafollicular cell) adenomas in the thyroid gland did not provide evidence to alter this position.

2.5 Genotoxicity

The results of studies on the genotoxicity of metalaxyl-M are summarized in Table 24

Table 24. Overview of genotoxicity tests with metalaxyl-M

End-point	Test system	Concentrations/doses tested	Purity	Results	Reference
		In vitro			
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537; *E. coli* WP2*uvrA*	**With and without S9:** 310, 620, 1200, 2500 and 5000 µg/plate in DMSO	97.3%	Negative	Hertner, 1994a
Chromosomal aberration	Chinese hamster ovary cells	**Without S9:** 18 h treatment: 30, 97.3, 250, 510 and 1000 µg/mL 42-h treatment: 250,510 and 1000 µg/mL **With S9:** 3 h treatment, 15 or 39 h recovery: 250, 510, 1000 and 2000 µg/mL Vehicle: DMSO	97.3%	Negative	Hertner, 1994b

Unscheduled DNA synthesis	Primary rat hepatocytes	**Test 1:** 4.9, 20, 78, 160, 310 and 620 µg/mL **Test 2** 20, 39, 78, 160, 310 and 620 µg/mL Vehicle: DMSO	97.1%	Negative	Ogorek, 2000
		In vivo			
Micronucleus formation	CD1 mice, 5/sex per dose, bone marrow	**Sampling time after 24 h** Single oral dose of 200, 400, 800 mg/kg bw for males and 125, 250, 500 mg/kg bw for females **Sampling time after 48 h** 500 mg/kg bw in both sexes Vehicle: 05% CMC 0.1% Tween 80	97.1%	Negative	Deparade, 1999

S9: Exogenous metabolic activation system from 9000 × *g* fraction of rat liver induced with Aroclor;
DMSO: Dimethyl sulfoxide; CMC: Carboxymethyl cellulose
Positive control substances were used in all assays and gave the expected results.

The results of studies on genotoxicity of metalaxyl are summarized in Table 25.

Table 25. Overview of genotoxicity tests with metalaxyl

End-point	Test object	Concentrations/doses tested	Purity	Results	Reference
		In vitro			
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537	20, 78, 313, 1250 and 5000 µg/plate in acetone	95.7%	Negative[a]	Despande & Arni, 1985
Mitotic gene conversion (reverse mutation)	*Saccharomyces cerevisiae* D7	100, 2000, 10 000 µg/mL in DMSO	94.1%	Negative[a]	Arni & Muller, 1982
Forward mutation	Mouse lymphoma L5178Y, *tk* locus	**With S9:** 0.062, 0.12, 0.25 and 0.5 mg/mL **Without S9:** 0.12, 0.25, 0.5 and 1 mg/mL in DMSO	94.1%	Negative[a]	Strasser & Muller, 1982
Chromosomal aberration	Chinese hamster ovary cells	**Without S9:** 18 h treatment, harvesting 2.5 h later **With S9:** 2 h treatment, harvesting 7.5 h later 150, 300, 900 and 1200 µg/mL in DMSO (both ± S9)	NR	**–S9:** positive at 1200 µg/mL (cytotoxicity) **+S9:** negative	Ivett, 1986
Chromosomal aberration	Human peripheral blood lymphocytes	**Without S9:** 24 h treatment **With S9:** 3 h treatment, harvesting 21 h later 10, 30, 100, 300, 1000 µg/mL in DMSO (both ± S9)	99%	**–S9:** dose-dependent increase **+S9:** slight increase at 300 µg/mL	Hrelia et al., 1996

Unscheduled DNA synthesis	Human fibroblasts	4, 20, 100 and 500 µg/mL in DMSO, noS9	94.1%	Negative[a]	Puri & Muller, 1982
Unscheduled DNA synthesis	Primary rat hepatocytes	16, 80, 400 and 2000 µg/mL in DMSO	94.1%	Negative[a]	Puri & Muller, 1985
Cell transformation	BALB/c 3T3 cells	50, 250 and 500 µg/mL in DMSO	99.9%	**Without S9:** positive at 500 µg/mL (cytotoxic) **With S9:** positive at all doses	Perocco et al., 1995
		In vivo			
Micronucleus formation	Tif:MAGf (SPF) mice, (5/sex per dose), bone marrow	Single oral dose of 78, 160 and 310 mg/kg bw in archis oil, sampling 16, 14, 48 h later	96.1%	Negative	Hertner & Arni, 1992
Micronucleus formation	CD-1 mice, 5 males per group, bone-marrow cells	Single intraperitoneal doses of 75, 150 and 300 mg/kg bw in corn oil; sampling at 24 h	99%	Negative	Hrelia et al., 1996
Nucleus anomalies	Chinese hamsters (5 males, 5 females per group); bone-marrow cells	Oral, 600, 1200 and 2400 mg/kg bw in CMC; 2 days; sampling 24 h after second treatment	98%	Negative	Langauer & Muller, 1979
Dominant lethal mutation	NMRI mice (20 males per group)	Single oral doses of 65 and 200 mg/kg bw in CMC	99.4%	Negative	Fritz, 1978a

S9: Exogenous metabolic activation system from 9000 × *g* fraction of rat liver induced with Aroclor;

DMSO: Dimethyl sulfoxide; CMC: Carboxymethyl cellulose; NR: Not reported:

Positive control substances were used in all assays and gave the expected results.

[a] With and without metabolic activation

2.6 Reproductive and developmental toxicity

(a) Multigeneration studies

In a multigeneration reproduction study, groups of male and female Charles River COBS CD rats received diets containing metalaxyl (purity 93.5%) at a concentration of 0, 50, 250 or 1250 ppm for three generations, resulting in intakes of 4.1 (3.2–4.7), 21 (17–23) and 96 (84–100) mg/kg bw per day for males, and 4.6 (3.6–5.1), 24 (20–27) and 150 (100–240) mg/kg bw per day for females. The values were calculated from data on food consumption and body weight for the F0, F1 and F2b generations before mating and corrected by the analytical food content. The litter sizes were not standardized. All terminated animals were subjected to macroscopic examination.

In the first generation (F0), 25 males and females in each group were mated. After weaning of the first litter (F1a), all the animals were mated again, and the resulting litters (F1b) were reared to day 21 post partum (PP), when 25 males and females were selected from each group to form the basis for the F2 generation. A third gestation of 15 F0 animals per dose was terminated after 20 days.The skeletons and viscera of the fetuses were examined for malformations. The F1b animals were mated when they were at least 90 days old. The resulting litters (F2a) were killed after weaning at 21 days old, and the parental animals were mated a second time. Ten dams per group were killed on gestation day (GD) 20, and the fetuses examined for malformations, while 14 dams were allowed to rear their litters. At weaning,

12 male and female pups from each group were kept on their diet for 90 days, and then their organs were weighed. The third generation was produced from another 12 males and 24 females from the F2b litters. These animals were also mated once more after production of the F3a generation. After weaning of the F3b pups the study was terminated. The organs from 10 pups of each sex from the control group and the 1250 ppm group were weighed and examined histologically.

No treatment-related effects were seen in the parental animals, with the possible exception of a slight retardation in body weight development in males of the F1b generation at the highest concentration during the first 10 weeks after selection. However, the finding was not reproduced in the F0 or F2 generations and was therefore considered to be of equivocal toxicological significance. The food consumption of females of the F0 and F1b generations at 50 and 250 ppm was lower than that of the other groups, but this effect was considered not to be toxicologically significant. Parameters of reproduction and fertility and of fetal and neonatal development remained unaffected in all groups and generations. At termination the mean liver weights of F2b females at 1250 ppm were slightly increased (111% of control values). This effect was considered not to be adverse, and reproductive performance and offspring development were unaffected, even at the highest concentration of 1250 ppm.

The NOAEL for parental, offspring and reproductive toxicity was 1250 ppm (equal to 96 mg/kg bw per day) the highest dose tested (Cozens, 1980).

(b) Developmental toxicity

Rat

Three studies of developmental toxicity were conducted in rats, one with metalaxyl-M and two with metalaxyl.

Study 1

In a developmental toxicity study, groups of 24 mated Sprague Dawley-derived Tif:Raif (SPF) rats were given metalaxyl-M (purity 97. l%) by gavage in an aqueous solution of CMC (0.5% w/w) at a dose of 0, l0, 50 or 250 mg/kg bw per day on GDs 6–15. The dams were killed on day 21 after conception, and fetuses removed by caesarean section for examination.

No clinical signs considered to be treatment-related were seen. One dam at the highest dose was killed when moribund on GD 12, the condition probably being due to an intubation error. In animals at the highest dose, body weight development was significantly reduced during treatment, body weight gain on days 6–11 being 73% of that in controls, and that on days 6–16 being 88% of control. However, no significant body weight decreases were observed on GDs 6–8. Body weight gain on days 16–21 was comparable to that of untreated controls. A slight, transient reduction in food intake was noted during the treatment period in dams receiving 50 and 250 mg/kg bw per day. The slight, transient reduction in food consumption in animals at 50 mg/kg bw per day was considered not to be adverse as, in that group no significant effect was noted on body weight development, nor was there any other sign of maternal toxicity. No treatment-related macroscopic findings were seen at necropsy of dams.

Treatment had no effect on the mean numbers of corpora lutea, implantation sites, early or late resorptions or post-implantation loss. There were no dead or aborted fetuses. Treatment also had no effect on the number of live fetuses, fetal sex ratio or fetal body weight. No treatment-related external skeletal or visceral malformations, anomalies or variations were observed among the fetuses. Metalaxyl-M thus had no embryotoxic or teratogenic potential in rats.

The NOAEL for maternal toxicity was 50 mg/kg bw per day, on the basis of a reduction in body weight gain (GDs 6–16) and feed consumption at the LOAEL of 250 mg/kg bw per day; the highest dose tested. The NOAEL for embryo/fetal toxicity was 250 mg/kg bw per day, the highest dose tested (Khalil, 1995).

Study 2

In a developmental toxicity study (not GLP compliant), groups of 25 successfully mated Sprague Dawley-derived Tif:RAI (SPF) rats received metalaxyl (purity 99.4%) in 2% aqueous CMC by gavage on GDs 6–15 at a dose of 0, 20, 60 or 120 mg/kg bw per day. The dams were killed on day 21 after conception. The study had been performed before GLP guidelines and EEC or OECD test guidelines were adopted. The major deviations from protocol OECD TG 414 (1981) concerned visceral

abnormalities, which were examined in only one-third of fetuses, and the results for body weight gain and food consumption, which were not outlined in detail. The study was considered to provide the Meeting with only additional information.

The dams at the highest dose showed mild apathy after administration of the substance from day 4 of treatment. In comparison with untreated controls, food consumption was slightly reduced in all treated groups during the first five days. A diagram (no numerical data were reported) indicated that the marginal decrease in food consumption of dams at 20 mg/kg bw per day was only transient at day 6. Body weight gain was slightly depressed at the two higher doses (no numerical data were reported).

The mean numbers of implantations sites and early or late resorptions were similar in all groups. No malformations were found in treated groups. In the original report, a marginally increased incidence of slightly retarded ossification of the fifth sternebrae in fetuses at the highest dose was reported to be related to slight maternal toxicity. As the increase was only slight (57% of treated fetuses in comparison with 44% of controls) and the sum of incidences of delayed ossification was not increased at 120 mg/kg bw per day, a relationship to treatment was considered to be doubtful. As no effect of treatment was seen on fetal body weight, it is unlikely that growth was retarded at the highest dose. The two instances (out of 91) of pulmonic hypoplasia observed in the low-dose group ,and the one instance each (out of 91) of pulmonic hypoplasia and abnormal ossification of a thoracic vertebra observed in the intermediate dose group, were considered to be of a spontaneous origin. Metalaxyl had no embryotoxic or teratogenic potential in rats under the experimental conditions of this study.

The NOAEL for maternal toxicity was 20 mg/kg bw per day, on the basis of a reduction in body weight gain at the LOAEL of 60 mg/kg bw per day and above. The NOAEL for embryo/fetal toxicity was 120 mg/kg bw per day, the highest dose tested. This study was not taken into account in the final evaluation (Fritz, 1978b).

Study 3

In a developmental toxicity study, groups of 27 or 38 (highest dose) mated Charles River COBS CD rats received matalaxyl (purity 96.8%) in 1% aqueous methylcellulose by gavage at a dose of 0, 50, 250 or (initially) 575 mg/kg bw per day on GDS 6–15. Owing to maternal deaths, the highest dose was reduced to 500 and then to 400 mg/kg bw per day after two days of treatment. The surviving dams were killed on GD 20, and the fetuses were removed by caesarean sections for examination.

Overt maternal toxicity was still seen at the two higher doses, evidenced most notably by death (Table 26), transient convulsions within minutes of dosing (majority of these convulsions occurred at the beginning of the treatment period; GD 6 and/or GD 7 in two animals at the high dose), loss of activity, ataxia (10 animals at the high dose), body tremors (one animal at the high dose), reduced or absent righting reflex and inadequate grooming. Animals at these doses also showed significantly depressed body weight gain during treatment (90% and 84% of control values at 250 and 400 mg/kg bw, respectively). Depressed body weight gains (–9 g and 0 g) were observed at 250 and 400 mg/kg bw per day respectively during GDs 6–9. A slight reduction in mean body weight gain was also seen in animals at the highest dose throughout gestation (days 0–20: 90% of control value). Food consumption remained comparable in all groups (Table 27).

Table 26. Group mean maternal and fetal observations at caesarean section

	Dose level (mg/kg bw per day)			
Observation	**0 (control)**	**50**	**250**	**400**
Animals on study	27	27	27	38
Animals pregnant	24	25	23	33
Animals died/killed in extremis – total	0	0	1	12
Animals died/killed in extremis- not pregnant	-	-	0	0
Animals died/killed in extremis- pregnant	-	-	1	11
Animals examined at caesarean section – total:	27	27	26	26
Non pregnant:	3	2	4	4
Pregnant:	24	25	22	22

Observation	Dose level (mg/kg bw per day)			
	0 (control)	50	250	400
Dams with resorptions only	0	0	0	0
Dams with viable fetuses	24	25	22	22
Mean number of viable fetuses/dam	12.7	12.6	12.5	13.0
Mean post-implantation losses/dam	1.0	1.5	1.0	0.8
Mean total implantations/dam	13.8	14.0	13.6	13.9
Mean number of corpora lutea/dam	15.4	15.5	15.0	15.7
Group mean pre-implantation loss (%)	10.8	9.3	9.4	11.8
Group mean post-implantation loss (%)	7.6	10.5	7.7	5.9
Mean fetal body weight (g)	3.4	3.3	3.2	3.2
Percentage male foetuses (%)	51.1	50.8	53.3	52.6

There were no statistically significant differences from control values

Source: Leng & Schardein, 1985

Table 27. Intergroup comparison of body weight gain (g)

Days of gestation	Dose level (mg/kg bw per day)			
	0 (control)	50	250	400
0–6	22	22	23	24
6–9	5	4	–3	0
9–12	11	13	10	10
12–16	22	23	23	23
16–20	55	57	53	50
6–15	38	40	34	32
0–20	115	119	111	104

Source: Leng & Schardein, 1985

No meaningful intergroup differences were seen in fetal parameters, however, some skeletal variations (presacral vertebrae, fewer than normal ribs, reduced ossification of skull, unossified pubic bones) were reported at 400 mg/kg bw per day. The incidences of most were within the range of other controls in the same laboratory, with the exception of unossified pubic bones, which occurred in 14% of the litters (probably secondary to maternal toxicity) and in only 0–6% of other, historical controls. Metalaxyl had no embryotoxic or teratogenic potential under the experimental conditions of this study.

The NOAEL for maternal toxicity was 50 mg/kg bw per day, on the basis of a reduction in body weight gain at higher doses and clinical signs, including convulsions, majority of which occurred at the beginning of the treatment period (GD 6 and/or 7) at 250 mg/kg bw per day. The NOAEL for developmental toxicity was 400 mg/kg bw per day, the highest dose tested (Leng & Schardein, 1985).

Rabbit

In a developmental toxicity study (not GLP compliant; predating regulation), groups of 20 mated Chinchilla SPF rabbits were administered metalaxyl (purity not specified) suspended in 2% aqueous CMC by gavage at a dose of 0, 5, 10 or 20 mg/kg bw per day on GDs 6–18. Major deviations from protocol OECD TG 414 (1981) were found with regard to the descriptions of body weight gain and food consumption, which were not given in detail. The study was considered to provide the Meeting only with additional information.

No clinical signs of toxicity were observed. Does at the two higher doses reacted to treatment with a dose-related reduction in food consumption and a slight reduction in body weight gain. Parameters of reproduction were comparable in all groups, and fetal development was not affected. The few instances of malformations observed in the group at 10 mg/kg bw per day (one agenesis of the left kidney and

ureter, one hypoplasia of kidneys) and at 20mg/kg bw per day (one agenesis of the right kidney and ureter) were considered to be spontaneous and unrelated to treatment. Renal maldevelopment was the most frequent type of malformation found in untreated controls in other studies with the breed of rabbits used in this study. Metalaxyl did not adversely affect embryonic or fetal development in rabbits under the experimental conditions of this study. No teratogenic potential was found.

The NOAEL for maternal toxicity was 5 mg/kg bw per day, on the basis of reductions in body weight gain and food consumption at higher doses. The NOAEL for developmental toxicity was 20 mg/kg bw per day, the highest dose tested. This study was not taken into account in the final evaluation due to study deficiencies (Fritz & Becker, 1978).

In a developmental toxicity study, groups of 18 inseminated Dutch Belted rabbits were given metalaxyl (purity 96.8%) suspended in 1% aqueous methylcellulose by gavage at a dose of 0, 30, 150 or 300 mg/kg bw per day on GDS 7–19. The dams were killed on GD 28 and the fetuses removed by caesarean sections.

Two control rabbits and one high-dose rabbit died in the middle of gestation interval but a definitive cause of death could not be established. Reduced faecal output was observed for seven rabbits at the highest dose. Animals at this dose lost weight during treatment (average, 71 g), and the overall body weight gain was 21% lower than the control value throughout the study. Food consumption was similarly reduced in the same group (77% of control value on days 7–20). In the absence of any effect on body weight or food consumption, the slightly decreased body weight gain (24% lower than control value on days 0–28) in animals at the intermediate dose was considered to be unrelated to treatment. Furthermore, the differences in body weight gain were more marked before and after the treatment period than during treatment. The variation in body weight gain might have been due to the slightly reduced initial body weight and thus to generally reduced body weight gain in this group.

No treatment-related differences were seen in reproduction parameters, fetal body weight or the incidences of fetal malformations or variations in treated groups compared to controls. Metalaxyl had no embryotoxic or teratogenic potential in rabbits under the experimental conditions of this study.

The NOAEL for maternal toxicity was 150 mg/kg bw per day on the basis of reductions in body weight gain and food consumption at the LOAEL of 300 mg/kg bw per day, the highest dose tested. The NOAEL for embryo/fetal toxicity was 300 mg/kg bw per day, the highest dose tested (Laughlin & Schardein, 1984).

2.7 Special studies

(a) Effects on drug metabolizing enzymes

The effects of metalaxyl on drug metabolizing enzymes were examined during a study of the kinetics and metabolism of this substance in rats (Uesugi, 1988). Five male rats were given ^{14}C metalaxyl (uniformly ring-labelled) orally at a dose of 40 mg/kg bw per day for seven days or 80 mg/kg bw per day for three or seven days. Controls received 0.6% methylcellulose solution for three or seven days. Phenobarbital was used as a positive control and was administered intraperitoneally at a dose of 80 mg/kg bw per day for three days. The animals were fasted for 20 hours after the final administration and killed. The livers were homogenized after which the supernatant and microsomal fractions were prepared according to standard methods in order to determine the concentrations and activity of microsomal enzymes.

The effects of metalaxyl and phenobarbital on drug metabolizing enzymes are shown in Table 28. In rats given 40 mg/kg bw per day for seven days, the activities of cytochrome P450 (CYP), aminopyrine *N*-demethylase, *p*-nitroanisole *O*-demethylase, *p*-nitrophenol UDP-glucuronyl transferase and dinitrochlorobenzene glutathione transferase were significantly increased compared with controls. Administration of 80 mg/kg bw per day for three or seven days significantly enhanced the activities of all these enzymes and also of NADPH-cytochrome c reductase. Administration of phenobarbital for three days significantly enhanced the activities of all enzymes examined when compared with controls. Thus, administration of metalaxyl at 40 or 80 mg/kg bw per day orally for 3–7 days significantly increased the concentration and/or the activity of several hepatic enzymes. The content of cytochrome b5 remained unaffected. In comparison with the positive control group treated with phenobarbital, the

enzyme-inducing effect of metalaxyl was relatively mild, about 50% of the activity compared to that seen in phenobarbital-treated animals for most of the enzymes (Uesugi, 1988).

Table 28. Effects of metalaxyl on metabolising enzymes in male rats

	Dose level etalaxyl (mg/kg bw per day)					Dose level phenobarbital (mg/kg bw/day)
Time post dose	0 (control)		40	80	80	80
	3 days dosing	7 days dosing	7 days dosing	3 days dosing	7 days dosing	3 days dosing
Body weight (g)	160.7	204.2	189.4	163.4	184.8	158.0
Liver wt (g/100 g body weight)	3.48	3.35	3.37	3.46	3.46	4.83
Cytochrome P 450 (nmol/mg protein)	0.54	0.55	0.65*	0.77*	0.73*	1.57**
Cytochrome b5 (nmol/mg protein)	0.13	0.13	0.15	0.18	0.12	0.35*
NADPH-cytochrome C reductase (µmol/min per mg protein)	0.030	0.031	0.030	0.039*	0.038*	0.070**
Aminopyrine *N*-demethylase (nmol/min/mg protein)	10.24	10.26	10.73*	10.91*	11.12*	14.41**
p-nitroanisole *O*-demethylase (nmol/min/mg protein)	0.23	0.24	0.29*	0.38**	0.42**	0.70**
p-nitrophenol UDP glucronyl transferase (nmol/min/mg protein)	25.2	25.3	31.5*	44.5**	35.5**	48.6**
dinitrochlorobenzene glutathione transferase (µmol/min per mg protein)	1.13	1.14	1.30*	1.28*	1.39**	2.20**

* $p < 0.05$, ** $p < 0.01$ Source: Uesugi, 1988

The ability of metalaxyl to affect specific biomarkers of nongenotoxic cocarcinogenesis was investigated in male and female Swiss Albino CD-1 mice given metalaxyl (purity 99.5%) dissolved in corn oil as a single intraperitoneal dose of 200 or 400 mg/kg bw or repeated doses of 200 mg/kg bw per day for three days. Controls received the vehicle only. Liver, kidney and lung were removed rapidly and processed separately, and a 9000 × *g* supernatant and microsomal fractions were prepared according to standard methods. The concentration or activity of microsomal enzymes was then determined. The study was considered by the Meeting to provide only additional information.

No significant changes in the absolute or relative weights of the liver, kidney or lung were observed after treatment. Although a single dose did not significantly affect the monooxygenases, a clear example of selective CYP3A induction was recorded in various tissues after repeated treatment. About a three-fold increase in CYP3A enzymes, as indicated by *N*-demethylation of aminopyrine, was observed in the livers of both sexes, and about a five-fold increase in the activity of this oxidase was found in the kidney. No significant change in the selected biomarkers was observed in lung. A weak but significant reduction in CYP2Bl activity was recorded in the liver of male animals. Liver and kidney CYP3A overexpression was corroborated by western immunoblotting with rabbit polyclonal antibodies to CYP3A l/2. Northern blotting with a CYP3A cDNA biotinylated probe showed that the expression of this enzyme in liver is regulated at the mRNA level (Paolini et al., 1996).

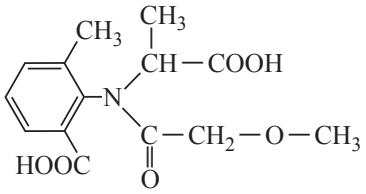
(b) Effects on cardiac activity in rats

The effect of metalaxyl (purity not reported) on cardiac activity was investigated in male rats given an intraperitoneal injection of 200, 250 or 300 mg/kg bw of metalaxyl. Metalaxyl decreased the heart rate at or near lethal doses (Naidu & Radhakrishnamurty, 1988, 1989).

(c) Studies on metabolites – acute toxicity

CGA 108906 (Metabolite 12; N-(2-carboxy-6-methylphenyl)-N-(methoxyacetyl)alanine; CAS 104390-56-9)

Fig. 3 Structure of CGA108906 (Metabolite 12)



In an acute oral toxicity study, groups of five male and five female Tif:RAIf (SPF) rats were given single oral gavage dose of CGA108906 (purity 99%) at a dose of 2000 mg/kg bw in distilled water containing 0.5% CMC and 0.1% polysorbate 80 and observed for 14 days.

There were no deaths and no effect on body weight. Piloerection, hunched posture and dyspnoea were seen, but the animals recovered within three days. At necropsy, no deviations from normal morphology were found in any animal.

The acute oral LD_{50} in rats was > 2000 mg/kg bw (Hartmann, 1994).

In an acute dermal toxicity study, a group of five male and five female Tif:RAIf (SPF) rats was dermally exposed to CGA108906 (purity 99%), in distilled water, in a single 24-hour application at a dose of 2000 mg/kg bw. A volume of 4 mL/kg bw was applied to an area (at least 10% of the body area) of shaved skin on the back, under a semi-occlusive dressing. After 24 hours, the dressing was removed and the skin cleaned with lukewarm water. The animals were observed for 14 days.

There were no mortalities and no clinical signs of systemic toxicity. Slight local erythema at the application site was seen in one male on days 5–11 and in two females on days 5 and 6. Body weight loss was recorded in one female during the second week after treatment. At autopsy no deviations from normal morphology were found.

The acute dermal LD_{50} in rats was > 2000 mg/kg bw (Winkler, 1996b).

CGA 107955 (Metabolite 6; N-(2,6-dimethylphenyl)-N-(hydroxyacetyl)alanine; CAS 104390-55-8 N)

In an acute oral toxicity study, groups of five male and five female Tif:RAIf (SPF) rats were given single gavage dose of CGA107955 (purity 95%) at a dose of 300, l000 or 3000 mg/kg bw in distilled water containing 0.5% CMC and 0.1% polysorbate 80 and were observed for 14 days before being killed. The experimental protocol was not fully in compliance with OECD TG 401, as body weights were not measured and necropsy was not performed.

There were no deaths. Dyspnoea, exophthalmia, ruffled fur and curved body weight position were seen. In addition, animals at the highest dose showed sedation 3–5 hours after the administration. The animals recovered within 10–12 days.

The acute oral LD_{50} in both male and female rats was > 3000 mg/kg bw (Sarasin & Gfeller, 1986).

CGA 62826 (Metabolite 1; enantiomer from metalaxyl-M is NOA 409045; N-(2,6-dimethylphenyl)-N-(methoxyacetyl)alanine; CAS 75596-99-5

In an acute toxicity study, group of five male and five female Tif:RAIf (SPF) rats received a single oral gavage dose of 2000 mg/kg bw of CGA62826 (purity 100%) at 2000 mg/kg bw in distilled water. Treated animals were observed for 14 days and necropsy was performed. There were no mortalities, no remarkable clinical signs, body weights were not affected and the necropsy examination did not reveal observable abnormalities.

The acute oral LD_{50} for CGA62826 was > 2000 mg/kg bw in rats (Winkler, 1996c).

In an acute dermal toxicity study, groups of five male and five female rats were given CGA62826 (purity 100%) in a dermal dose of 2000 mg/kg bw (limit test) and were observed for 14 days before

termination. The experimental protocol was in compliance with OECD TG 402 (1987). There were no deaths, no treatment-related clinical signs and no effects on body weight. There were no remarkable findings at the site of skin application. At necropsy no deviations from normal morphology were found in any animal (Winkler, 1996d).

(d) Studies on metabolites – short-term studies of toxicity

CGA108906 (Metabolite 12)

In a 28-day toxicity study, groups of five male and five female Sprague Dawley-derived Tif:RAif (SPF) rats, were given CGA108906 (purity 99%) suspended in water containing 0.5% CMC and 0.1% Tween 80 by gavage at a dose of 0, 10, 50, 200 or 1000 mg/kg bw per day for 28 days. An additional group of five rats of each sex from the control and the highest-dose groups were kept for a four-week recovery period before termination. Overt signs of toxicity were recorded daily, and body weight, food consumption and water consumption were recorded weekly throughout the study. Detailed clinical observations were performed before treatment and once weekly thereafter. A battery of functional observational tests (FOB) and motor activity tests were conducted at weeks 4 and 8 (recovery groups only). Haematological, blood chemistry and urine analyses were carried out on all surviving animals at the end of treatment and on animals kept for evaluation of reversibility. After they were killed, the animals were examined macroscopically and organ weights recorded. Organs and tissues were collected and prepared for histopathological evaluation. The protocol complied with OECD TG 407 (1995) and TM B7 from Annex V of Directive 92/69/EEC.

Daily and weekly clinical observations showed no relevant changes related to treatment. There were no deaths attributable to treatment. Body weight gain and food and water consumption were similar in treated and control groups. Haematological examination revealed no changes that could be attributed to treatment. Slightly increased plasma glucose and potassium concentrations were recorded in males at the highest dose after four weeks of treatment, but these findings were completely reversed after the recovery period. Urine analysis showed a reduced pH value for animals at the highest dose, but after the recovery period no differences were noted between treated rats and controls. No treatment-related changes were found in the FOB or motor activity tests.

At the end of treatment the mean and relative weights of the heart were slightly increased (6% and 11%, respectively) in males at the highest dose. This finding was reversed within the four-week recovery period. Macroscopic and microscopic examination revealed no treatment-related changes.

Treatment with CGA108906 was well tolerated up to the limit dose of 1000 mg/kg bw per day. Only minor and completely reversible changes were observed at the highest dose, which were considered not to be adverse in the absence of any histopathological findings.

The NOAEL for CGA108906 (Metabolite 12) was 1000 mg/kg bw per day; the highest dose tested (Gerspach, 1997).

CGA62826 ***(Metabolite 1)***

In a 28-day toxicity study, groups of five male and five female Sprague Dawley-derived Tif:RAif (SPF) rats, were given CGA62826 (purity 100%) by gavage, suspended in water containing 0.5% CMC and 0.1% Tween 80 at a dose of 0, 10, 50, 200 or 1000 mg/kg bw per day for 28 days. Additional groups of five rats of each sex from the control and the highest-dose groups were kept for a four-week recovery period before sacrifice. Overt signs of toxicity were recorded daily, and body weight, food consumption and water consumption recorded weekly throughout the study. Detailed clinical observations were performed before treatment and once weekly thereafter. In the case of recovery groups only, FOB and motor activity tests were conducted at weeks 4 and 8. Haematology, blood chemistry and urine analysis were carried out on all surviving animals at the end of the treatment period and at the end of the recovery period on animals kept for evaluation of reversibility. At termination, animals were examined macroscopically, and organ weights were recorded. Organs and tissues were collected and prepared for histopathological evaluation.

Daily and weekly clinical observations revealed no change of toxicological relevance. Nodeaths occurred. Body weight gain, and food and water consumptions were similar in treated and control groups. Haematological, blood chemistry and urine analyses revealed no changes that could be attributed to treatment. No treatment-related changes were found in the FOB and motor activity tests.

At the end of treatment, the relative weight of the liver in males at the highest dose was minimally increased, by 7%. A tendency to increased liver weights was also noted in females at 50 and 1000 mg/kg bw per day (by 7% and 6%, respectively). These changes were reversed within the recovery period. No treatment-related changes were observed at necropsy. Microscopic examination showed increased incidences of minimal hypertrophy of liver hepatocytes in males at 200 and 1000 mg/kg bw per day and in females at 50, 200 and 1000 mg/kg bw per day. This treatment-related effect disappeared during the recovery period.

Treatment with CGA62826 was well tolerated, with no signs of overt toxicity. There was no indication that CGA62826 had neurotoxic potential. The changes in the liver indicated a weak hepatotrophic effect in rats due to the metabolite. However, in view of the minimal degree of these effects and their complete reversibility, no toxicological importance was attributed to these findings.

The NOAEL for CGA62826 (Metabolite 1) was l000 mg/kg bw per day; the highest dose tested (Frankhauser, 1997).

(e) Studies on metabolites – genotoxicity

Table 29. Genotoxicity of metabolites

End-point	Test object	Concentrations/doses tested	Purity	Results	Reference
	CGA108906; Met. 12; *N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)alanine				
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537; *E. coli* WP2*uvrA*	310–5000 µg/plate in DMSO	99%	Negative	Ogorek, 1997
Forward mutation	Chinese hamster V79 cells *hprt* locus	**Test 1** +S9: 74–2000 µg/mL –S9: 37–1000 µg/mL **Test 2** +S9: 56–1500 µg/mL –S9: 37–1000 µg/mL **Test 3** +S9: 400–1350 µg/mL –S9: 900–1200 µg/mL Solvent, DMSO	99%	Negative	Deparade, 1998
Forward mutation	Mouse lymphoma L5178Y, *tk* locus	±S9: 500–3000 µg/ml in DMSO		Negative	Clay, 2001
Chromosomal aberration	Chinese hamster ovary cells (V79 cells)	**With S9:** (1) 4 h exposure, harvesting 14 h later (2) 18 h exposure, direct harvesting (3) 28 h exposure, direct harvesting **Without S9:** (1) 4 h exposure, harvesting 14 h later (2) 4 h exposure, harvesting 24 h later All 750, 1500 and 3000 µg/mL in DMSO	99%	Negative	Czich, 2001

CGA62826; Met. 1; *N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)alanine					
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537; *E. coli* WP2*uvrA*	310–5000 µg/plate in DMSO	100%	Negative	Deparade, 1997
Forward mutation	Chinese hamster V79 cells *hprt* locus	**Test 1** +S9: 92–2500 µg/mL –S9: 150–4000 µg/ml **Test 2** ± S9: 111–3000 µg/mL In DMSO	100%	Negative	Ogorek, 1998

S9: Exogenous metabolic activation system from 9000 × *g* fraction of rat liver induced with Aroclor;
DMSO: Dimethyl sulfoxide; Positive control substances were used in all assays and gave the expected results

Based on available toxicological data on metabolite CGA108906 (Metabolite 12) and evidence that all other plant metabolites considered in the FAO evaluation for JMPR 2021 occur in rats, the Meeting concluded that the toxicity of these metabolites will be covered by the ADI and ARfD for the parent compound, metalaxyl.

3. Observations in humans

No data was provided. However, JMPR 2002 reported that no adverse effects have been revealed in routine medical examinations, including anamnesis, physical examination covering blood pressure and comprehensive blood and urine analysis, of employees who had handled metalaxyl-M since 1999 in laboratories, production and formulation plants or field trial projects. Apart from local dermal effects, no compound-related adverse effects on human health had been reported in workers involved in the production of metalaxyl between 1986 and 1994.

No cases of poisoning have been reported in the open literature involving workers in the production and formulation or field use of metalaxyl or metalaxyl-M.

Comments

Biochemical aspects

Studies of the biokinetics and metabolism of both metalaxyl and metalaxyl-M have been performed. Absorption, distribution and excretion of the two compounds were similar, and both were rapidly absorbed and eliminated after oral administration, with no potential for accumulation observed. In rats, maximum blood concentrations of radioactivity were detected 0.5–1 hour after administration for both metalaxyl and metalaxyl-M, irrespective of dose, except that the maximum concentrations for females in the higher dose group of metalaxyl occurred at 4 h (Muller, 1997). The decline in radioactivity was biphasic, with half-lives of l.5 h and 125 h in males, and 3 h and 96 h in females for the first and second phases, respectively (Uesugi, 1988). Rats eliminated 90–100% of the total administered dose of either substance within 72 hours, with the majority eliminated within 24 hours (Muller, 1997). The extent of urinary excretion of radioactivity was about 1.5 times higher in females than in males, whereas the faecal elimination extent was about 1.5-fold to 2-fold higher in males than in females (Hamboeck, 1977). The similarity in excretion pattern of radioactivity after oral and intravenous administration of metalaxyl indicates that the compound was well absorbed (Uesugi, 1988). In a study with bile duct-cannulated rats, elimination of metalaxyl in the bile was substantial. Approximately 66–71% of the administered dose (AD) was excreted in bile within 24 hours in males and females at the low dose of 2 mg/kg bw and in males at the high dose of 80 mg/kg bw. At this dose, excretion was 54.5% in females (Uesugi, 1988). Based on the biliary excretion study, oral absorption of metalaxyl is estimated to be greater than 90% within 24 hours, except in high-dose females where it is 70% (Uesugi, 1988). The concentration of

residues of both compounds in organs and tissues was about 0.01 ppm at seven days after the low dose. The pattern of distribution was similar at the higher dose but approximately 100-fold greater.

The total residues remaining after seven days at the low dose represented 0.16–0.55% of the AD for both metalaxyl and metalaxyl-M in males and females. The total residues remaining after seven days at the high dose amounted to 0.17–0.43% of AD for metalaxyl-M and metalaxyl in males and females (Muller, 1977). The terminal half-life for tissue depletion was variable, with the shortest estimate in the plasma and longest in the lungs, 1.0 day and 37.4 days respectively. The terminal half-lives for brain, heart, kidneys, liver, muscle, spleen and whole blood were 17.11, 24.11, 18.44, 18.35, 10.06, 24.12 and 7.41 days, respectively (Shaw & Montgomery, 2011).

Metalaxyl and metalaxyl-M were both extensively metabolized, showing a similar pattern of metabolites irrespective of sex or administered dose. Metabolism involved hydrolysis of side-chains and oxidation of the phenyl ring. Most of the phase I metabolites were excreted as conjugates with glucuronic acid and sulfate (Muller, 1997). Treatment with metalaxyl resulted in modest induction of hepatic and renal P450 enzymes and some other drug-metabolizing enzymes (Paolini et al., 1996; Uesugi, 1988).

Toxicological data

Median lethal dose (LD_{50}) values in rats treated orally with metalaxyl-M were 953 and 375 mg/kg bw for males and females, respectively (Schoch, 1994a). The LD_{50} value in rats after dermal application was > 2000 mg/kg bw (Schoch, 1994b). The median lethal concentration (LC_{50}) in rats treated by inhalation for four hours was >2.3 mg/L, the highest achievable concentration (Arts, 1995). Metalaxyl was nonirritating to the skin of rabbits but severely irritating to the eyes of rabbits (Marty, 1994a, b). It was negative for sensitization in Guinea pigs in Magnusson & Kligman and Buehler tests (Marty, 1994c; Glaza, 1995).

Metalaxyl-M and metalaxyl showed similar toxicological properties. A comparative 28-day study in rats given metalaxyl-M and metalaxyl by gavage confirmed the toxicological equivalence of the *R*-enantiomer and the racemate, as both the nature of their effects and their dose–effect relationships were similar. In a comparative toxicity study in rats via gavage for 28 days, the NOAEL was 300 mg/kg bw per day, the highest dose tested for metalaxyl and metalaxyl-M (Gerspach, 1994).

In studies with mice, rats and dogs treated orally, both substances had low toxicity, and treatment was well tolerated, even at relatively high doses. Dog, where red blood cells were the target, was the most sensitive species. Increased liver weights were observed in rats and dogs, but in this case the effect was considered not to be adverse.

In a 28-day toxicity study in rats via gavage, metalaxyl at escalating doses up to 600 mg/kg bw per day produced liver effects (increased absolute and relative liver weights) which were not considered adverse (Sachsse, 1979).

In a 90-day toxicity study with rats, metalaxyl-M was administered via the diet at concentrations of 0, 25, 50, 250, 625 or 1250 ppm (equal to 0, l.7, 3.5, 17, 45 and 91 mg/kg bw per day for males, 0, 1.9, 3.7, 18, 49 and 95 mg/kg bw per day for females). The NOAEL was 1250 ppm (equal to 91 mg/kg bw per day) the highest dose tested (Gerspach, 1995).

In a separate 90-day toxicity study in rats, metalaxyl was administered via the diet at a concentration of 0, 50, 250 or 1250 ppm (equal to 0, 3.2, 16 and 79 mg/kg bw per day for males, 0, 3.5, 18 and 86 mg/kg bw per day for females). The NOAEL was 1250 ppm (equal to 79 mg/kg bw per day) the highest dose tested. Liver cell hypertrophy observed at 1250 ppm was considered not to be adverse (Drake, 1977).

The toxicity of metalaxyl-M in dogs was investigated in a 13-week dietary administration study. Metalaxyl was tested in a six-month study with administration via the diet and in a two-year study with administration via capsules.

In a 90-day toxicity study in dogs, metalaxyl-M was administered via the diet at a concentration of 0, 50, 125, 250 or 1250 ppm (equal to 0, 1.6, 4.1, 7.3 and 39 mg/kg bw per day for males, 0, 1.6, 4.3, 7.9 and 40 mg/kg bw per day for females). The NOAEL was 1250 ppm (equal to 39 mg/kg bw per day)

the highest dose tested, as the increased alkaline phosphatase activity and increased liver weights seen at 1250 ppm were not considered adverse (Altmann, 1995).

In a six-month toxicity study in dogs, metalaxyl was administered via the diet at a concentration of 0, 50, 250 or 1000 ppm (equal to 0, 1.6, 7.8 and 31 mg/kg bw per day for males, 0, 1.7, 7.4 and 32 mg/kg bw per day for females). The NOAEL was 250 ppm (equal to 7.8 mg/kg bw per day), on the basis of decreases in red blood cell (RBC) count, haematocrit and haemoglobin in males (marginal but consistent throughout the study and also observed in a two-year toxicity study in dogs) at 1000 ppm (equal to 31 mg/kg bw per day) (Beck & deWard, 1981).

In a two-year toxicity study in dogs, metalaxyl was administered in gelatine capsules at a dose of 0, 0.8, 8 or 80 mg/kg bw per day. The NOAEL was 8 mg/kg bw per day based on RBC toxicity at the LOAEL of 80 mg/kg bw per day (Harada, 1984).

In a study of carcinogenicity in mice, metalaxyl was administered via diet for 104 weeks at a concentration of 0, 50, 250 or 1250 ppm (equal to 0, 4.5, 22.7 and 119 mg/kg bw per day for males, 0, 5.0, 21.5 and 132 mg/kg bw per day for females). The NOAEL for systemic toxicity was 1250 ppm (equal to 119 mg/kg bw per day) the highest dose tested. The NOAEL for carcinogenicity was 1250 ppm (equal to 119 mg/kg bw per day) the highest dose tested (McSheehy, Macrae & Whitney, 1980a, b).

In a study of chronic toxicity and carcinogenicity in rats, metalaxyl was administered for 105 weeks via the diet at a concentration of 0, 50, 250 or 1250 ppm (equal to 0, 1.7, 8.7 and 43 mg/kg bw per day for males, 0, 2, 10 and 55 mg/kg bw per day for females). The NOAEL for systemic toxicity was 1250 ppm (equal to 43 mg/kg bw per day) the highest concentration tested; the mild liver changes seen at this concentration were considered not to be adverse. The NOAEL for carcinogenicity was 1250 ppm (equal to 43 mg/kg bw per day) the highest dose tested (Ashby & Whitney, 1980a, b).

Since technical grade metalaxyl contains approximately 50% of the *R*-enantiomer, the results also apply to metalaxyl-M.

The Meeting concluded that metalaxyl and metalaxyl-M are not carcinogenic in mice or rats.

Metalaxyl was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found (Arni & Muller, 1982; Despande & Arni, 1985; Fritz, 1978a; Hertner & Arni, 1992; Hrelia et al., 1996; Ivett, 1986; Langauer & Muller, 1979; Perocco et al., 1995; Puri & Muller, 1982, 1985; Strasser & Muller, 1982).

The Meeting concluded that metalaxyl and metalaxyl-M are unlikely to be genotoxic.

In view of the lack of carcinogenicity in mice and rats, and that both compounds are unlikely to be genotoxic, the Meeting concluded that metalaxyl and metalaxyl-M are unlikely to pose a carcinogenic risk to humans.

In a three-generation study of reproductive toxicity in rats, metalaxyl was administered via the diet at concentrations of 0, 50, 250 or 1250 ppm This resulted in intakes of 4.1 (3.2–4.7), 21 (17–23) and 96 (84–100) mg/kg bw per day for males, and for females 4.6 (3.6–5.1), 24 (20–27) and 150 (100–240) mg/kg bw per day. The NOAEL for parental systemic toxicity, reproductive toxicity and offspring toxicity was 1250 ppm (equal to 96 mg/kg bw per day) the highest dose tested (Cozens, 1980).

In a developmental toxicity study in rats, metalaxyl-M was administered once daily by gavage at 0, l0, 50 or 250 mg/kg bw per day on gestation days (GDs) 6–15. The NOAEL for maternal toxicity was 50 mg/kg bw per day, on the basis of a reduction in body weight gain and feed consumption at the LOAEL of 250 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 250 mg/kg bw per day, the highest dose tested (Khalil, 1995).

In a separate developmental toxicity study in rats, metalaxyl was administered once daily by gavage at 0, 50, 250 or (initially) 575 mg/kg bw per day on GDs 6–15. Owing to maternal deaths, the highest dose was reduced to 500 and then to 400 mg/kg bw per day after two days of treatment. The NOAEL for maternal toxicity was 50 mg/kg bw per day, on the basis of a reduction in body weight gain at higher doses and clinical signs (including convulsions), the majority of which occurred at the beginning of the treatment period (GD 6 and/or GD 7) at 250 mg/kg bw per day. The NOAEL for

developmental toxicity was 400 mg/kg bw per day, the highest dose tested (Leng & Schardein, 1985).

In a developmental toxicity study in rabbits, metalaxyl was administered once daily by gavage at 0, 30, 150 or 300 mg/kg bw per day on GDs 7–19. The NOAEL for maternal toxicity was 150 mg/kg bw per day, on the basis of reductions in body weight gain and food consumption at the LOAEL of 300 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 300 mg/kg bw per day, the highest dose tested (Laughlin & Schardein, 1984).

The Meeting concluded that metalaxyl and metalaxyl-M are not teratogenic.

No neurotoxicity studies were available, however no evidence of neurotoxicity or neuropathology were observed in any of the systemic toxicity studies.

The Meeting concluded that metalaxyl and metalaxyl-M are unlikely to be neurotoxic.

No evidence of direct immunotoxic effects was observed in the available toxicity studies.

The Meeting concluded that metalaxyl and metalaxyl-M are unlikely to be immunotoxic.

Toxicological data on metabolites and/or degradates

The metabolism of metalaxyl and metalaxyl-M is similar in animals and plants. There appear to be three major plant metabolites of metalaxyl:

- *N*-(2-6-dimethylphenyl)-*N*-(methoxyacetyl)alanine (Met. 1; CGA62826),
- *N*-(2,6-dimethylphenyl)-*N*-(hydroxyacetyl)alanine (Met. 6; CGA107955) and
- *N*-(2-carboxy-6-methylphenyl)-*N*-methoxyacetyl)alanine (Met. 12; CGA108906)

Their acute toxicity was studied after oral administration. These metabolites showed little toxicity, with LD_{50} values > 2000 mg/kg bw (Hartmann, 1994; Sarasin & Gfeller, 1986; Winkler, 1996c, d).

NOAELs in 28-day studies were 1000 mg/kg bw per day, the highest doses tested for CGA108906 (Gerspach, 1997) and CGA62826 (Frankhauser, 1997).

Metabolites CGA108906 and CGA62826 had no mutagenic potential in vitro (Clay, 2001; Czich, 2001; Deperade, 1997, 1998; Ogorek, 1997, 1998).

Metabolites CGA108906 (Met. 12) and CGA62826 (Met. 1; found in rat metabolism study) are expected to be no more toxic than the parent compound based on available toxicity studies.

The Meeting concluded that the toxicity of plant and animal metabolites Met. 1, Met. 3, Met. 6, Met. 7, Met. 8 and Met. 12 would be covered by the acceptable daily intake (ADI) and acute reference dose (ARfD) established for metalaxyl.

Microbiological data

No data were available.

Human data

No cases of adverse effects were reported in personnel involved in the production and formulation of metalaxyl or metalaxyl-M or in the field use of these products.

The Meeting concluded that the existing database was adequate to characterize the potential hazards of metalaxyl and metalaxyl-M to fetuses, infants and children.

Toxicological evaluation

The Meeting re-affirmed the group ADI of 0–0.08 mg/kg bw for metalaxyl and metalaxyl-M (alone or in combination) established by the 2002 Meeting on the basis of the NOAEL of 8 mg/kg bw per day in the two-year toxicity study in dogs, based on red blood cell toxicity at the LOAEL of 80 mg/kg bw per day, and using a safety factor of 100.

The Meeting established a group ARfD of 0.5 mg/kg bw for metalaxyl and metalaxyl-M (alone or in combination) on the basis of the NOAEL of 50 mg/kg bw per day observed in the developmental toxicity study in rats with metalaxyl, based on ataxia, tremors, hypoactivity and hypothermia, and using a safety factor of 100 (Leng & Schradein, 1985). In addition, clinical signs of toxicity were observed consistently in acute oral toxicity studies and after the first dose in a number of repeat-dose toxicity studies, with a LOAEL of around 200 mg/kg bw. The ARfD applies to all age groups.

Levels relevant to risk assessment of metalaxyl and metalaxyl-M

Species	Study	Effect	NOAEL	LOAEL
Mouse	104-week study of toxicity and carcinogenicity[a]	Toxicity	1250 ppm, equal to 119 mg/kg bw per day[d]	-
		Carcinogenicity	1250 ppm, equal to 119 mg/kg bw per day[d]	-
	Two-year study of toxicity and carcinogenicity[a]	Toxicity	1250 ppm, equal to 43 mg/kg bw per day[d]	-
		Carcinogenicity	1250 ppm, equal to 43 mg/kg bw per day[d]	-
Rat	Three-generation study of reproductive toxicity[a]	Reproductive toxicity	1250 ppm, equal to 96 mg/kg bw per day[d]	-
		Parental toxicity	1250 ppm, equal to 96 mg/kg bw per day[d]	-
		Offspring toxicity	1250 ppm, equal to 96 mg/kg bw per day[d]	-
	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[d]	-
Rabbit	Developmental toxicity study[b]	Maternal toxicity	150 mg/kg bw per day	300 mg/kg bw per day
		Embryo/fetal toxicity	300 mg/kg bw per day[d]	-
Dog	Six-month studies of toxicity[a]	Toxicity	250 ppm, equal to 7.8 mg/kg bw per day	1000 ppm, equal to 31 mg/kg bw per day
	Two-year study of toxicity[c]	Toxicity	8 mg/kg bw per day	80 mg/kg bw per day[d]
Metabolite CGA 62826 (M-1)				
Rat	Four-week study of toxicity[b]	Toxicity	1000 mg/kg bw per day[d]	
Metabolite CGA 108906 (M-12)				
Rat	Four-week study of toxicity[b]	Toxicity	1000 mg/kg bw per day[d]	

[a] Dietary administration [b] Gavage administration [c] Capsule administration [d] Highest dose tested

Acceptable daily intake (ADI); group ADI for metalaxyl and metalaxyl-M (alone or in combination) and for M-1, M-3, M-6, M-7, M-8 and M-12

0–0.08 mg/kg bw

Acute reference dose (ARfD); group ARfD for metalaxyl and metalaxyl-M (alone or in combination) and for M-1, M-3, M-6, M-7, M-8 and M-12

0.5 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 metalaxyl and metalaxyl-M

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	About 70% excreted within 24 hours; >90% oral absorption based on biliary excretion study
Dermal absorption	No data provided
Distribution	Widely distributed
Potential for accumulation	None
Rate and extent of excretion	95–100% after 72 hours; slightly higher urinary excretion for females
Metabolism in animals	Extensive metabolism via hydrolysis of the methyl ester and methyl ether groups; oxidation of the 2-(6)-methyl group; oxidation of the phenyl ring; *N*-dealkylation. Conjugation to glucuronides and sulfates
Toxicologically significant compounds in animals and plants	Parent compound
Acute toxicity	
Rat, LD_{50}, oral	375 mg/kg bw
Rat, LD_{50}, dermal	>2000 mg/kg bw
Rat, LC_{50}, inhalation	>2.3 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Severely irritating
Guinea pig, dermal sensitization	Not sensitizing (Magnusson & Kligman and Buehler)
Short-term studies of toxicity	
Target/critical effect	Red blood cells
Lowest relevant oral NOAEL	8 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	1000 mg/kg bw per day, highest dose tested (rat and rabbit)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	None
Lowest relevant NOAEL	34 mg/kg bw per day, highest dose tested (rat)
Carcinogenicity	Not carcinogenic in mice and rats[a]
Genotoxicity	No evidence of genotoxicity[a]
Reproductive toxicity	
Target/critical effect	No reproductive effects were observed
Lowest relevant parental NOAEL	96 mg/kg bw per day, highest dose tested (rat)
Lowest relevant offspring NOAEL	96 mg/kg bw per day, highest dose tested (rat)
Lowest relevant reproductive NOAEL	96 mg/kg bw per day, highest dose tested (rat)
Developmental toxicity	
Target/critical effect	Not teratogenic in rats and rabbits (clinical signs, reduced body weight gains and mortality)
Lowest relevant maternal NOAEL	50 mg/kg bw per day (rat)
Lowest relevant embryo/fetal NOAEL	250 mg/kg bw per day, highest dose tested (rat)

Neurotoxicity	
Acute neurotoxicity NOAEL	No data
Subchronic neurotoxicity NOAEL	No data
Developmental neurotoxicity NOAEL	No data
Immunotoxicity	No data
Mechanism studies	Weak inducer liver, kidney and lungs
Studies on toxicologically relevant metabolites	
CGA 108906 (M-12)	
Acute oral LD_{50}	> 2000 mg/kg bw (rat)
28-day toxicity	1000 mg/kg bw per day, highest dose tested (rat)
Genotoxicity	Negative (in vitro)
CGA 62828 (M-1)	
Acute oral LD_{50}	> 2000 mg/kg bw (rat)
28-day toxicity	1000 mg/kg bw per day, highest dose tested (rat)
Genotoxicity	Negative (in vitro)
CGA 107955 (M-6)	
Acute oral LD_{50}	> 3000 mg/kg bw (rat)
Microbiological data	Not applicable
Human data	No adverse effects on health of manufacturing personnel

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

Summary

	Value	Study	Safety factor
ADI[a]	0–0.08 mg/kg bw	Two-year study of toxicity (dog)	100
ARfD[a]	0.5 mg/kg bw[a]	Developmental toxicity study (rat)	100

[a] Applies to metalaxyl and metalaxyl-M (alone or in combination), and to M-1, M-3, M-6, M-7, M-8 and M-12)

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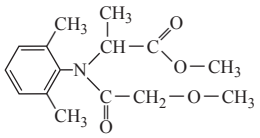
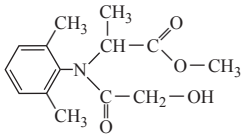
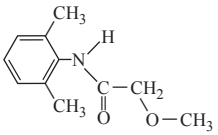
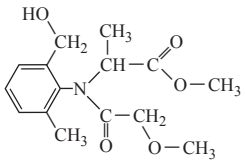
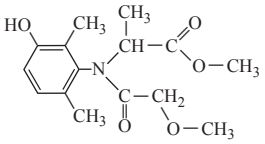
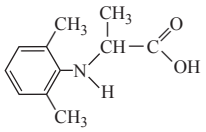
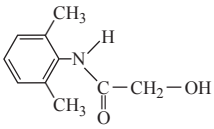
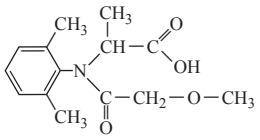
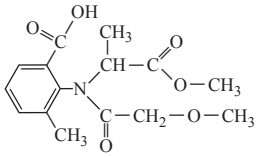
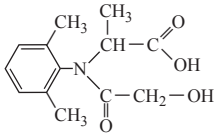
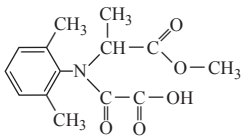
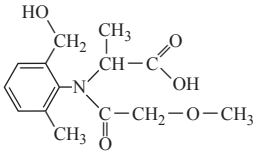
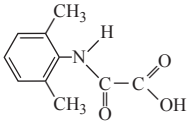
Appendix 1. Identification of [*phenyl*-^{14}C]metalaxyl metabolites in rats

Metabolite	Structure
CGA48988 Metalaxyl *N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)alanine methyl ester	
CGA67869 Metabolite 3 *N*-(2,6-dimethylphenyl)-*N*-(hydroxyacetyl)alanine methyl ester	
CGA67868 Metabolite 10 *N*-(2,6-dimethylphenyl)-2-methoxyacetamide	
CGA94689: Isomers A and B Metabolite 8 *N*-[(2-hydroxymethyl)-6-methylphenyl]-*N*-(methoxy acetyl)alanine methyl ester	
CGA100255; *meta*-hydroxyl isomers; Metabolite 7 *N*-(3-hydroxy-2,6-dimethylphenyl)-*N*-(methoxyacetyl)alanine methyl ester	
CGA67867 Metabolite 11 *N*-(2,6-dimethylphenyl)alanine	
CGA37734 Metabolite 5 *N*-(2,6-dimethylphenyl)-2-hydroxyacetamide	
CGA62826 Metabolite 1 *N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)alanine	
CGA108905: mixed isomers Metabolite 9 2-[(methoxyacetyl)(2-methoxy-1-methyl-2-oxo-ethyl) amino]-3-methyl benzoic acid	
CGA107955 Metabolite 6 *N*-(2,6-dimethylphenyl)-*N*-(hydroxyacetyl)alanine	

Metabolite	Structure
CGA79353 Metabolite 4 *N*-(carboxycarbonyl)-*N*-(2,6-dimethylphenyl)alanine methyl ester	
Metabolite 2 *N*-[(2-hydroxymethyl)-6-methylphenyl]-*N*-(methoxyacetyl)alanine	
CGA68124 Metabolite 14 [(2,6-dimethylphenyl)amino]oxoacetic acid	

PYRASULFOTOLE

First draft prepared by
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Explanation

Pyrasulfotole is the ISO-approved common name for (5-hydroxy-1,3-dimethyl-1*H*-pyrazol-4-yl)(α,α,α-trifluoro-2-mesyl-*p*-tolyl)methanone (IUPAC), Chemical Abstracts Service number 365400-11-9.

The primary mode of action (MOA) of pyrasulfotole is as an inhibitor of the enzyme 4-hydroxyphenylpyruvate dioxygenase (4-HPPD) in susceptible plants, thereby disrupting the synthesis of carotenoids that are produced by plants to protect against oxidative and photolytic damage.

Pyrasulfotole 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 guidelines, unless otherwise specified. No additional information from a literature search was identified that complemented the toxicological information submitted for the current assessment.

Overall, the Meeting considered that the database was adequate for the risk assessment.

Evaluation for acceptable intake

1 Biochemical aspects

1.1 Absorption, distribution and excretion

In a preliminary study (Totis, 2002) the toxicokinetics and metabolism of pyrasulfotole were investigated following a single oral administration by gavage to male and female Wistar rats at a nominal dose of 100 mg/kg body weight (bw) of [*phenyl-U-*^{14C}]pyrasulfotole. Metabolite profiling and identification were performed in order.

The experiment demonstrated that elimination of radiolabel via urine was greater than via faeces for both sexes. In males urine:faeces was 74.71%:11.55%, in females 84.39%/8.00%. Therefore there was no sex-related difference in the route of elimination. At 72 hours post dosing, tissue residues were very low; the mean recovery of radioactivity in the animals' tissues accounted for 0.27% of administered dose (AD) for the males and 0.37% AD for the females. The tissue concentrations of [^{14C}]pyrasulfotole equivalents found in the male and female tissues demonstrated that the highest concentration of radioactive residues was present in the liver (1.336 µg equiv./g for males and 1.587 µg equiv./g for females).

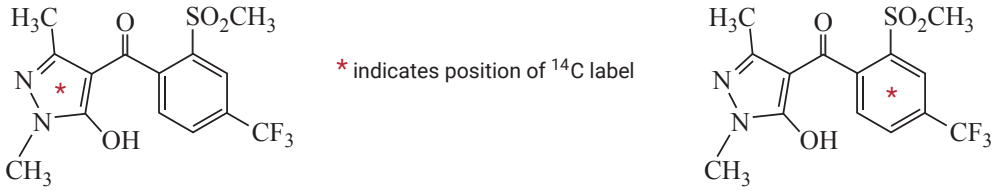
In both sexes the only other tissues possessing detectable radioactive residues were the kidneys (0.424 µg equiv./g in males and 0.844 µg equiv./g in females), the gastrointestinal tracts and contents (0.230 µg equiv./g for both males and females) and the carcass (0.215 µg equiv./g in males, 0.310 µg equiv./g in females). These results indicated that the distribution of radioactivity was very similar in males and females, with the liver consistently containing the highest mean concentration. All other sampled tissues of both sexes (brain, fat, testes, ovaries, cardiac blood, plasma and muscle) presented levels of radioactivity below the limit of detection at 72 hours after dosing.

Blood radioactivity concentrations were determined following a single oral administration of [^{14}C]pyrasulfotole to male and female rats at the nominal dose level of 100 mg/kg bw. The results obtained indicated a sex difference in the maximum mean whole blood concentration for pyrasulfotole, the maximum concentration (C_{max}) value being higher for males (75.48 µg equiv./g) than females (56.84 µg equiv./g). The absorption of radioactivity was very rapid, with mean time to maximum concentration (T_{max}) interpolated values of 0.5 h for both sexes. The elimination half-life ($t_{½\ elim}$) values based upon the terminal data points also indicated a slight sex difference; the values obtained being around four hours for males and around six hours for females. The estimated area under the concentration–time curve (AUC) indicated a higher bioavailability of the radiolabel in males (80.54 µg hour/g) than in females (58.35 µg hour/g).

Pyrasulfotole was found not to be extensively metabolized in the rat with only five radioactive fractions in urine and just four radioactive fractions in faecal extracts. Moreover pyrasulfotole itself was observed to be the major radioactive component in the urine and faeces, representing ca 82% and 89% of the total excreted radioactivity in males and females respectively.

In the main study the absorption, distribution, metabolism and elimination (ADME) of total radioactivity was investigated in male Wistar Hanover rats (five rats for each radiolabel) following a single oral administration of 10.0 mg/kg bw of [*phenyl-UL-*^{14}C]pyrasulfotole or 9.88 mg/kg bw of [*pyrazole-3-*^{14}C]pyrasulfotole.

Figure 1. Chemical structure of radiolabelled pyrasulfotole



[*Pyrazole-3*-^{14}C]pyrasulfatole

[*Phenyl-UL*-^{14}C]pyrasulfatole

Additionally in two separate experiments, male Wistar Hanover rats with surgically implanted jugular cannulae were dosed intravenously with [*phenyl-UL*-^{14}C]pyrasulfotole (four rats) or [*pyrazole-3*-^{14}C]pyrasulfotole (five rats) at dose rates of 9.81 and 9.60 mg/kg bw respectively to investigate the absorption of pyrasulfotole.

Throughout all the experiments urine and faeces were collected in separate containers cooled with dry ice. The weight of urine at each interval was measured and aliquots radio-assayed. Each faecal sample was weighed, homogenized and aliquots of the faecal homogenate were oxidized and radio-assayed. Rats were housed in metabolism cages equipped with a flow-through system which allowed for the separation and collection of respired gases (^{14}C-labelled carbon dioxide and all other volatile compounds) for radiolabels from both sources for just the oral dose experiments. Sampling was maintained for the first 24 hours only. For the oral dose experiment with [*phenyl-UL*-^{14}C]pyrasulfotole sampling was continued until 52 hours post dose; for all other experiments sampling finished at 48 hours post dose. Following completion of each experiment, each metabolism cage was washed with methanol/water, and the washes from each cage were separately radio-assayed. Collection containers for urine and faeces were also washed with methanol/water, and the combined washes from these containers for each rat were radio-assayed. At the conclusion of each experiment, individual rats were humanely killed and triplicate blood samples taken and tissues/organs processed for radio-assay. Identification and quantitation of the metabolites in the urine samples and faecal extracts were accomplished using reverse phase high-performance liquid chromatography (HPLC) and liquid chromatography with tandem mass spectrometry (LC–MS–MS).

Absorption

The [*phenyl-UL*-^{14}C]pyrasulfotole was readily absorbed following oral dosing, with 62% of the dose recovered in the urine within six hours and a total of 73% of the dose recovered in the urine at 52 hours, the time of sacrifice. Following intravenous dosing with [*phenyl-UL*-^{14}C]pyrasulfotole 10% of the dose was found in the faeces at the time of sacrifice (48 hours).

The [*pyrazole-3*-^{14}C]pyrasulfotole was also readily absorbed following oral dosing, with 57% of the dose recovered in the urine within six hours and a total of 75% of the dose recovered in the urine at 48 hours, the time of sacrifice. Following intravenous dosing with [*pyrazole-3*-^{14}C]pyrasulfotole, 8% of the dose was found in the faeces at the time of sacrifice (48 hours).

Distribution

In all experiments, < 2% of AD remained in the carcass and tissues at sacrifice, and the highest levels of residue were found in the liver and kidney.

Table 1. Distribution of radioactivity in male rat tissues/organs after administration of [phenyl-UL-^{14}C]- or [pyrazole-3-^{14}C]-labelled pyrasulfotole at ca 10 mg/kg bw via oral or intravenous dosing[a]

Tissue/organ	Pyrasulfotole residue levels; group means shown (µg equiv./g tissue)			
	[*phenyl-UL*-^{14}C] label		[*pyrazole-3*-^{14}C] label	
	Oral	Intravenous	Oral	Intravenous
Bone	0.004	0.006	0.012	0.006
Brain	0.003	0.005	0.006	0.002
Carcass	0.014	0.064	0.026	0.116
Fat	0.005	0.004	0.018	0.006
Gastrointestinal tract	0.052	0.078	0.098	0.047
Heart	0.006	0.005	0.011	0.005
Kidney	0.326	0.346	0.410	0.353
Liver	1.770	1.538	1.793	1.660
Lung	0.014	0.014	0.021	0.013
Muscle	0.006	0.006	0.009	0.006
Skin	0.010	0.018	0.023	0.014
Spleen	0.005	0.005	0.015	0.005
Testes	0.005	0.004	0.008	0.004
Thyroid	0.016	0.013	0.028	0.005

[a] Reported results are averages from all rats from the dosing group for the experiment Source: Fischer & Roensch, 2005

Excretion

Following dosing with [*phenyl-UL*-^{14}C] or [*pyrazole-3*-^{14}C]pyrasulfotole, by either oral or intravenous routes, excretion was rapid (96–111% of AD was excreted within 24 hours). Mass balances for the oral and intravenous experiments were all within an acceptable range (100–108% of AD). Irrespective of route or label, the majority of the radioactivity was excreted in the urine (73.1–90.7%), with less excreted in the faeces (8.0–32.0%). Much of the AD (56.8–84.1%) was rapidly excreted in the urine within six hours of dosing.

Table 2. Recovery of radioactivity in tissues and excreta of male rats after administration of [phenyl-UL-^{14}C]- or [pyrazole-3-^{14}C]-labelled pyrasulfotole at ca 10 mg/kg bw via oral or intravenous dosing[a]

Sample	Radioactive dose recovered (% of administered dose; group means)			
	[*phenyl-UL*-^{14}C] label		[*pyrazole-3*-^{14}C] label	
	Oral	Intravenous	Oral	Intravenous
Expired air	<0.1	NA	<0.1	NA
Tissues	1.0	0.8	1.0	1.0
Carcass	0.1	0.4	0.2	0.8
Cage wash[b]	2.3	2.0	2.5	3.1
Urine				
0 to 6 hours	62.4	82.7	56.8	84.1
6 to 12 hours	8.2	1.8	15.2	3.6
12 to 24 hours	1.3	1.5	2.5	1.8
24 to 48 hours	1.1	0.7	0.5	1.2
48 to 52 hours	0.1	NA	NA	NA

Sample	Radioactive dose recovered (% of administered dose; group means)			
	[*phenyl-UL*-^{14}C] label		[*pyrazole-3*-^{14}C] label	
	Oral	Intravenous	Oral	Intravenous
Total for urine	73.1	86.6	74.9	90.7
Faeces				
0 to 24 hours	30.0	9.6	31.1	7.4
24 to 48 hours	1.2	0.8	0.8	0.6
48 to 52 hours	<0.1	NA	NA	NA
Total for faeces	31.2	10.4	32.0	8.0
Total	**108**	**100**	**111**	**106**

NA = Not applicable; Source: Fischer & Roensch, 2005

[a] Reported results are averages of all rats from the dosing group for the experiment

[b] Cage wash includes the rinses from the metabolism cages, the urine and faeces collection containers

1.2 Biotransformation

In the main study described above in section 1.1, all individual residues which accounted for > 5% of AD were identified, as were some components present in smaller quantities. Identification of the residues was accomplished by comparison of the mass-spectral data to that of authentic reference standards when available. Some residues were tentatively identified by comparison of their HPLC retention times to those of authentic reference standards, or components which had been identified in other matrices.

Table 3. Metabolite profile in excreta of male rats after administration of [phenyl-UL-^{14}C]- or [pyrazole-3-^{14}C]-labelled pyrasulfotole at ca 10 mg/kg bw via oral or intravenous dosing

Sample	Radioactive dose recovered (% of AD; group means)			
	[*phenyl-UL*-^{14}C] label		[*pyrazole-3*-^{14}C] label	
	Oral	Intravenous	Oral	Intravenous
Pyrasulfotole	89.7	86.7	95.4	90.0
Pyrasulfotole-hydroxymethyl	2.0	1.5	2.3	1.4
Pyrasulfotole-desmethyl (AE 1073910)	8.1	6.4	8.0	6.2
AE-B197555 (RPA 203328)	1.4	1.4	ND	ND
Total identified	101	96.1	106	97.6
U-1	ND	ND	ND	<0.1
U-2	ND	ND	1.1	0.1
Expired air	<0.1	NA	<0.1	NA
Tissues	1.0	0.8	1.0	1.0
Carcass	0.1	0.4	0.2	0.8
Cage wash	2.3	2.0	2.5	3.1
Faeces extracts and extracted solids	0.8	0.9	0.3	0.2
Faeces samples not extracted	1.2	0.8	0.8	0.6
Total unidentified[a]	5.5	5.0	6.0	5.8
Total accounted for	106	101	112	103

Source: Fischer & Roensch, 2005

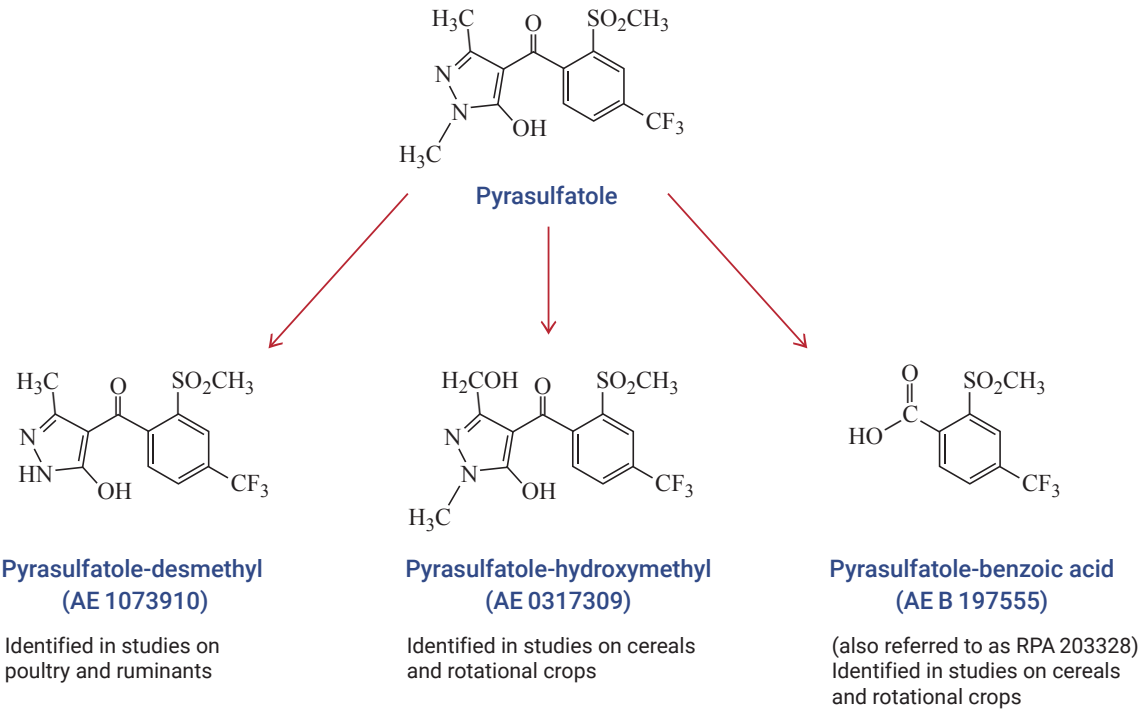
[a] Includes U-1, U-2, expired air, tissues, carcass, cage cash, faeces extracts and extracted solids, plus faeces samples not extracted

From all experiments, 87% to 95% of AD was excreted unchanged as pyrasulfotole.

Pyrasulfotole-hydroxymethyl, pyrasulfotole-desmethyl, and AE B197555 (RPA 203328) were observed as minor metabolites in the urine and faeces. Greater than 96% of AD in each experiment was identified. The major metabolic pathway occurred via *N*-demethylation of pyrasulfotole.

The proposed metabolic pathway of pyrasulfotole in the rat is shown in below in Fig. 2.

Figure 2. Proposed metabolic pathways of pyrasulfotole in rats



Notes: Pyrasulfotole-desmethyl (Bayer code AE 1073910) has been identified in studies in poultry and ruminants. Pyrasulfotole-hydroxymethyl (Bayer code AE 0317309) has been identified in studies in cereals and rotational crops. Pyrasulfotole-benzoic acid (Bayer code AE-B197555 also referred to as RPA 203328) has been identified in studies in cereals and rotational crops. (Fischer & Roensch, 2005)

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

Regarding acute toxicity for pyrasulfotole, only an acute oral toxicity study was submitted. Since no acute toxicity was observed in this study and JMPR evaluation is undertaken with reference to consumer exposure, these limited data were considered acceptable. The result of the acute oral toxicity study is summarized in Table 4.

Table 4. Study of acute the toxicity of pyrasulfotole

Species	Strain	Sex	Route	Purity	LD_{50}	Reference
Rat	Wistar	Female	Oral	95.7%	>5000 mg/kg bw[a]	Schüngel, 2004

LD_{50} Median lethal dose.

[a] No clinical signs, effects on body weight or pathological findings were observed

2.2 Short-term studies of toxicity

(a) Oral administration

Mouse

Study 1

In an exploratory 14-day toxicity study, pyrasulfotole (purity 99.0%) was administered orally by gavage to C57BL mice, in groups of five males and five females, at dose levels of 0, 100, 300 or 1000 mg/kg bw per day for 14 days. Clinical signs were recorded daily, body weights were recorded on days 1, 7 and 14 and food consumption was measured on days 7 and 14. Blood samples were taken before necropsy for plasma chemistry determinations. All surviving animals were necropsied, selected organs weighed and a range of tissues were taken, fixed and examined microscopically.

Three mice treated at 1000 mg/kg bw per day were found dead (two females on days 4 and 7, and one male on day 14). One of the females showed prostration, reduced motor activity and absence of grasping reflex on the day before death. No clear cause of death could be established for the females. Histopathological examination of the decedent male revealed blood in the trachea and the alveoli of the lung, possibly suggesting a gavage error. The only clinical signs observed were, at 1000 mg/kg bw per day, reduced motor activity in two females for several days at the end of the second week of treatment. The overall body weight evolution and food consumption of the animals were unaffected by treatment.

In clinical chemistry, a dose-related statistically significant increase in the mean total cholesterol concentrations was observed in both sexes at 300 and 1000 mg/kg bw per day (+25% and +64% males, +26% and +62% in females, compared to the controls). In addition, a lower mean alkaline phosphatase (ALP) activity was seen in both sexes at 1000 mg/kg bw per day (–34% and –42% compared to controls for males and females respectively). Mean absolute and relative kidney weights were higher in females treated at 1000 mg/kg bw per day (+19% for absolute weight and +15% for relative weight compared to controls). No clear effect on kidney weight was observed in the males. Mean absolute and relative liver weights compared to controls were higher in males (+11%) and in females (+14% absolute, +16% relative) treated at 1000 mg/kg bw per day. No treatment-related macroscopic findings were noted in either premature decedents or animals sacrificed at study termination. Histopathological examination of the premature decedents revealed treatment-related kidney lesions, specifically multifocal cortical tubuloepithelial degeneration and/or multifocal basophilic tubules. Treatment-related lesions found during histopathological examination of the animals sacrificed at study termination were seen in the kidney and in the liver. In the kidney, multifocal basophilic (regenerative) tubules, were observed in both sexes at 1000 and 300 mg/kg bw per day; in females this effect was also seen at 100 mg/kg bw per day. The change was slightly increased in severity at 1000 mg/kg bw per day, when compared to the other dosed groups. Associated with this change, in a proportion of males and females at 1000 and 300 mg/kg bw per day, cortical tubules were multifocally dilated. The renal lesions in decedent and surviving mice appear to indicate an effect on the tubular epithelium, with early degeneration (seen in decedents at days 4 and 7) and later regeneration. In the liver, a slight diffuse centrilobular hepatocellular hypertrophy was seen in all surviving males at 1000 mg/kg bw per day.

The NOAEL for this study could not be determined based on effects in the kidneys (multifocal cortical tubuloepithelial degeneration and/or multifocal basophilic tubules) which were seen at all dose levels (Langrand-Lerche, 2001a).

Study 2

In a 28-day toxicity study, pyrasulfotole (purity 97.4%) was administered in the diet to ten C57BL/6J mice/sex per dose at dose levels of 0, 200, 1000 or 5000 ppm (equal to 0, 35.8, 192 and 961 mg/kg bw per day in males, 0, 45.0, 233 and 1082 mg/kg bw per day in females). Clinical signs were monitored twice daily on weekdays and once daily on weekends and holidays. Body weight and food consumption were measured weekly throughout the study. Animals were fasted overnight prior to necropsy and blood was collected for clinical chemical analysis (total bilirubin, urea, total protein, albumin, total cholesterol, aspartate aminotransferase, alanine aminotransferase and ALP sctivities). Selected organs were weighed, and organs/tissues were preserved for histopathological examination.

There were no mortalities during the study and no clinical signs considered related to treatment. No significant effect on mean body^weight was noted during the study, although a lower cumulative body^weight gain compared to the control group was observed at 5000 ppm on week 4 (reductions of 12.1% for males and 15.2% for females compared to controls). No significant effects on food consumption were observed during the study. A slight reduction (13.3% lower than controls) was observed for females at 5000 ppm during study week 4 only. This was largely due to one animal in the group that showed a lower than average food consumption during this period, and may also be influenced by one female in the control group with a higher than average food consumption.

There were no alterations in any clinical chemistry parameters in either males or females at any dose level. There were no treatment-related alterations in organ weights in either males or females. Gritty content was found in the urinary bladder of two males at 5000 ppm: similar findings were noted in repeat-dose studies with rats and dogs. Analysis of this material in the urinary tract revealed high concentrations of pyrasulfotole. In three males at 5000 ppm, examination of the urinary bladder revealed diffuse urothelial hyperplasia, diffuse submucosal granulation tissue, and diffuse suburothelial mixed cell infiltrate. Two of these three animals displayed gritty contents in the urinary bladder at necropsy. Pale livers were noted in females at 5000 ppm (5/10 females), but this was considered not to be of toxicological concern as there were no histopathological correlates and there was no clear dose–response relationship.

The NOAEL was 1000 ppm (equal to 192 mg/kg bw per day) based on gritty content in the urinary bladder and histopathological findings including urothelial hyperplasia in the urinary bladder seen in males only, at 5000 ppm (equal to 961 mg/kg bw per day) (McElligott, 2002).

Study 3

In a 90-day toxicity study, pyrasulfotole (purity 95.7%) was administered continuously to C57BL/6J mice (10/sex per dose) via the diet at 0, 100, 750, 1500 or 3000 ppm (equal to 0.0, 16.5, 124, 259 and 500 mg/kg bw per day in males, 0.0, 19.7, 152, 326, and 617 mg/kg bw per day in females) . Animals were checked twice daily on weekdays and once daily on weekends /holidays. Body weight and food consumption were measured at weekly intervals throughout the study. Prior to necropsy, blood samples for clinical chemistry were taken following an overnight fast. Urine samples were collected overnight on the night prior to blood sampling and necropsy. All animals were diet-fasted, but one group of mice was also water-fasted in error. Following gross necropsy selected organs were weighed, and organ and tissue samples preserved for histopathological examination.

One male mouse at 100 ppm was sacrificed for humane reasons on day 29. Prior to sacrifice the mouse had shown reduced motor activity, prostration and wasting. Necropsy revealed a marked hydrocephalus which was considered to be a spontaneous developmental defect and not related to treatment. There were no other mortalities.

There were no treatment-related clinical signs in either males or females. There were no effects on body weight, body weight gain or food consumption. There were no treatment-related changes in any clinical chemistry parameters. Urine pH was increased with statistical significance at 3000 ppm in females (pH was 6.3 compared to 6.0 in controls), but no statistical analysis was performed on male data due to the small number of values obtained. There were no treatment-related effects of pyrasulfotole on organ weights. No treatment-related changes were observed on gross or histopathological examinations.

The NOAEL for dietary administration of pyrasulfotole in male and female mice was 3000 ppm, the highest dose tested (equal to 500 mg/kg bw per day in males and 615 mg/kg bw per day in females) (Steiblen, 2003).

Rat

In an exploratory 14-day toxicity study, pyrasulfotole (purity 99.0%) was administered to Wistar rats (5/sex per group) in the diet at dose levels of 0, 400, 2000 or 7500 ppm for fourteen days (equal to 0.0, 27.4, 135 and 497 mg/kg bw per day in males, 0.0, 32.0, 157, and 547 mg/kg bw per day in females). A satellite subgroup of three rats per sex was added to the high-dose and control groups for interim sacrifice after three days of treatment. The satallite groups were just used to assess liver weight, hepatic cellular proliferation and liver histopathology. Animals were observed daily for mortality and clinical signs. Body weight was recorded on days 1, 7, 14 and before necropsy, and food consumption was measured weekly. Ophthalmological examination was performed on all animals before the start of treatment and in the control and top-dose groups at the end of the study (days 13 and/or 14). Blood samples were taken before final necropsy for haematology and clinical chemistry determinations. At study termination, brain, kidney, liver, ovaries, spleen, testes and thyroid gland were weighed; duodenum, kidney, liver, ovaries, spleen, testes and thyroid gland were collected from the five remaining males and females in each group (day 15). Liver, kidney, spleen, ovaries, testes and thyroid gland were examined microscopically in the high-dose and control groups. Hepatic cellular proliferation was assessed using a specific immunostaining technique. The remaining portions of the liver were homogenized for

microsomal preparations in order to determine their cytochrome P-450 isoenzyme profile.

There were no mortalities and no clinical signs. Body weight was slightly (but with statistical significance) decreased in males at 400 and 7500 ppm during the first and second weeks of treatment; at the mid dose no effect on body weight was observed. Therefore, in the absence of a dose-related effect the body weight effect was not considered to be treatment-related. Body weight gain was decreased by 59% compared to controls (statistically significant) in high-dose males after one week. At this time point, decreases in body weight gain were also seen in mid- and low-dose males, however, without an apparent dose–response realionship and without reaching statistical significance. After 14 days no significant changes in body weight gain were seen in males, even though the high-dose group showed a decrease of 29% compared to controls. In females no effect on body weight gain was seen, either on day 7 or on day 14. Food consumption was unaffected in males; in females at 7500 ppm food consumption was decreased only during the first week. No changes were observed at the ophthalmological examination. No treatment-related effects were observed in haematology. Total cholesterol was increased in females in all dose levels, reaching statistical significance at the mid and high doses (values: 1.52, 2.00, 2.17* and 2.29* at 0, 400, 2000 and 7500 ppm respectively). No effects on clinical chemistry were observed in males. In males, relative kidney weight was significantly increased in all dose groups, by 11–12% compared to controls, however, without a dose–response relationship. No effect on absolute kidney weight was seen. In females no effect was seen on organ weights. No effect was seen on gross pathology or histopathology in either sex. Regarding hepatoxicity testing, the total cytochrome P-450 content was not affected, enzyme activities were not altered (benzoxyresorufin-*O*-dealkylase, pentoxyresorufin-*O*-deethylase, lauric acid hydroxylation), and no effect on hepatocellular proliferation was found.

The NOAEL was 2000 ppm (equal to 135 mg/kg bw per day) based on decreased body weight gain in males at 7500 ppm (equal to 497 mg/kg bw per day) (Langrand-Lerche, 2001b).

In a 90-day toxicity study, pyrasulfotole (purity 97.4%) was administered continuously via the diet at 0, 2, 30, 1000, 7000, or 12 000 ppm (equal to 0.0, 0.13, 1.96, 66, 454, and 830 mg/kg bw per day for males and 0.0, 0.15, 2.32, 77, 537, 956 mg/kg bw per day in females) to Wistar rats (10/sex per dose). Cage-side observations were conducted twice daily on weekdays and once daily on weekends, detailed clinical examinations were conducted at least weekly during the study. Neurological (reflex) examinations were carried out in the week preceding the initiation of compound administration and during week 12 of the study. Body weight was measured during acclimatization, on the first day of the feeding period, and weekly for the remainder of the study. Food consumption was measured on a weekly basis. Ophthalmoscopic examinations were carried out prior to the start of the study and in weeks 2, 4, 8 and 12. Blood was collected from overnight-fasted animals in the last week of the study for haematology and clinical chemistry parameters, and urine was also collected from fasted animals in week 13 for urinalysis. All animals were subjected to macroscopic examination, and tissues were collected for histological examination. Organs were weighed only in those animals surviving until terminal sacrifice.

At 7000 ppm, one male was found dead on day 8, one male was sacrificed for humane reasons on day 70, and one male was killed for humane reasons on day 83. At 12 000 ppm, one female was sacrificed on day 13 for humane reasons. Four males in this treatment group were found dead (days 15, 41, 45 and 72), and two males were killed for humane reasons (days 43 and 64), prior to unscheduled termination of the remaining males in this treatment group on day 72. No deaths occurred in other treatment groups. Based on early mortality at 12 000 ppm and on histological findings found at 7000 ppm at terminal sacrifice, histological examination was not conducted on either sex at 12 000 ppm. Therefore the top dose group at which histological examination was performed was 7000 ppm.

At 12 000 and 7000 ppm in both sexes treatment-related clinical signs were yellow (intense) colored urine, few or no faeces, wasted appearance, coldness to touch, piloerection, reduced motor activity, laboured respiration, hunched posture, increased salivation and soiling around the mouth. At 1000^ ppm coloured urine was also noted for all males on a few days and one female presented a white area on the eyes. There were no effects noted at examination of reflexes in week 12.

Body weight was decreased in both males and female at 7000 ppm and 12 000 ppm, occasionally achieving statistical significance, with a decrease in body weight gain greater than 10% in females. There were occasional slight decreases in food consumption in both males and females at 7000 and

12 000 ppm, but there was no alteration in food consumption at concentrations of l000 ppm or below.

Ophthalmological examinations revealed corneal opacity (snowflake appearance) associated with neovascularization of the cornea.

There were no biologically significant effects on haematological parameters at any dose in either males or females. In males, bilirubin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), urea, and creatinine were increased at 7000 ppm, while cholesterol and triglycerides were increased at 1000 and 7000 ppm. Alkaline phosphatase was decreased at 1000 ppm and above when compared to controls. Very few of these parameters showed any treatment-related response in females.

At urinalysis, ketone levels were increased at 1000 ppm and above in both males and females. This was considered likely to be due to detection of the diketone structure of pyrasulfotole, as a significant proportion of the parent molecule is excreted unchanged in the urine. The pH was significantly lower in 1000 and 7000 ppm males and in 1000, 7000 and 12 000 ppm females. There was an increased incidence of occult blood, erythrocytes, leukocytes, and epithelial cells in the urine in both males and females at 7000 ppm and in females at 12 000 ppm (but males in the 12 000 ppm group did not survive until the end of the study so urine had not therefore been collected for analysis).

Mean absolute and relative liver and kidney weights were significantly increased, in a biological and statistical sense, in both males and females at 1000 ppm and above.

Table 5. Results in male and female rats administered pyrasulfotole in the diet for 13 weeks

	Dose group (ppm)					
	0	**2**	**30**	**1000**	**7000**	**12 000**
Ophthalmology				**Males**		
	0	0	0	0	2	1
				Females		
	0	0	0	1	0	4
Body weight and body weight gain (g)						
				Males		
Body weight: week 0	203	202	203	202	202	201
week 2	309	305	310	302	280*	270**
week 3	346	342	349	342	318	300**
week 7	454	455	462	459	433	396**
week 13	517	510	532	524	489	ND
Body weight gain (% of control)	314 (100)	308 (98.1)	329 (104.8)	323 (102.9)	287 (91.4)	ND
				Females		
Body weight: week 0	128	176	174	174	174	174
week 2	(100)	224	218	216	201**	204*
week 3	234	236	230	233	216**	220
week 13	303	306	301	301	286	281
Body weight gain (% of control)	128 (100)	130 (101.6)	127 (99.2)	127 (99.2)	112 (87.5)	108* (84.4)
Clinical chemistry						
				Males		
Bilirubin, µmol/L	1.9	2.0	1.5	1.8	2.3	ND
ASAT, IU/ L	54	88	55	56	110	ND
ALAT, IU/L	27	62	24	32	54	ND
ALP, IU/L	79	85	69	63*	63	ND

	Dose group (ppm)					
	0	**2**	**30**	**1000**	**7000**	**12 000**
Urea, mmol/L	4.88	5.08	5.26	5.01	7.89	ND
Creatinine, µmol/L	33	34	36	34	48	ND
Cholesterol, mmol/L	1.82	2.14	1.94	2.64**	2.74**	ND
Triglycerides, mmol/L	0.82	1.02	0.81	1.74**	1.38**	ND
				Females		
Bilirubin (µmol/L)	2.0	2.3	2.1	1.8	2.0	1.8
ASAT (IU/L)	49	48	54	55	58	50
ALAT (IU/L)	22	23	22	23	22	24
ALP (IU/L)	48	43	46	47	47	48
Urea (mmol/L)	5.33	5.19	5.10	4.64	6.00	7.98
Creatinine (µmol/L)	35	38	37	34	40	51
Cholesterol (mmol/L)	1.99	3.04	1.98	2.29	2.39	2.59*
Triglycerides (mmol/L)	0.60	0.99	0.63	0.91	0.81	0.69
Organ weights (g)						
				Males		
Liver, absolute [% change from control]	11.6	11.7	12.1	14.2** [+22]	13.4** [+15.5]	ND
Liver, relative [% change from control]	2.35	2.39	2.36	2.84** [+20.8]	2.87** [+22]	ND
Kidney, absolute [% change from control]	2.82	2.66	2.83	2.95 [+5]	3.59* [+27]	ND
Kidney, relative [% change from control]	0.572	0.543	0.555	0.591 [+3]	0.785** [+37]	ND
				Females		
Liver, absolute	6.7	6.9	6.9	7.3	7.1	6.9
Liver, relative [% change from control]	2.30	2.35	2.40	2.54** [+10.4]	2.59** [+12.6]	2.52* [+9.6]
Kidney, absolute	1.81	1.89	1.83	1.96	2.16	2.22
Kidney, relative [% change from control]	0.625	0.644	0.636	0.681 [+9]	0.792* [+26.7]	0.819 [+31]

ND: No data; this group was sacrificed on humane grounds on day 72; Source: Langrand-Lerche, 2003

ASAT: Aspartate aminotransferase; ALAT: Alanine aminotransferase; ALP: Alkaline phosphatase

* $p < 0.05$, ** $p < 0.01$

From gross necropsy of the male rats either found dead or sacrificed prior to the end of the study (three at 7000 ppm and 10 at 12 000 ppm), the principal cause of death was considered to be related to calculi in the urinary tract. Treatment-related macroscopic findings seen in these animals included dilation of the renal pelvis and gritty content therein, obviously large kidneys, pale or mottled colour of the kidneys, and foci on the kidneys, and red or gritty content in the urinary bladder, distension of the urinary bladder, and red foci in the bladder, along with an obviously large liver. Other findings which were less clearly related to treatment included dilation, dark content, and black foci of the stomach, soiled fur, and dark content in the intestines.

In the surviving animals, treatment-related macroscopic findings were seen at 1000 ppm and above. In both males and females from 1000 ppm, these findings included corneal opacities, abnormal shape of the kidneys, mottled kidneys, dilation of the renal pelvis and gritty contents therein, gritty content and distension of the urinary bladder, and gritty content of the ureters. A few females at 7000

and 12 000 ppm were noted with obviously small kidneys, and one female at 12 000 ppm showed gritty content in the urethra. A few males had obviously large livers at 1000 ppm, with prominent lobulation at 1000 and 7000 ppm. One male had an enlarged thyroid at l000 ppm. Microscopic pathology was not conducted on the 12 000 ppm as this was found to be above the maximum tolerated dose (MTD) in the rat. Additionally, as 30 ppm was observed to be free of treatment-related effects, histopathological examination was not conducted on the 2 ppm dose group. The treatment-related changes observed in animals surviving at 90 days were seen in the urinary tract and the liver in both sexes, and the eyes and thyroid gland in males.

Table 6. Treatment-related macroscopic and microscopic findings in male and female rats surviving to the end of the 90-day study

Organ	Finding	Dose group (ppm)					
		0	**2**	**30**	**1000**	**7000**	**12 000**
Macroscopic findings							
					Males		
Number of animals		10	10	10	10	7	ND
Eye(s)	Opacity	0	0	0	0	2	ND
Kidneys	Abnormal shape	0	0	0	0	1	ND
	Pelvic dilatation	0	1	0	0	2	ND
	Mottled	0	0	0	0	2	ND
	Pelvis: gritty content	0	0	0	0	4	ND
Liver	Obviously large	0	0	0	3	0	ND
	Prominent lobulation	0	1	0	2	1	ND
Thyroid gland	Obviously large	0	0	0	1	0	ND
Urinary bladder	Gritty content	0	0	0	0	4	ND
	Distended	0	0	0	0	1	ND
Ureter(s)	Gritty content	0	0	0	0	3	ND
					Females		
Number of animals		10	10	10	10	10	9
Eye(s)	Opacity	0	0	0	1	0	2
Kidneys	Abnormal shape	0	0	0	0	2	2
	Pelvic dilatation	0	0	0	0	4	2
	Obviously small	0	0	0	0	2	2
	Mottled	0	0	0	0	1	1
	Pelvis: gritty content	0	0	0	0	4	4
Urinary bladder	Gritty content	0	0	0	0	1	2
	Distended	0	0	0	0	1	1
Ureter(s)	Gritty content	0	0	0	0	5	4
Urethra	Gritty content	0	0	0	0	0	1
Microscopic findings							
					Males		
Number of animals		10		10	10	7	ND
Eye(s)	Keratitis, mixed cellular, focal, unilateral	0		0	0	2	ND
Liver	Hepatocellular hypertrophy, centrilobular, diffuse	0		0	9	6	ND

Organ	Finding	Dose group (ppm)					
		0	2	30	1000	7000	12 000
Kidney(s)	Urolithiasis, pelvis	0		0	0	1	ND
	Urothelial hyperplasia, simple, diffuse	0		0	1	1	ND
	Urothelial hyperplasia, simple, focal/multifocal	0		0	0	4	ND
	Dilated renal pelvis, unilateral	0		1	2	2	ND
	Dilated renal pelvis, bilateral	0		0	0	2	ND
	Interstitial fibrosis, multifocal to diffuse	0		0	0	3	ND
	Dilated tubules, cortical, focal/multifocal	0		0	1	3	ND
	Urolithiasis	0		0	0	2	ND
Urinary bladder	Urothelial hyperplasia, simple, multifocal to diffuse	0		0	0	4	ND
Thyroid gland	Follicular cell hypertrophy/ hyperplasia, diffuse	0		0	5	2	ND
	Loss of colloid, diffuse	0		1	9	5	ND
Ureter(s)	Urolithiasis	0		0	0	3	ND
	Urothelial hyperplasia, simple, diffuse	0		0	0	2	ND
	Ureteritis, mixed cellular, diffuse	0		0	0	1	ND
					Females		
Number of animals		10		10	10	10	ND
Liver	Hepatocellular hypertrophy, centrilobular, diffuse	0		0	0	1	ND
	Periportal vacuolation, hepatocellular, diffuse	0		0	3	8	ND
Kidney(s)	Urolithiasis, pelvis	0		0	0	3	ND
	Urothelial hyperplasia, simple, diffuse	0		1	0	5	ND
	Urothelial hyperplasia, simple, focal / multifocal	1		1	1	2	ND
	Dilated renal pelvis, unilateral	0		0	0	5	ND
	Dilated renal pelvis, bilateral	0		1	0	0	ND
	Interstitial fibrosis, multifocal to diffuse	0		0	0	2	ND
Urinary bladder	Urothelial hyperplasia, simple, multifocal to diffuse	1		1	0	4	ND
Ureter(s)	Urolithiasis	0		0	0	4	ND
	Urothelial hyperplasia, simple, diffuse	0		0	0	5	ND
	Ureteritis, mixed cellular, diffuse	0		0	0	2	ND
	Serosal mixed cellular infiltrate, local	0		0	0	2	ND

ND: No data; this group was sacrificed on humane grounds on day 72; Source: Langrand-Lerche, 2003

The NOAEL for dietary administration of pyrasulfotole was 1000 ppm (equal to 66 mg/kg bw per day) based on mortality, clinical signs, increased liver and kidney weight, effects on the eyes, macroscopic findings in the bladder and ureter, histopathological findings in kidney, bladder and ureter at 7000 ppm (equal to 454 mg/kg bw per day) (Langrand-Lerche, 2003). Please note that eye effects are further discussed in section 2.6(c) mechanistic studies.

Dog

Study 1

In a 28-day toxicity study, pyrasulfotole (purity 98.1%) was administered continuously via the diet at 0, 5000, 13 000 or 26 000 ppm to groups of three beagle dogs/sex per dose (equal to 0, 174, 469 and 860 mg/kg bw per day in males, 0, 171, 440, and 782 mg/kg bw per day in females).

Mortality and clinical signs were checked daily. Body weight and food consumption were measured weekly. Weekly physical examinations were conducted during the study, as well as a detailed neurological examination. Ophthalmic examinations were performed on all animals during the acclimatization period and at the end of the treatment period. Overnight urine samples were collected six days prior to dosing and on day 25 for urinalysis. Blood was collected four days prior to dosing and on day 28 prior to feeding for haematology and clinical chemistry. Macroscopic examination of all organs and tissues were conducted, selected organs weighed and samples of organs and tissues taken for histopathological examination.

There were no mortalities during the course of the study. There were no treatment-related clinical signs in males at any dietary concentration of pyrasulfotole. In females, treatment-related clinical signs were limited to intense yellow urine at 26 000 ppm, observed after two weeks treatment on two occasions for one female and several occasions for a second female.

There was no effect of dietary pyrasulfotole administration on body weight in either male or female dogs. There was no effect on food consumption in males at any dose. In females, food consumption was slightly reduced at 13 000 and 26 000 ppm as compared to controls (–16% and –9%, respectively). There were no treatment-related ophthalmological findings in either males or females. In two out of three males at 26 000 ppm, the activated partial thromboplastin time (APTT) was slightly lower than in controls (–15% and –18% respectively). Triglyceride concentrations were increased in both sexes in a dose-related manner, at all doses. In females at 26 000 ppm, ALP activity was increased compared to pre-test values. Urinalysis revealed no treatment-related changes in either males or females at any dose.

Liver weights were increased to a biologically significant extent in both males and females, although in females this did not show a dose–response relationship. Macroscopic examinations revealed two males and three females at 26 000 ppm with gritty material in the kidney and renal pelvis at necropsy. One male and one female at this dose also had urinary stone(s) in the urinary bladder. One male at 13 000 ppm had urinary stone(s) and congestion in the urinary bladder. There were no other treatment-related findings. Microscopic findings were limited to the kidney in both males and females and were likely a consequence the urinary stones seen at gross necropsy.

Table 7. Results from the 28-day study in dogs administered pyrasulfotole

	Dose group (ppm)							
	Males				Females			
	0	**5000**	**13 000**	**26 000**	**0**	**5000**	**13 000**	**26 000**
Clinical chemistry (day 26)								
Triglyceride (mmol/L) [% of pre-test value]	0.34 [–11%]	0.72 [+36%]	0.91 [+72%]	1.23 [+151%]	0.74 [+9%]	0.98 [+63%]	1.27 [+95%]	1.95 [+298%]
ALP (IU/L) [% of pre-test value]	88 [–22%]	118 [+27%]	210 [+150%]	185 [+108%]	109 [–14%]	168 [+66%]	150 [+36%]	257 [+59%]

	Dose group (ppm)							
	Males				Females			
	0	5000	13 000	26 000	0	5000	13 000	26 000
Organ weights								
Terminal bw (kg)	8.21	7.78	8.02	8.37	8.54	8.51	8.14	8.17
Brain (g)	71.7	73.5	70.7	73.2	72.7	74.3	73.5	69.5
Liver, absolute (g) [% diff. from control]	286.3	331.0 [+16%]	417.7 [+46%]	438.5 [+53%]	314.5	363.1 [+16%]	398.9 [+27%]	367.1 [+17%]
Liver, relative to bw [% diff. from control]	3.5	4.3 [+23%]	5.2 [+49%]	5.2 [+49%]	3.7	4.3 [+16%]	4.9 [+32%]	4.5 [+22%]
Liver/brain weight [% diff. from control]	399.2	450.6 [+12%]	592.1 [+48%]	601.5 [+50%]	431.9	492.0 [+15%]	553.9 [+29%]	533.5 [+24%]
Histopathological findings in kidneys								
Number of animals	3	3	3	3	3	3	3	3
Inclusions:								
intracytoplasmic, globular, eosinophilic, tubular, multifocal	0	0	1	2	0	0	0	0
Infiltrate, sub- and intra-urothelial, mixed cell, multifocal	0	0	0	2	0	0	1	2
Mineralization, pelvic, multifocal	0	0	0	1	0	0	0	1
Erosion, urothelial, focal	0	0	0	1	0	0	0	0

ALP: Alkaline phosphatase; bw: Body weight; Source: Kennel, 2002

Dietary administration of pyrasulfotole to beagle dogs at doses of up to 26 000 ppm resulted in effects on the liver (increased weight and changes to clinical chemistry) and kidneys (gritty content and/or stones in the urinary tract, with associated histopathological findings). In addition, intense yellow urine in two females at 26 000 ppm was noted lon imited occasions during the latter half of the study. The NOAEL was less than 5000 ppm (equal to < 171 mg/kg bw per day) based on clinical chemistry findings at all dose levels tested (Kennel, 2002).

Study 2

In a non-GLP investigative 29-day study, pyrasulfotole (purity 95.7%) was administered continuously via the diet at 0, 1500, 9000 or 18 000 ppm to groups of four beagle dogs/sex per dose (test substance intakes not reported). The animals were approximately 4–6 months old at the start of the dosing period. Clinical signs were monitored at least once a day, with twice-daily checks for moribundity and morbidity. Body weights were measured weekly and food consumption measured on a daily basis. Ophthalmological examinations were conducted on all animals prior to the beginning of dosing and prior to termination of the study. Animals were fasted overnight prior to blood collection for clinical chemistry and haematology, during acclimatization and during study week 3. Urine was collected over a 24-hour period ending on study day 24. The study was intended to be a 90-day exposure study, however, because of excessive, unexpected toxicity the study was terminated after 29 days. Following sacrifice, the dogs were subjected to gross necropsy. Gross lesions, liver and any eyes with ophthalmological abnormalities were preserved, but no histopathological studies were carried out.

One male dog at 9000 ppm was euthanized for humane reasons on study day 23, while two males at 18 000 ppm were euthanized on study day 28 in a moribund condition. The remainder of the animals were euthanized on study day 29. Red coloration was noted in the urine of some male dogs in all dose groups. In the three males sacrificed prior to the termination of the study, decreased activity, reduced food consumption and lethargy were noted. The 9000 ppm male sacrificed on day 23 lost 605 g during

the study. One of the 18000 ppm males sacrificed on day 28 lost 694 g prior to sacrifice. Other than these two animals, there were no effects on body weight.

With the exception of the three animals sacrificed early, there was no effect on food consumption. The 9000 ppm male stopped eating on day 14 prior to sacrifice on day 23. One of the 18 000 ppm males ate 1–10 g of food on study days 12–14 and showed markedly decreased food consumption on study days 22, 23, and 25–27. The other 18 000 ppm male showed markedly decreased food consumption on study days 22, 23, and 25–27.

There were no treatment-related ophthalmological findings.

There was no effect on haematological parameters in either males or females. Urinalysis revealed blood in the urine which was increased (statistically significant) on study day 24 in all male treatment groups. Urinary protein was elevated to a biologically significant extent at 9000 and 18 000 ppm in males due to the one or two dogs (respectively) at these doses that were euthanized early. One female at 9000 ppm also showed increased urinary protein. In high-dose females there was a significant decrease in urine creatinine on study day 24.

The 9000 ppm dog euthanized on study day 23 was found to be dehydrated and to have multiple yellow calculi in its urethra, bloody urine in the bladder, bilateral enlarged kidneys, bilateral moderate renal pelvis dilatation, and other urinary tract findings. One of the 18 000 ppm males sacrificed on study day 28 was found to have yellow calculi in its urethra, bilateral dark red kidney capsule with haemorrhage in the sub-capsule, bilateral moderate renal pelvis dilatation, bilateral dark gray/discoloured/necrotic renal pelvis, and a discoloured/dark red urinary bladder with a very thick wall. The other 18 000 ppm male was found to have multiple calculi in its urinary bladder, bilateral marked dilated renal pelvis, bilateral dilated ureter, bloody urine in the bladder, and a urinary bladder with a very thick wall. There were no gross observations in non-moribund animals.

The dietary administration of pyrasulfotole to male beagle dogs produced precipitation of pyrasulfotole in the urinary tract, with the precipitate forming into urinary tract stones. It was considered that the moribundity, clinical signs, and necropsy findings in the moribund animals were likely to be due to the presence of these urinary tract stones. Although no stones were observed in females, one female at 9000 ppm did show increased urinary protein. Based on the lack of data in the report, a NOAEL cannot be derived (Eigenberg, 2004).

Study 3

In a 90-day study in the dog, pyrasulfotole (purity 95.7%) was administered continuously via the diet at 0, 100, 500 or 1000 ppm to groups of four beagle dogs/sex per dose (equal to 0, 3, 17 and 40 mg/kg bw per day in males, 0, 3, 17, and 33 mg/kg bw per day in females). The animals were approximately 6–7 months old at the start of the dosing period. Clinical signs were monitored at least twice daily. Body weights were measured on a weekly basis, and food consumption was determined daily. Ophthalmological examinations were conducted prior to the first day of dosing and just prior to the end of the study. Blood was collected from fasted animals prior to the beginning of the study and again in weeks 4, 8, and 13 for clinical chemistry and haematological determinations. The period of urine collection was 24 hours. At the end of the study the dogs were subjected to gross necropsy, selected organs weighed, and organ and tissue samples were collected for histopathological examination.

There were no mortalities or clinical signs of toxicity. There were no treatment-related clinical signs in either males or females. There were no effects on body weight, body weight gain or food consumption. There were no treatment-related changes in any clinical chemistry or urine parameter. A significant decrease on day 28 in creatine phosphokinase activity was noted in females of all dose groups, but not indicating any dose–response relationship. A slight decrease in AST was noted in low- and mid-dose females on day 28. These effects were not dose-dependent and were not considered compound-related. There were no ophthalmological findings at any dose level which could be considered treatment-related. There were no treatment-related haematological findings at any dose level. There were no treatment-related effects on organ weights due to pyrasulfotole. A significantly increased relative adrenal weight was found in males in the mid- and high-dose groups, which is considered incidental as there was no dose–response relationship, the magnitude of the difference was slight and there was no statistical difference in absolute adrenal weights. No treatment-related changes were observed on gross or histopathological examinations.

The NOAEL for this study was 1000 ppm, the highest dose tested (equal to 33 mg/kg bw per day), in the absence of any adverse treatment-related findings (Eigenberg, 2005).

Study 4

In a one-year toxicity study, pyrasulfotole (purity 95.7%) was incorporated into diet and administered for one year to groups of four male and four female beagle dogs at doses of 0, 250, 1000 or 3000 ppm (equal to 0, 7, 34, and 101 mg/kg bw per day in males, 0, 9, 33, and 93 mg/kg bw per day in females). The animals were 7–8 months old at the start of the study. Body weights were measured on a weekly basis and immediately prior to necropsy, and food consumption was determined daily. Clinical signs were monitored at least twice daily on weekdays and once daily on weekends and holidays. Ophthalmological examinations were conducted prior to the start of the study and just before the end of the study. Blood for haematology and clinical chemistry was drawn after overnight fasting once during the prestudy acclimatization phase and in weeks 13, 26, 39 and 52. Urine was collected overnight at these same times. At the end of the study the dogs were sacrificed and subjected to a gross necropsy. Selected organs were weighed, and organs and tissues collected for microscopic examination.

There were no mortalities or treatment-related clinical signs of toxicity. There were no effects on body weight, body weight gain or food consumption.

There were no treatment-related alterations in clinical chemistry parameters in either males or females in any treatment group. There were occasional differences from controls which were judged not to be related to administration of pyrasulfotole for various reasons, including a lack of consistency over time, comparable values within the same treatment group in the pre-treatment period as well as during the study, coverage by historical control data, or marked alteration in one animal only which led to an unrepresentative alteration in the group mean.

Table 8. Selected findings in male and female beagle dogs administered pyrasulfotole in thediet for one year

	Dose group (ppm)							
	Males				Females			
	0	**250**	**1000**	**3000**	**0**	**250**	**1000**	**3000**
Absolute organ weights (g)								
Terminal body weight	9769.9	11259.5	10273.8	10734.8	8771.0	9085.0	9843.3	9658.8
Liver weight [% change from control]	259.00	340.85 [+32%]	321.17 [+24%]	381.48 [+47%]	242.40	281.60 [+16%]	299.65 [+24%]	362.05 [+49%]
Kidney weight [% change from control]	50.720	62.121 [+22%]	62.791 [+24%]	69.975 [+38%]	39.815	43.778 [+10%]	43.173 [+8%]	47.262 [+19%]
Thyroid weight [% change from control]	0.782	0.938 [+20%]	0.982 [+26%]	0.901 [+15%]	0.719	0.769 [7%]	0.771 [+7%]	0.937 [+30%]
Organ weights, relative to body weight (%)								
Terminal bw (kg) [% change from control]	2.65	2.99 [+13%]	3.12 [+18%]	3.56 [+34%]	2.771	3.102 [+12%]	3.055 [+10%]	3.766 [+36%]
Brain (g) [% change from control]	0.526	0.547 [+4%]	0.610 [+16%]	0.665 [+26%]	0.454	0.483 [+6%]	0.438 [-4%]	0.492 [+8%]
Liver, absolute (g) [% change from control]	0.0081	0.0085 [+5%]	0.0096 [+18%]	0.0084 [+4%]	0.0082	0.0084 [+2%]	0.0079 [-4%]	0.0097 [+18%]
Histopathology								
Liver, hepatocytomegaly	0	0	0	2	0	0	0	3
Kidney, dilatation [average severity] [a]	1 [1.0]	1 [1.0]	3 [2.0]	4 [2.0]	0	0	0	0

[a] Severity ranked from 1 (minimal) to 5 (severe); * $p < 0.05$ Source: Eigenberg, 2006a

There were no treatment-related alterations in urinalysis parameters in any group. There were occasional alterations which were judged to be not related to administration of pyrasulfotole for various reasons, including a lack of consistency over time, comparable values within the same treatment group in the pretreatment period as well as during the study, coverage by historical control data, or marked alteration in one animal only which led to an unrepresentative alteration in the group mean.

No ophthalmological findings at any dose level were considered treatment-related. There were no treatment-related haematological findings at any dose level. There were occasional alterations which were judged not to be related to administration of pyrasulfotole for various reasons, including a lack of consistency over time, comparable values within the same treatment group in the pre-treatment period as well as during the study, coverage by historical control data, or marked alteration in one animal only which led to an unrepresentative alteration in the group mean. Treatment-related changes in organ weights were noted for liver, thyroid, and kidney at 3000 ppm only, specifically increased absolute and relative liver weight in males and females, increased absolute and relative kidney weight in males, and increased absolute and relative thyroid weight in females.

There were no observations at gross necropsy which were considered to be related to administration of pyrasulfotole. In the liver of male and female dogs at 3000 ppm there was an increased incidence of centrilobular and/or midzonal hepatocytomegaly. Minimal to moderate dilatation of the proximal convoluted tubules was present in male kidneys from the 1000 and 3000 ppm dose groups. Proteinaceous material (coded as debris) was present in males at all dose levels but the severity grade increased in the dilated tubules of the animals administered 1000 and 3000 ppm pyrasulfotole. There was no indication of tubular necrosis or cast formation.

The NOAEL for this study was 250 ppm for males (equal to 7 mg/kg bw per day) based on increased incidence and severity of tubular dilation in the kidneys accompanied by increased kidney weight seen at 1000 ppm (equal to 34 mg/kg bw per day) (Eigenberg, 2006a).

(b) Dermal application

No study available.

(c) Exposure by inhalation

No study available.

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

In a mouse chronic toxicity/carcinogenicity study, pyrasulfotole (purity 95.7%) was incorporated into rodent diet and administered at concentrations of 0, l00, 1000 or 4000 ppm to groups of 60 male C57BL/6J mice for up to 78 weeks. Groups of 60 females received 0, 100, 1000 or 6000 ppm for the first 11 weeks of the study. In due course, mortality at 6000 ppm was considered to be excessive and this dose was decreased to 4000 ppm from week 12 onwards. These concentrations resulted in doses equal to 0, 13.6, 137 and 560 mg/kg bw per day for males and 0, 16.7, 168 and 713 mg/kg bw per day for females. Ten animals/sex per dose were assigned to satellite toxicity groups for sacrifice at 52 weeks.

Body weights were measured on the first day of treatment, weekly for the first 13 weeks of the study, and then at four-week intervals until the end of the study. Clinical signs and mortality were monitored twice daily on weekdays and daily on weekends and holidays. Food consumption was recorded weekly during the first 13 weeks of treatment and once every four weeks thereafter. Ophthalmological examinations were conducted on all animals during the acclimatization phase and on all surviving animals at the end of the study. At 3, 6, 9, and 12 months, ophthalmoscopy was conducted on 24 animals per sex and treatment group. At the end of the one-year treatment period, blood was collected for haematology from the surviving animals in the one-year sacrifice group, and from the first 10 surviving animals of the carcinogenicity group. At the end of the study (78 weeks), blood was collected from the first 20 surviving animals of the terminal sacrifice group. At both time points, blood was collected after overnight fasting. At the 12-month and 78-week sacrifices gross necropsy was conducted, selected organs weighed, and organ/tissue samples were taken for histopathological examination.

There was a treatment-related increase in mortality in males at 4000 ppm and females at 6000↓4000 ppm. There were a number of treatment-related clinical signs seen in these animals, most likely related to the excretion of parent compound in the urine. Treatment-related clinical signs included hardness in the area of the urinary bladder, soiled fur, reduced motor activity, laboured or rapid respiration and red urine.

Body weight and body weight gain were reduced in males at 4000 ppm and females at 6000↓4000. Although terminal body weights compared to controls were unaffected in males at 1000 ppm, there were statistically significant reductions in body weight gain compared to controls at a number of time points with these males. This contradiction was probably a result of the males in the 1000 ppm treatment group being slightly heavier at the beginning of the study compared to concurrent controls, therefore the decreased body weight gain over the course of the study resulted in similar terminal body weights. Overall, it was considered that there had been a treatment-related effect on male body weights at 1000 ppm.

There was no effect of treatment on food consumption at any dose level. There were no ophthalmological findings at any dose level. Red cell count, haemoglobin (Hb), hematocrit (Ht), and mean corpuscular Hb concentration were decreased in females at 6000↓4000 ppm at 18 months, with decreases in most parameters also seen at 12 months. Mean corpuscular volume was slightly increased in females at 4000 ppm at 18 months. In males at 4000 ppm, similar haematological effects were generally only observed at 18 months.

Table 9. Selected findings in the mouse administered pyrasulfotole in the diet for 78-weeks

	Dose group (ppm)							
	Males				Females			
	0	**100**	**1000**	**4000**	**0**	**100**	**1000**	**4000**
Mortality (% of unscheduled animal deaths)								
Up to day 367	5%	7%	10%	23%	8%	7%	10%	35%
Up to end of study	14%	16%	16%	50%	30%	16%	16%	60%
Clinical signs								
Soiled fur, either general or localised	1	0	2	43	2	0	3	9
Reduced motor activity	1	0	2	8	3	1	3	12
Prostration	1	0	0	5	0	0	1	3
Tremors	0	1	1	4	0	1	0	1
Red urine	0	0	0	4	0	0	0	1
Laboured respiration	0	0	0	4	0	0	0	2
Rapid respiration	1	0	1	0	0	0	0	4
Cold to touch	1	0	0	6	3	0	1	5
Abdomen distended	0	1	1	9	0	1	1	3
Hardness in the urinary bladder area	0	0	0	52	0	1	0	33
Body weight (g)								
Day 1	20.9	21.3	21.4	21.3	17.8	17.8	17.8	17.9
Day 8	21.8	21.8	24.9	21.5	17.9	17.7	17.6	17.5
Day 92	27.4	27.9	27.5	26.8	22.2	22.6	22.5	22.3
Day 176	29.7	30.2	29.8	28.7**	24.4	24.7	24.3	24.0
Day 372	32.2	32.5	31.5	29.9**	27.3	27.3	27.7*	26.8
Day 540	32.0	32.5	32.0	29.4**	28.0	27.8	27.4	26.5**

	Dose group (ppm)							
	Males				Females			
	0	100	1000	4000	0	100	1000	4000
Body weight gain (g) [% change from control]								
Days 1–8	0.9	0.6** [–33]	0.5** [–45]	0.3** [–67]	0.1	–0.1	–0.2	–0.4**
Days 1–92	6.5	6.7	6.1* [–7]	5.5** [–15]	4.4	4.8	4.7	4.4
Days 92–176	2.4	2.3	2.3	1.9** [–21]	2.0	2.1	1.7*	1.7
Days 176–344	2.4	2.4	1.7** [–29]	1.3** [–46]	3.0	2.6	3.5	2.8
Days 344–540	–0.3	0.0	0.6**	–0.4	0.5	0.4	–0.2*	–0.2
Days 1–540	11.0	11.3	10.7	8.0** [–27]	10.0	9.9	9.6	8.6*
Haematology								
RBC count (10^{12}/L)								
12 months	9.60	9.53	9.46	9.21	9.50	9.44	9.40	9.00
18 months	9.85	9.79	9.61	9.14*	9.17	8.97	8.82	7.70**
Haemoglobin (g/dL)								
12 months	14.2	14.3	14.2	14.0	14.5	14.4	14.5	13.6**
18 months	13.9	14.2	13.7	13.0	13.8	13.6	13.2	11.6**
Haematocrit (l/l)								
12 months	0.471	0.472	0.470	0.463	0.481	0.478	0.478	0.461*
18 months	0.448	0.455	0.448	0.428	0.441	0.435	0.429	0.385**
MCV (fl)								
12 months	49	50	50	50**	51	51	51	51
18 months	46	47	47	47**	48	49	49	50**
MCH (pg)								
12 months	14.7	15.0	15.0*	15.2**	15.3	15.3	15.5	15.1
18 months	14.1	14.5*	14.3	14.2	15.1	15.1	15.0	15.1
MCHC (g/dL)								
12 months	30.1	30.3	30.2	30.2	30.1	30.1	30.4	29.5**
18 months	30.9	31.1	30.6	30.2*	31.4	31.2	30.8	29.9**
WBC count (10^9/L)								
12 months	3.3	3.2	3.2	2.1*	3.2	3.5	3.7	3.7
18 months	2.8	2.2	2.4	2.1	2.6	3.0	3.0	24.3
Organ weights; 12-month sarcifice (g)								
Terminal body weight	27.6	28.5	26.5	26.1	22.3	23.4	23.3	22.9
Brain weight, absolute	0.460	0.463	0.456	0.450	0.470	0.477	0.76	0.463
Kidney weight, absolute	0.419	0.432	0.402	0.762*	0.342	0.347	0.358	0.376
Kidney weight, relative to body weight	1.52	1.52	1.52	2.91**	1.54	1.49	1.54	1.64
Kidney weight, relative to brain weight	91.0	93.2	88.3	170.1*	73.0	72.7	75.1	81.3

	Dose group (ppm)							
	Males				Females			
	0	100	1000	4000	0	100	1000	4000
Organ weights; terminal sacrifice (g)								
Terminal doy weight	28.1	28.6	28.0	25.9*	24.7	24.4	24.1	23.2**
Brain weight, absolute	0.462	0.463	0.456	0.446	0.481	0.480	0.474	0.470
Liver weight, absolute	1.19	1.25*	1.29*	1.22	1.34	1.36	1.39	1.39
Liver weight, relative to body weight	4.23	4.38*	4.61**	4.73**	5.43	5.59	5.77**	6.01**
Liver weight, relative to brain weight	257.1	270.3	282.8**	273.4*	279.1	283.8	294.6	297.3
Kidney weight, absolute	0.474	0.472	0.465	0.901**	0.405	0.405	0.420	0.432
Kidney weight, relative to body weight	1.687	1.656	1.663	3.464**	1.643	1.662	1.742	1.865
Kidney weight, relative to brain weight	102.6	101.9	102.0	203.8**	84.1	84.3	88.8	92.2
Macroscopic findings; interim sacrifice								
Kidney								
Obviously large	0/9	1/9	0/10	7/9	0/10	0/9	1/9	2/7
Obviously small	0/9	0/9	0/10	1/9	0/10	0/9	0/9	4/7
Stone(s)	0/9	0/9	0/10	1/9	0/10	0/9	0/9	4/7
Pelvic dilatation	0/9	0/9	0/10	6/9	0/10	0/9	0/9	3/7
Pale	0/9	0/9	0/10	1/9	0/10	0/9	0/9	3/7
Cyst(s)	0/9	0/9	0/10	1/9	0/10	0/9	0/9	2/7
Urinary bladder								
Gritty content (stones)	0/9	0/9	0/10	8/9	0/10	0/9	0/9	6/7
Distended	0/9	0/9	0/10	8/9	0/10	0/9	0/9	4/7
Macroscopic findings; terminal sacrifice (unscheduled and scheduled deaths combined)								
Kidney								
Obviously large	0	0	0	6	0	0	1	1
Obviously small	0	0	0	5	0	0	0	13
Stone(s)	0	0	0	14	0	0	0	23
Pelvic dilatation	0	0	1	20	0	0	1	13
Pale	0	2	0	16	1	0	4	15
Cyst(s)	0	0	1	23	0	0	1	14
Urinary bladder								
Stone(s)	0	0	0	46	0	0	0	29
Distended	1	2	1	23	0	1	0	11

RBC: Red blood cell; MCV: Mean corpuscular volume; Source: Steiblen, 2006

MCH: Mean corpuscular haemoglobin; MCHC: Mean corpuscular haemoglobin concentration; WBC: White blood cell;

* $p < 0.05$, ** $p < 0.01$

At 12 months, males at 4000 ppm showed a statistically significant increase in absolute and relative kidney weights compared to controls. At 18 months, absolute and relative liver weights at 1000 and 4000 ppm were increased in males with a slight effect in females, and absolute and relative kidney weights were increased at 4000 ppm in males only.

Prior to the 12-month sacrifice one male and three females in the 12-month satellite group at 4000 ppm were found dead (male on day 240, females on days 60, 61, and 75). Macroscopic examination of these animals revealed significant yellow urinary bladder stones. In the 12-month satellite animals sacrificed as scheduled, macroscopic examination revealed stone(s) in the renal pelvis in one male and four out of seven females at 4000 ppm. Both males at 4000 ppm and females at 6000↓4000 ppm showed enlarged or small kidneys, dilation of the renal pelvis, pale kidneys or renal cyst(s). Stones or gritty content were also observed in the urinary bladders of nearly all top-dose animals sacrificed at 12 months, along with bladder distension in the majority of these animals.

Among the animals in the 18-month study group, the majority of those which died unscheduled at 4000 ppm were found to have died as a result of acute or chronic renal failure, due to urinary tract blockage or chronic kidney and/or urinary bladder inflammation. Stones were found in the kidney and/or urinary bladder of these animals. Other findings at necropsy of unscheduled deaths were enlarged or small kidneys, renal pelvic dilation, pale kidneys, renal cyst(s) and distension of the urinary bladder. Similar findings were observed in animals sacrificed on schedule at 18 months.

Microscopic examination was not conducted at 12 months. At 18 months, histopathologic findings included increased incidence in males and females at 1000 and 6000↓4000 ppm of centrilobular hepatocellular hypertrophy. In both males and females at all doses but in the absence of a dose–response relationship, there was a slight increase in the incidence of gall bladder epithelial hyperplasia. Treatment-related histopathological findings in the urinary tract were limited to top-dose males and females and included epithelial hyperplasia, fibrosis and scarring, and inflammatory cell infiltration. These findings were related to the urinary tract calculi observed at this dose level in the majority of animals. Separate analysis of the gallstones revealed them to be composed primarily of cholesterol.

Table 10. Treatment-related non-neoplastic and neoplastic microscopic findings in all male and female mice in the carcinogenicity phase of the feeding study with pyrasulfotole

	Dose group (ppm)							
	Male				Female			
	0	100	1000	4000	0	100	1000	4000
	Non-neoplastic changes							
Liver								
Centrilobular hepatocellular hypertrophy	0	1	14**	25**	0	0	3	7**
Hepatocellular vacuolation: mainly periportal: diffuse	0	0	0	7	0	0	0	6
Interstitial mixed cell infiltrate: focal/multifocal	14	19	18	26**	19	20	25	17
Gall bladder								
Gallstones	4	19**	22**	16**	0	5	14**	5**
Epithelial hyperplasia: focal/ multifocal	1	2	6	3	0	4	1	3
Kidney								
Pelvic stones: unilateral	0	0	0	10**	0	0	0	21**
Pelvic stones: bilateral	0	0	0	3	0	0	0	3
Collecting duct hyperplasia: unilateral	0	0	0	7**	2	0	2	13**
Pelvic epithelium hyperplasia: unilateral, focal/multifocal	0	0	1	10**	0	0	1	11**
Papillary fibrosis/atrophy: unilateral	0	0	2	13**	0	0	1	19**
Papillary fibrosis/atrophy: bilateral	0	0	0	12**	0	0	0	4**

	Dose group (ppm)							
	Male				Female			
	0	100	1000	4000	0	100	1000	4000
Atrophy/fibrosis/scar: cortex/medulla: unilateral	0	0	1	18**	7	10	20**	26**
Atrophy/fibrosis/scar: cortex/medulla: bilateral	0	1	0	21**	1	0	1	9**
Suburothelial mixed cell infiltrate: focal/multifocal	0	0	0	8*	0	0	0	5**
Papillary necrosis: unilateral/bilateral: focal/multifocal	1	0	1	7*	0	1	2	4
Collecting duct concretions: unilateral/bilateral	0	0	0	22**	1	3	3	15**
Arteritis/periarteritis: focal/multifocal	0	0	0	6*	0	0	0	6**
Urinary bladder								
Stones: intraluminal	0	0	0	17**	0	0	0	8**
Urothelial hyperplasia: simple: multifocal/diffuse	0	0	1	20**	0	0	0	31**
Urothelial hyperplasia: nodular/ glandular: multifocal/diffuse	0	0	0	40**	0	0	0	13**
Urothelial hyperplasia: squamous: multifocal/diffuse	0	0	0	28**	0	0	0	9**
Distension	11	7	5	37**	4	4	5	19*
Intramuscular inflammatory cell infiltrate: focal/multifocal	3	0	0	27**	1	1	1	20**
Sub urothelial mixed cell infiltrate: focal/multifocal	1	0	1	11*	0	0	1	4
Interstitial mixed cell infiltrate: focal/multifocal	0	0	0	17**	0	0	0	8**
Serosal mixed cell infiltrate: focal/multifocal	0	1	1	11**	0	0	0	12**
Ureters								
Stones	0	0	0	2	0	0	0	0
Urothelial hyperplasia: simple: multifocal/diffuse	0	0	0	3	0	0	0	1
Urothelial hyperplasia: nodular/ glandular: multifocal/diffuse	0	0	0	2	0	0	0	2
	Neoplastic changes							
Urinary bladder								
M – transitional cell carcinoma	0	0	0	8**	0	0	0	2
B – transitional cell papilloma	0	0	0	3	0	0	0	2
Urethra (prostatic)								
M – urethral transitional cell carcinoma	0	0	0	1	-	-	-	-

* $p < 0.05$; ** $p < 0.01$ Fisher's Exact test (one tailed); Source: Steiblen, 2006

Treatment-related neoplastic findings were limited to the urinary tract of males and females and comprised transitional cell carcinomas and papillomas of the urinary bladder and urethra. In all cases, these tumours were found in the presence of urinary tract stones. Therefore, these tumours were considered to be the result of a non-genotoxic proliferative mechanism due to the concurrent presence of secondary inflammation and hyperplastic findings in the same tissues, induced by the urinary stones.

Administration of pyrasulfotole to male and female mice via dietary incorporation, at 4000 ppm led to urinary tract stone formation, increased mortality generally due to either acute or chronic renal failure secondary to urinary tract stone development, decreased body weight and body weight gain, increased kidney and liver weights in males, renal pelvis dilation, and urothelial hyperplasia throughout the urinary tract. Liver weight and centrilobular hepatocellular hypertrophy were increased compared to controls in males at 1000 and 4000 ppm, and centrilobular hepatocellular hypertrophy was increased in females at 4000 ppm only. Gallbladder stones, shown to consist chiefly of cholesterol, were observed in both males and females at all doses, with no clear relationship to dose. Treatment-related neoplastic findings of the urinary tract were related to the presence of urinary tract stones and were only observed at 4000 ppm. These neoplasias were considered to be secondary to the administration of high doses of pyrasulfotole, as the urinary tract stones were shown to be composed of unmetabolised pyrasulfotole, and the neoplasias were due to a non-genotoxic proliferative mechanism.

In this study no systemic NOAEL could be set. The lowest dose tested (100 ppm, equal to 13.6 mg/kg bw per day in males, 16.7 mg/kg bw per day in females) was considered a LOAEL based on gallbladder stones. Regarding carcinogenicity, the NOAEL was 1000 ppm (equal to 137 mg/kg bw per day) based on urinary tract carcinoma and papilloma observed at 4000 ppm (equal to 560 mg/kg bw per day). These tumours were seen in the presence of urinary tract stones/calculi (Steiblen, 2006).

Rat

In a chronic toxicity/oncogenicity study, pyrasulfotole (purity 95.7%) was administered in the diet at 0, 25, 250, 1000 or 2500 ppm to separate groups of Wistar rats (75/sex per dose). These concentrations provided final doses of 0, 1.0, 10, 41 and 104 mg/kg bw per day for males and 0, 1.4, 14, 57 and 140 mg/kg bw per day for females. After 28 weeks, 10 males and 10 females from each group were allocated to the six-month subchronic phase of the study and were necropsied at the first scheduled interim sacrifice. After 52 weeks another 10 males and 10 females from each group were allocated to the chronic (12 months) phase and necropsied at the second scheduled interim sacrifice. The remaining 55 animals/sex per group were allocated to the carcinogenicity (24 months) phase of the study. Body weight and food consumption were measured weekly for the first 13 weeks of the study, then every four weeks through the remainder of the study until necropsy. Food consumption was recorded twice weekly for the first six weeks, then weekly up to week 13, then every four weeks thereafter. Ophthalmological examinations were conducted on all animals during acclimatization and at months 3, 6, 12, 18 and 24 of treatment. Blood for haematological and clinical chemical examination was collected after 6, 12 and 24 months from overnight-fasted rats. Urine was collected overnight at 3, 6, 12, 18, and 24 months from animals fasted overnight of food and water; urinalysis was performed on all surviving animals of the 6- and 12-month interim sacrifice groups, whereas with the terminal sacrifice group only the first ten surviving rats were examined. At 6, 12, and 24 months animals were sacrificed and subjected to gross necropsy. Selected organs were weighed, and organs/tissues preserved for histopathological examination.

A treatment-related increase in mortality was observed in males at 2500 ppm at 6, 12, and 24 months. There was no treatment-related effect on mortality in other male treatment groups or in females at any dose level. Treatment-related clinical signs of toxicity included white area on the eye and soiled fur in one or more areas. An increased incidence of these findings was observed at all time points in both males and females receiving 250, 1000 or 2500 ppm.

Body weight and body weight gain were reduced in males and females at the higher doses of pyrasulfotole; males were more affected than females. There was no effect on food consumption at any dose in either males or females. Ophthalmological examination revealed a number of treatment-related findings; corneal opacity, neovascularization of the cornea, oedema of the cornea, and "snowflake" corneal opacities. These were seen in both males and females after 6, 12, and 24 months of treatment. At 6 and 12 months, increased incidence of these findings was seen only at 250, 1000 and 2500 ppm.

At 24 months frequency of these findings was compared to controls at all doses in males. However, at 25 ppm there was only a slight increase in corneal opacity, neovascularization of the cornea, and oedema of the cornea in males, and in neovascularization of the cornea in females.

There was no treatment-related effect on haematology at any dose or time point in either male or female rats. The only treatment-related clinical chemical finding was increased plasma cholesterol, with statistically significant increases at all dose levels at month 7, reaching statistically significance for the increases in 1000 and 2500 ppm groups at 18 months. Plasma cholesterol was increased with statistical significance in males at 25 ppm at the seven-month time point: cholesterol concentrations in this dose group returned to control levels by the 12-month time point.

Urinalysis revealed higher levels of ketones at all collection periods in both sexes at 1000 ppm and 2500 ppm, and in males at 250 ppm at months 19 and 24 only. These increases were considered due to the diketone form of the pyrasulfatole, which is largely excreted unchanged in the urine. Urine pH was decreased at 250 ppm and above in males at all time points, while in females at 250 ppm and above urine pH was decreased only at the three-month time point.

Table 11. Selected findings in male and female rats administered pyrasulfotole in the diet

	Dose group (ppm)									
	Males					**Females**				
	0	**25**	**250**	**1000**	**2500**	**0**	**25**	**250**	**1000**	**2500**
					Mortalities, (*n*)					
6 months	1	1	0	1	4	0	0	0	0	0
[%]	[1.3]	[1.3]	[0]	[1.3]	[5.3]	[0]	[0]	[0]	[0]	[0]
12 months	2	6	2	2	8	2	1	0	1	0
[%]	[3.1]	[9.2]	[3.1]	[3.1]	[12.3]	[3.1]	[1.5]	[0]	[1.5]	[0]
24 months	30	38	25	29	40*	28	19	25	22	26
[%]	[54.5]	[69.1]	[45.5]	[52.3]	[72.7]	[50.9]	[34.5]	[45.5]	[40.0]	[47.3]
					Clinical signs, (*n*)					
White area eye										
6 months	0	1	38	41	48	0	1	5	36	52
12 months	1	1	48	60	59	0	2	10	41	57
24 months	6	7	46	50	48	4	6	13	40	43
Soiled anogenital region										
6 months	0	0	0	1	2	0	0	1	1	25
12 months	0	0	0	4	16	0	0	1	9	35
24 months	0	2	7	6	15	3	1	0	8	20
					Body weight (g)					
Day 1	287	287	288	289	286	196	193	193	194	193
Day 8	336	336	330	329	327*	214	216	212	210	209
Day 92	548	565	539	526*	529	297	302	300	296	299
Day 176	632	546	624	602**	597**	320	328	330	327	331
Day 372	704	714	688	664*	661**	367	367	369	357	336
Day 540	737	733	688*	664**	647**	437	432	423	410	423
Day 708	651	636	624	619	576	452	443	423	409	415

	Dose group (ppm)									
	Males					Females				
	0	25	250	1000	2500	0	25	250	1000	2500
Body weight gain (g)										
Days 1–8	49	50	42**	40**	41**	17	22**	20	17	16
Days 1–92	261	279*	252	237**	243**	102	109**	107	102	105
Days 92–176	83	76	86	77	70**	22	26	30**	31**	33**
Days 176–372	70	70	64	67	65	47	36	40	29**	33*
Days 372–540	41	29	1**	–2**	–14**	70	65	57	53	57
Days 540–708	–73	–82	–47	–38	–60	23	21	12	4	–2
Overall	365	353	339	331	298	254	249	231	217	223
Ophthalmology (N)										
Corneal opacity										
6 months	0	1	32	39	47	0	0	0	18	91
12 months	1	1	40	47	44	0	0	2	25	39
24 months	1	4	20	24	15	1	1	3	18	23
Neovascularization of cornea										
6 months	0	1	38	41	47	0	0	4	34	53
12 months	1	1	45	49	45	0	0	5	37	49
24 months	1	4	26	27	17	1	2	9	28	27
Oedema of the cornea										
6 months	0	0	32	36	45	0	0	0	16	32
12 months	0	0	41	48	44	0	0	1	25	39
24 months	2	3	25	27	17	0	1	3	24	26
"Snowflake" corneal opacity										
6 months	0	0	6	3	2	0	0	10	17	22
12 months	0	0	6	3	3	0	0	8	12	12
24 months	0	0	9	5	2	0	0	7	11	8
Clinical chemistry										
Total cholesterol (mmol/L)										
7 months **[%]**[a]	1.90	2.32** [+22]	2.40** [+26]	2.42** [+27]	2.47** [+30]	1.86	2.02	2.23** [+20]	2.15* [+16]	2.13* [+15]
12 months **[%]**[a]	2.23	2.61	3.40** [+52]	3.29** [+48]	3.69** [+65]	2.01	2.21	2.39	2.17	2.24
18 months **[%]**[a]	2.71	2.91	3.47 [+28]	3.92** [+45]	4.24** [+56]	2.18	2.61	2.62	2.77	2.57 [+18
24 months	3.61	3.45	4.30 [+19]	4.81 [+33]	5.32 [+47]	2.60	3.34 [+28]	3.09 [+19]	3.04 [+17]	4.23 [+63]
Urinary protein[b]										
Prestudy			*N* = 30					*N* = 24		
0			0					2		
1			7					12		
2			23					8		
3			0					2		
4			0					0		

	Dose group (ppm)									
	Males					Females				
	0	25	250	1000	2500	0	25	250	1000	2500
Month 3	*N*=30	*N*=29	*N*=29	*N*=30	*N*=30	*N*=27	*N*=29	*N*=29	*N*=27	*N*=30
0	0	0	0	0	0	4	4	4	3	6
1	0	1	0	1	2	8	2	9	10	11
2	17	12	11	13	14	13	18	13	12	13
3	9	12	12	11	7	2	4	2	2	0
4	4	4	6	5	7	0	1	1	0	0
Month 6	*N*=27	*N*=30	*N*=29	*N*=29	*N*=29	*N*=26	*N*=30	*N*=30	*N*=30	*N*=28
0	0	0	0	0	0	3	1	4	3	3
1	0	0	0	0	0	11	5	7	8	8
2	14	16	6	5	6	9	21	17	17	17
3	10	5	11	12	8	3	2	1	1	0
4	3	9	12	12	15	0	1	1	1	0
Month 12	*N*=20	*N*=20	*N*=17	*N*=18	*N*=17	*N*=18	*N*=20	*N*=19	*N*=17	*N*=20
0	0	0	0	0	0	5	2	5	3	5
1	0	0	0	0	0	2	8	5	3	4
2	9	4	0	0	0	9	6	4	9	8
3	5	4	0	1	3	2	3	4	1	3
4	6	12	17	17	14	0	1	1	1	0
Month 24	*N*=10	*N*=9	*N*=10	*N*=10	*N*=10	*N*=9	*N*=8	*N*=10	*N*=9	*N*=10
0	0	0	0	0	0	1	0	0	0	0
1	0	0	0	0	0	1	0	0	1	1
2	2	0	0	0	0	2	0	2	2	0
3	3	1	0	0	0	3	4	3	1	1
4	5	8	10	10	10	2	4	5	5	8
Organ weights (g)										
6 months										
Terminal body weight	598.7	617.8	597.4	577.6	581.0	316.1	306.3	327.9	299.1	311.5
Brain	2.20	2.21	2.19	2.18	2.17	2.11	2.06	2.02	2.04	2.00
Liver	11.87	13.04	14.49**	14.03*	14.84**	6.93	6.89	7.27	7.49	7.26
Liver rel. to bw	1.980	2.113	2.425**	2.426**	2.551**	2.192	2.253	2.221	2.521**	2.332
Kidney	2.92	3.14	3.36	3.56**	4.10**	1.94	1.97	1.96	2.01	2.01
Kidney rel. to bw	0.494	0.509	0.564	0.628**	0.713**	0.614	0.644	0.600	0.676	0.648
Thyroid	0.0219	0.0231	0.0246	0.0269	0.0284	0.0167	0.0152	0.0155	0.0142	0.0184
Thyroid rel. to bw	0.0037	0.0038	0.0041	0.0046	0.0049*	0.0053	0.0049	0.0047	0.0047	0.0060
12 months										
Terminal body weight	658.9	668.8	661.8	654.7	642.7	324.5	331.1	349.4	351.1	336.4
Brain	2.32	2.31	2.21	2.26	2.27	2.05	2.05	2.10	2.07	2.06
Liver	13.45	14.05	16.10*	16.43**	15.96**	7.16	7.69	8.27	8.59*	8.07

	Dose group (ppm)									
	Males					Females				
	0	25	250	1000	2500	0	25	250	1000	2500
Liver rel. to bw	2.042	2.102	2.430**	2.511**	2.486**	2.201	2.328	2.369	2.442	2.392
Kidney	3.44	3.67	3.99	4.17**	4.21**	2.24	2.31	2.50	2.48	2.36
Kidney rel. to bw	0.524	0.550	0.607**	0.634**	0.656**	0.691	0.697	0.718	0.709	0.701
Thyroid	0.0257	0.0245	0.0289	0.0317	0.0308	0.0157	0.0215	0.0201	0.0206	0.0173
Thyroid rel. to bw	0.0039	0.0037	0.0044	0.0048	0.0048	0.0048	0.0065	0.0058	0.0058	0.0052
24 months										
Terminal body weight	639.6	621.9	594.2	590.8	565.2	405.6	408.6	380.2	376.5	384.0
Brain	2.37	2.37	2.31	2.26	2.25	2.12	2.16	2.12	2.12	2.08
Liver	12.93	13.28	15.53** +20	15.83** +22	15.09 +17	10.18	10.31	10.29	10.45	10.57
Liver rel. to bw	2.035	2.154	2.626** +29	2.694** +32	2.676** +31	2.520	2.542	2.747	2.794	2.781
Kidney	3.84	4.01	4.46* +16	5.01** +30	4.98* +30	2.91	3.38	3.11	3.02	3.14
Kidney rel. to bw	0.608	0.654	0.753** +24	0.854** +40	0.882** +45	0.728	0.837	0.837	0.821	0.830
Thyroid	0.0378	0.0309	0.0343	0.0308	0.0325	0.0258	0.0270	0.0260	0.0245	0.0256
Thyroid rel. to bw	0.0060	0.0050	0.0058	0.0052	0.0058	0.0065	0.0067	0.0069	0.0066	0.0067
Macroscopic findings[c]										
Eyes, opacity										
6 months	0/10	1/10	3/10	3/10	3/10	0/10	0/10	0/10	0/10	2/10
12 months	0/10	0/10	1/10	5/10	5/10	0/10	1/10	0/10	5/10	7/10
24 months -US[c]	2/32	3/38	9/26	14/29	26/40	1/28	5/20	2/25	14/23	28/14
24 months -S[d]	3/23	1/17	22/29	22/26	12/15	3/27	5/35	8/30	24/32	24/27
Liver, obviously large										
6 months	1/10	2/10	4/10	1/10	3/10	0/10	0/10	0/10	0/10	0/10
12 months	0/10	0/10	3/10	3/10	3/10	0/10	0/10	0/10	0/10	0/10
Liver, prominent lobulation										
6 months	1/10	3/10	4/10	4/10	2/10	1/10	0/10	1/10	0/10	1/10
Kidney, irregular surface										
24 months -US[c]	2/32	2/38	4/26	9/29	16/40	1/28	1/20	2/25	1/23	2/28
24 months -S[d]	0/23	1/17	2/29	14/26	7/15	1/27	1/35	1/30	2/32	4/27
Kidney, pale										
24 months -US[d]	2/32	3/38	2/26	5/29	8/40	0/28	1/20	0/25	0/23	2/28
24 months -S[e]	1/23	0/17	1/29	5/26	3/15	2/27	2/35	1/30	2/32	2/27

* $p < 0.05$, ** $p < 0.01$; Source: Wason, 2006a

[a] % increase compared to control; [c] For 6 and 12 months: unscheduled and scheduled sacrifices are summed;
[d] 24-US = unscheduled sacrifice at 24 months; [e] 24-S = scheduled sacrifice at 24 months.

[b] Protein scores: 0 = negative 0g/L, 1 = trace, 2 = 1 + 0.3g/L, 3 = 2 + 1g/L, 4 = 3 + ≥3g/L

There was a statistically significant increase in liver weight in males at 250 ppm and above in this study at all time points, however without a clear dose–response relationship; kidney weights were increased in males at 1000 ppm and above at all time points. Thyroid weights showed a slight (not statistically significant) increase at 1000 ppm and above at the six- and 12-month time points. There were no biologically significant effects on organ weights in any females group at any time point.

Treatment-related macroscopic findings were observed at different time points in the eyes, liver, and kidney. Eye opacities were seen at all time points in both males and females. These findings are discussed further in section 2.6(c) mechanistic studies, . Based on MOA eye effects were related to increased tyrosinemia and were considered not to be relevant to human risk assessment. Enlarged liver was observed in males at 12 months at 250 ppm and above. At 24 months, there was an increased incidence among males of pale kidneys or kidney displaying an irregular surface at 1000 and 2500 ppm.

Treatment-related non-neoplastic microscopic findings were observed in the eyes, liver, pancreas, thyroid gland, and kidneys at all time points, however, most effects only reached statistical significance at the 24 month time point. At 24 months statistically significant increases in eye effects were seen at 250 ppm and above; these included inflammation, hyperplasia, atrophy and neovascularization of the cornea. In the liver, a significant increase in hepatocellular hypertrophy was seen in males a 250 ppm and above after 24 months, however without a dose–response relationship. In the pancreas diffuse degeneration/atrophy was seen in males a 1000 ppm and above and focal degeneration/atrophy in females a 2500 ppm. In the kidney, hyperplasia of collecting ducts was increased in males at 1000 and 2500 ppm after 24 months. Also in the kidneys, increased severity of chronic progressive nephropathy was observed with increasing dose levels. In the thyroid an increase was seen in follicular cell hypertrophy in males and females. The effects on the thyroid are further discussed in an expert review, in which it is concluded that findings were not adverse.

Table 12. Treatment-related non-neoplastic microscopic findings in male and female rats administered pyrasulfotole by dietary incorporation

	Dose group (ppm)									
	Males					Females				
	0	**25**	**250**	**1000**	**2500**	**0**	**25**	**250**	**1000**	**2500**
					Eyes					
6 months										
Number examined	9	10	10	10	10	10	10	10	10	10
Inflammation, cornea, unilateral or bilateral	0	0	6	8	8	0	0	0	6	7
Hyperplasia, cornea, regenerative, unilateral or bilateral	0	0	5	8	7	0	0	0	5	6
Neovascularization, cornea, unilateral or bilateral	0	0	3	7	6	0	0	0	3	6
12 months										
Number examined	10	10	9	10	8	9	10	10	9	10
Inflammation, cornea, unilateral or bilateral	0	0	6	9	8	0	1	0	5	7
Hyperplasia, cornea, regenerative, unilateral or bilateral	0	0	6	9	8	0	1	0	4	6
Neovascularization, cornea, unilateral or bilateral	0	1	5	8	8	0	1	0	4	7

	Dose group (ppm)									
	Males					Females				
	0	25	250	1000	2500	0	25	250	1000	2500
24 months										
Number examined	55	55	55	55	55	55	55	54	55	55
Inflammation, cornea, unilateral or bilateral	1	3	39**	49**	44**	0	1	2	28**	32**
Hyperplasia, cornea, regenerative, unilateral or bilateral	0	0	31**	38**	32**	1	0	2	24**	25**
Neovascularization, cornea, unilateral or bilateral	2	3	46**	49**	53**	0	0	13**	41**	42**
Vacuolation, cornea, focal	0	0	2	0	2	1	0	0	5	5
Metaplasia, mucous, cornea	0	0	2	9**	11**	0	0	0	0	0
Atrophy, cornea	0	0	11**	25**	24**	0	0	0	5*	6*
Atrophy, retina, peripheral, unilateral or bilateral	2	3	17**	15**	17**	1	4	8*	20**	13**
Liver										
6 months										
Number examined	9	10	10	10	10	10	10	10	10	10
Hypertrophy, hepatocellular, centrilobular	0	8	10	10	10	0	0	3	4	5
12 months										
Number examined	10	10	9	10	8	9	10	10	9	10
Hypertrophy, hepatocellular, centrilobular	0	0	6	10	6	0	0	0	0	0
24 months										
Number examined	55	55	55	55	55	55	55	54	55	55
Hypertrophy, hepatocellular, centrilobular	0	0	17**	11**	13**	0	0	0	0	1
Pancreas										
6 months										
Number examined	9	10	10	10	10	10	10	10	9	10
Degeneration/atrophy, acinar, diffuse	0	0	0	0	0	0	0	0	1	8
12 months										
Number examined	10	10	9	10	8	9	10	10	9	10
Degeneration/atrophy, acinar, diffuse	0	0	0	1	1	0	0	0	0	7
Fibrosis, interstitial, diffuse	0	0	0	0	5	0	0	0	0	3

	Dose group (ppm)									
	Males					Females				
	0	25	250	1000	2500	0	25	250	1000	2500
24 months										
Number examined	55	55	54	54	54	55	55	54	55	55
Degeneration/atrophy, acinar, diffuse	0	0	1	8**	9**	0	1	2	4	4
Degeneration/atrophy, acinar, focal	34	34	34	30	30	18	20	25	26	34**
Thyroid										
6 months										
Number examined	9	10	10	10	10	10	10	10	10	10
Altered colloid, basophilic deposits	1	4	8	6	8	0	0	0	0	0
Increased follicular diameter	0	0	1	1	5	0	0	0	0	0
Pigment, brown, follicular cells	0	0	1	1	4	0	0	0	0	0
12 months										
Number examined	10	10	9	10	8	9	10	10	9	10
Hyperplasia, follicular cell, focal	0	0	1	0	4	0	0	0	0	0
Hypertrophy, follicular cell, diffuse	0	0	4	5	7	0	0	1	2	1
Altered colloid, basophilic deposits	2	4	8	10	8	0	0	1	1	1
Increased follicular diameter	0	0	5	5	8	0	0	0	0	0
Pigment, brown, follicular cells	1	1	6	8	8	0	0	1	1	0
24 months										
Number examined	55	55	54	55	55	55	55	54	55	55
Colloid alteration	21	24	36**	30	33*	4	3	9	13**	8
Pigment, brown, follicular cells	3	14**	39**	33**	38**	0	7**	14**	14**	18**
Hypertrophy, follicular cell, diffuse	2	2	5	8*	4	0	0	3	7**	2
Hyperplasia, follicular cell, focal	3	2	9	12*	8	0	1	0	1	1
Kidneys										
6 months - nephropathy										
Number examined	9	10	10	10	10	10	10	10	10	10
progressive chronic	2	2	2	3	5	0	0	0	0	0
minimal	2	2	2	2	4	-	-	-	-	-
slight	0	0	0	1	1	-	-	-	-	-

	Dose group (ppm)									
	Males					Females				
	0	25	250	1000	2500	0	25	250	1000	2500
12 months - nephropathy										
Number examined	10	10	9	10	8	9	10	10	9	10
progressive chronic	3	5	9	10	8	0	0	1	1	0
minimal	2	4	7	10	5			1	1	
slight	1	1	2	0	2			0	0	
moderate	0	0	0	0	1			0	0	
24 months - nephropathy										
Number examined	55	55	55	55	55	55	55	54	55	55
progressive chronic	44	51*	54**	52*	51*	37	42	45	45	42
minimal	37	37	27	18	16	29	38	36	42	31
slight	3	8	16	8	9	6	3	5	1	6
moderate	2	3	7	14	13	2	1	2	1	3
marked	1	0	1	6	7	0	0	1	1	0
severe	1	2	3	6	6	0	0	1	0	2
Hyperplasia,										
collecting ducts	5	11	11	19**	19**	7	9	8	7	5

* $p < 0.05$, ** $p < 0.01$ Source: Wason, 2006a

The only treatment-related neoplastic finding was that of squamous cell tumours (one papilloma, one carcinoma) observed in the cornea of two males in the 2500 ppm dose group. These tumours were considered to have resulted from the corneal inflammation and regenerative hyperplasia, a consequence of tyrosinemia, and therefore were not relevant to human risk assessment. Other tumours which were observed showed no relationship to dose or were considered to be consistent with those tumours normally found in ageing rats, so were not considered to be treatment-related.

The systemic NOAEL was 25 ppm (equal to 1.0 mg/kg bw per day) based on effects on the eyes and increased plasma cholesterol at 250 ppm (equal to 10 mg/kg bw per day). It is noted that increased cholesterol was also observed at the low dose at seven months. However, this finding was not considered adverse as it was found in isolation. The NOAEL for carcinogenicity was 1000 ppm (equal to 41 mg/kg bw per day) based on neoplastic findings in the cornea in males at 2500 ppm (equal to 104 mg/kg bw per day). These findings were considered to be related to the chronic stimulation of the cornea and thus a non-genotoxic mechanism (Wason, 2006a). Eye effects are further discussed in section 2.6(c) mechanistic studies.

An expert review of the histopathological thyroid sections from male and female rats in the chronic toxicity/oncogenicity study in the rat (Wason, 2006a) was conducted by an independent panel of senior pathologists to determine whether or not the morphologic changes observed in the thyroid represented an adverse effect of pyrasulfotole on the thyroid.

The pathology expert group noted that colloid alterations were present in all groups including controls and that the morphology was similar in controls and thyroids from treated groups; the primary difference was an increase for the treated groups in the number of follicles affected. Additionally, colloid changes were seen in the absence of follicular cell hypertrophy and were not considered to indicate a persistent alteration in thyroid function in this study. Similarly, the brown pigment observed in the follicular cells was considered to be similar in morphology amongst control and treated animals. This pigment was judged to be suggestive of lipofuscin, which is a normal pigment often associated with aging and seen in a number of organs under untreated conditions. A summary of the expert pathologists' findings is shown in Table 13.

Table 13. Incidence and severity of colloid alteration and pigment deposition in the thyroid of male and female rats administered pyrasulfotole for 24 months

	Dose group (ppm)									
	Male					Female				
Finding	**0**	**25**	**250**	**1000**	**2500**	**0**	**25**	**250**	**1000**	**2500**
Number examined	55	55	54	55	55	55	55	54	55	55
Colloid alteration: total	18	24	36	30	31	2	2	9	13	8
Minimal	11	20	21	11	18	2	2	6	8	5
Slight	7	4	15	15	10	0	0	3	5	3
Moderate	0	0	0	4	3	0	0	0	0	0
Pigment deposition: total	3	14	39	33	38	0	7	14	14	18
Minimal	3	14	14	13	23	0	7	14	9	16
Slight	0	0	25	20	15	0	0	0	5	2

Source: Mann, 2005

It was the opinion of the pathology expert group that the colloid alteration and pigment deposition observed in rats administered pyrasulfotole for two years were representative of normal age-related physiologic changes specific to the rat, and that these findings were not adverse. Thus, the NOAEL for colloid alteration and pigment deposition in the thyroid was considered to be above 2500 ppm (equivalent to 104 mg/kg bw per day) (Mann, 2005).

2.4 Genotoxicity

The results of studies on the genotoxicity of pyrasulfotole are summarized in Table 14.

Table 14. Overview of genotoxicity tests with pyrasulfotole

End-point	Test object	Concentrations/doses tested	Purity	Results	Reference
		In vitro			
Bacterial gene mutation	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537; *Escherichia coli* WP2uvrA	**1st experiment;** plate incorporation, (± S9): 16, 50, 158, 500, 1581 and 5000 µg/plate in DMSO **2nd experiment;** pre-incubation, (± S9): 16, 50, 158, 500, 1581 and 5000 µg/plate in DMSO	95.7%	Negative	Herbold, 2004
Chromosome aberration[a]	Chinese hamster V79 cells	**Without S9 activation:** 4 h exposure/18 h harvest: 500, 1000 and 2000 and *2500* µg/mL in DMSO 4 h exposure/30 h harvest: *1500*, 2000 and *2500* µg/mL in DMSO 18 hour exposure/18 hour harvest: 200, *400*, 600, *800* and 1000 µg/mL in DMSO **With S9 activation:** 4 h exposure/18 hour harvest: 500, *1000*, 1500, *2000* and 2500 µg/mL in DMSO 4 h exposure/30 h harvest: *1500*, *2000*, 2500 µg/mL in DMSO	95.7%	Negative	Thum, 2004

End-point	Test object	Concentrations/doses tested	Purity	Results	Reference
Mammalian gene mutation	Chinese hamster V79 cells (*HPRT* locus)	30, 60, 120, 240, 480, and 960 µg/mL in DMSO (± S9)	95.7%	Negative	Herbold, 2004
		In vivo			
Micronucleus induction in mice	NMRI mouse	125, 250 and 500 mg/kg bw (females 250, 500 and 1000 mg/kg bw) in 0.5% aqueous Cremophor (IP)[b]	95.7%	Negative[c]	Herbold, 2003

DMSO: Dimethyl sulfoxide, expt: experiment; IP: Administered by intraperitoneal injection.

[a] Based on survival index and mitotic index, concentrations in italics were not further evaluated in the study report. The current OECD 473 guideline (2016) indicates that at least 300 metaphases should be scored. In the study only 200 metaphases were scored, however, this is in line with the version of the guideline in place at time the study was conducted (OED 473, 1997).

[b] The test substance was administered via two injections 24 hours apart.

[c] Clinical signs of systemic exposure were observed at all dose levels, including apathy, roughened fur, weight loss, staggering gait, spasm, twitching, stretching of the body, decreased body temperature, and difficulty breathing. At a dose of 1000 mg/kg bw 8/10 females died during the test period. In the report it was concluded that there were no relevant differences in toxicity between male and female mice, therefore, only males were evaluated. In males exposed to 500 mg/kg bw a significantly altered ratio between polychromatic and normochromatic erythrocytes was found indicating bone marrow exposure. The current OECD 474 guideline (2016) indicates that at least 4000 immature erythrocytes per animal should be scored. In the study only 2000 erythrocytes were scored, however, this is in line with the version of the guideline in place at time the study was conducted (OECD 474, 1997)

Pyrasulfotole was tested in an adequate range of tests and was conidered unlikely to be genotoxic.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Rat

In a two-generation study of toxicity, groups of 25 male and 25 female Wistar rats were given diets containing pyrasulfotole (purity 95.7%) at a concentration of 0, 30, 300 or 3000 ppm. In the F0 generation, these doses were equal to 2.5, 26.3 and 272 mg/kg bw per day in males, 3.1, 32.6 and 346 mg/kg bw per day in females during premating. In the F0 dams during gestation these doses were equal to 2.0, 22.3, 229 mg/kg bw per day, and during lactation equal to 3.1, 30.9, 294 mg/kg bw per day. In the F1 generation premating, the doses were 3.7, 34.1 and 354 mg/kg bw per day in males, 4.2, 38.9, 393 mg/kg bw per day in females. During gestation doses for F1 dams were equal to 2.2, 23.2 and 250 mg/kg be per day and during lactation 3.2, 29.9 and 263 mg/kg bw per day.

The groups of male and female F0 rats were fed a diet containing pyrasulfotole for approximately 12 weeks prior to mating, with a three-week time period for determination of estrous cyclicity. F1 offspring were nursed up to an age of four weeks. Some of the offspring were selected for further treatment and for breeding the F2 generation. With the F1 parental animals, 25 male and 25 female weanlings in each dietary group were fed diets containing pyrasulfotole for at least a 10-week premating phase, and again a three-week time period was used to determine estrous cyclicity. Clinical signs were monitored daily. Body weights were measured in males and females on the first day of the study prior to provision of diet containing the test compound, and then weekly until necropsy in males. In females, body weights were measured weekly during premating, and on gestation days (GDs) 0, 7, 14 and 20, on lactation days (LDs) 0, 7, 14, 21 and 28, and at necropsy. Food consumption was measured weekly in males except during mating, and in females weekly during premating, and for GD periods 0–7, 7–14 and 14–20, and for LD periods 0–4 and 4–7. Spermatological investigations (sperm motility and viability, morphology, determination of spermatozoa in epididymis, and determination of homogenization-resistant spermatid heads in the testis) were conducted at necropsy in the control and 3000 ppm males of the F0 and F1 generations. Near, or on the day of sacrifice, the eyes of adult males and females were examined ophthalmologically.

Litters were culled, where possible, to four male and four female pups LD 4. The number of live pups, pup weight, external findings, and number of dead pups were determined on LDs 0, 4, 7, 14,

21 and 28. For the F2 litters, anogenital distance was measured on LD 0, and the sex of each pup was determined on LDs 0 and 4. Ophthalmological examinations were conducted in postweaning animals in both F1 and F2 generations. ln the F1 litters, one male and one female weanling from each litter were randomly selected wherever possible for use as F1 adults. These selected animals were then monitored for preputial separation or vaginal opening, and bodyweight was measured on the day of landmark attainment.

At necropsy, the F0 and F1 adult animals were subjected to a gross necropsy. Implantation sites were counted, selected organs weighed, and samples of organs/tissues were preserved for histopathological examination. Lung flotation was performed for pups found dead at the first litter inspection to determine whether or not they had breathed at birth. Macroscopic examination was conducted on all animals and selected organs were weighed from one male and one female per litter.

Parental findings

There was no treatment-related increase in mortality in either the F0 or the F1 males or females at any dose level. Two F1 females at 3000 ppm were sacrificed in a moribund condition, but their deaths were not considered treatment-related.

The only treatment-related clinical sign was an increased incidence of eye opacities in F0 and F1 animals at 3000 ppm during the premating, gestation and lactation phases. Ophthalmology confirmed the incidence of either diffuse or reticulate corneal opacitiy and corneal neovascularization was increased in both F0 and F1 males and females at 300 and 3000 ppm.

There was no treatment-related effect of pyrasulfotole administration on body weight in the F0 males or females. Body weight in 300 ppm males showed a decrease in males (statistically significant) at termination of the study, but as there was no dose–effect relationship this was considered not to be treatment-related.

In the F1 adults, body weight and body weight gain were reduced to a statistically significant extent in males at 3000 ppm, and body weight was statistically significantly reduced in females at 3000 ppm with a final reduction in body weight at the end of the pre-mating period at 3000 ppm of 12% in males and 7% in females. There was no statistically significant effect on body weight at any dose during gestation in either the F0 or F1 dams. During LDs 0–4, body weight gain of F0 dams at 3000 ppm and F1 dams at 300 and 3000 ppm was reduced compared to controls with statistical significance. However, regarding the F1 females at 300 ppm, these animals already had a higher body weight at the start of the lactation period compared to controls therefore th decreased body weight gain at 300 ppm was not considered a treatment-related effect.

There was no effect on food consumption during the premating period in either male or female rats of the F0 or F1 generations. Food consumption during LDs 0–4 was slightly reduced at 3000 ppm in F0 dams and at 300 and 3000 ppm in F1 dams. This effect on food consumption was evaluated as being related to treatment.

Assessments of reproductive function indicated no treatment-related effect on either estrous cyclicity in females or on any sperm analysis parameters in males from either the F0 or F1 generation.

Assessments of reproductive performance revealed no treatment-related effects on any index of reproductive performance in either the F0 or F1 adults. Both gestation and rearing indices were slightly decreased at 3000 ppm in the F1 adults, but these decreases were due to deaths that were not related to administration of pyrasulfotole, and therefore were in themselves not related to treatment.

In F0 males, absolute kidney and liver weights were increased at 3000 ppm, and absolute thyroid weight was increased at 300 and 3000 ppm. Relative kidney, liver and thyroid weights were increased in F0 males at 300 and 3000 ppm. There were slight increases in F0 females at 300 and 3000 ppm in absolute and relative kidney and liver weights, but not that achieved statistical significance. ln the F1 males, there were increases in absolute liver, kidney and thyroid weights at all doses, but these showed no relationship to dose; however, relative liver and kidney weight did show a dose-related increase. In F1 females, a dose-related increase in the absolute weight of liver and adrenals was seen only at 3000 ppm. Relative kidney, liver and adrenal weights were increased in females at 3000 ppm.

Table 15. Selected findings in F0 and F1 male and female adult rats administered pyrasulfotole

		Dose group (ppm)							
		Male				Female			
		0	30	300	3000	0	30	300	3000
Eye findings in F0 generation – clinical signs and ophthalmology (n)									
Number examined (*N*)		25	25	25	25	25	25	25	25
Clinical finding[a]	Eye opacity	0	0	0	15	0	0	0	4
Opthal-mology	Diffuse corneal opacity	0	0	3	16	1	0	2	18
	Reticulate corneal opacity	0	0	2	11	0	0	13	18
	Cornea neo-vascularization	0	0	2	16	0	0	2	17
Eye findings in F1 generation – clinical signs and ophthalmology (n)									
Number examined (*N*)		25	25	25	25	25	25	25	23
Clinical finding[a]	Eye opacity	0	0	0	15	0	0	0	8
Opthal-mology	Diffuse corneal opacity	0	0	5	20	1	0	5	19
	Reticulate corneal opacity	0	0	6	20	0	0	9	20
	Cornea neo-vascularization	0	0	5	20	0	0	3	19
Body weight and body weight gain (g) during pre-mating phase									
F0 generation		-	-	-	-	-	-	-	-
BW week 1		124.8	123.4	126.1	124.2	117.8	118.8	118.6	119.8
BW week 12		412.45	402.5	395.3	402.0	235.2	231.4	229.1	231.1
BWG weeks 1–12		287.6	279.1	269.2	277.8	117.4	112.6	110.5	111.2
F1 generation									
BW week 5		94.9	95.5	93.5	80.9**	86.9	89.5	86.2	77.1**
BW week 14		384.3	383.4	367.4	337.6**	219.7	225.9	218.7	205.3**
BWG weeks 5–14		289.4	288.0	284.0	256.6**	132.8	136.4	132.5	128.2
Body weight and body weight gain (g) during gestation									
F0 generation									
BW day 0 postcoitum		-	-	-	-	239.3	230.3	227.2	227.8
BW day 14 postcoitum		-	-	-	-	285.5	273.1	281.3*	271.5
BW day 20 postcoitum		-	-	-	-	338.9	323.2	326.3	328.9
BWG days 14–20		-	-	-	-	53.4	50.0	55.0	57.3
F1 generation									
BW day 0 postcoitum		-	-	-	-	218.8	232.6	223.1	213.1
BW day 14 postcoitum		-	-	-	-	263.6	279.8*	267.2	255.4
BW day 20 postcoitum		-	-	-	-	309.8	333.7**	318.3	308.9
BWG day 14–20		-	-	-	-	46.2	53.9*	51.1	53.5*

	Dose group (ppm)							
	Male				Female			
	0	30	300	3000	0	30	300	3000
Body weight and body weight gain (g) during lactation								
F0 generation								
BW day 0 postcoitum	-	-	-	-	255.9	250.9	251.2	258.4
BW day 4 postcoitum	-	-	-	-	270.6	259.5	259.1	260.0
BWG days 0–4	-	-	-	-	14.7	8.7	8.0	1.6**
F1 generation								
BW day 0 postcoitum	-	-	-	-	239.6	255.3	250.2	238.9
BW day 4 postcoitum	-	-	-	-	250.6	263.3	249.0	239.5
BWG day 0–4	-	-	-	-	11.1	8.0	–1.2**	0.7*
Absolute organ weight								
F0 generation								
Terminal bw (g)	478.7	473.0	457.1*	461.6	266.1	256.8	265.4	267.7
Kidneys (mg) [% of control]	3063	3032 [99]	3107 [101]	3436** [112]	2170	2120	2245	2240
Liver (g) [% of control]	17.01	17.35 [102]	17.49 [103]	19.20** [112]	13.67	12.68	13.84	14.29 [105]
Thyroid (mg) [% of control]	14	16 [114]	18** [129]	19** [136]	11	12	12	12
F1 generation								
Terminal bw (g)	457.7	461.5	436.2	407.9**	257.7	268.8	261.2	251.0
Kidneys (mg)	2883	3071*	3090*	2975	1975	2150	2091	2210
Liver (g)	16.88	17.62	17.05	16.58	13.05	13.52	13.15	13.50
Thyroid (mg)	12	16**	16**	15	12	12	11	11
Relative organ weight (mg/100 g bw)								
F0 generation								
Kidneys [% of control]	640.1	641.6 [100]	680.1** [106]	745.1** [116]	816.1	827.1	848.1	838.1
Liver [% of control]	3552	3664 [103]	3821** [108]	4160** [117]	5139.8	4919.4	5213.4	5347.9
Thyroid [% of control]	2.9	3.4 [117]	4.0** [138]	4.0** [138]	4.1	4.7	4.6	4.5
F1 generation								
Kidneys % [% of control]	630.7	665.3* [105]	709.6** [113]	725.5** [115]	766.8	800.7 [104]	800.5 [104]	880.6** [115]
Liver [% of control]	3688	3813 [103]	3909* [103]	4069** [110]	5061	5031	5041	5387
Thyroid [% of control]	2.7	3.6** [133]	3.7** [137]	3.7** [137]	4.6	4.5	4.2	4.5

[a] During all phases; BW: body weight; BWG: body weight gain; Source: Eiben, 2005

* $p < 0.05$, ** $p < 0.01$.

There were no treatment-related macroscopic findings in F0 male or female rats at any dose. In F1 males, one rat at 300 ppm and 14 males at 3000 ppm showed eye opacities. Two F1 females at 3000 ppm also showed eye opacities. There were no other treatment-related findings in F1 male or female rats.

There were no treatment-related effects on organs of the reproductive tract. Treatment-related effects of pyrasulfotole administration were observed in the eyes and thyroid of both males and females of the F0 and F1 generations, and in pituitary, kidneys, and liver of F0 and F1 males only. Findings in the eyes at 300 and 3000 ppm in both males and females of the F0 and F1 generations included keratitis and vascularization, correlating with in-life observations.

Thyroid findings included colloid alteration at all doses in male F0 and F1 animals and F1 females; also in F0 females at 300 ppm and above. Pigment deposition occurred at all doses in F0 and F1 males and at 300 ppm and above in F0 and F1 females. Increased follicular cell hypertrophy was seen in F0 males at 3000 ppm and in F1 males at 300 ppm and above, also in F0 females at 3000 ppm. Eosinophilic inclusions were increased in the anterior pituitary of males at 300 and 3000 ppm in both the F0 and F1 generations.

Findings in the liver included hepatocellular hypertrophy, cellular alteration, and slight increases in periportal fat accumulation in F0 and F1 males at 300 and 3000 ppm. The kidneys of males in the F0 and F1 generation had increased incidence and/or severity of basophilic tubules and tubular dilation, in a dose-related manner from 300 ppm.

Table 16. Treatment-related histopathological findings in male and female F0 and F1 rats administered pyrasulfotole in the diet

		Dose group (ppm)							
		F0 animals				F1 animals			
Parameter	**Finding**	**0**	**30**	**300**	**3000**	**0**	**30**	**300**	**3000**
		Males				**Males**			
Eyes	Keratitis	0	0	2	16	0	0	6	18
	Epithelial hyperplasia	0	0	3	16	0	0	6	16
	Corneal vascularization	0	0	2	14	0	0	6	15
Thyroid	Follicular cell hypertrophy	4	0	4	11	3	4	9	11
	Colloid alteration	11	25	25	25	12	23	25	25
	Pigment deposition	1	11	24	25	0	10	23	24
Liver	Hepatocellular hypertrophy	0	0	2	17	0	0	9	15
	Cytoplasmic change	0	0	5	17	0	0	15	19
	Fat positive reaction (total)	22	23	24	23	18	18	17	19
	Grade 1	9	13	8	11	15	15	13	10
	Grade 2	12	10	14	8	3	3	4	9
	Grade 3	1	0	2	4	0	0	0	0

		Dose group (ppm)							
		F0 animals				F1 animals			
Parameter	Finding	0	30	300	3000	0	30	300	3000
Kidneys	Basophilic tubules (total)	21	25	25	24	24	24	25	25
	Grade 1	16	17	8	0	11	10	5	7
	Grade 2	4	7	9	15	8	13	10	8
	Grade 3	1	1	8	9	5	0	10	10
	Grade 4	0	0	0	0	0	1	0	0
	Tubular dilation (total)	17	15	21	24	17	16	20	21
	Grade 1	12	10	10	5	7	8	4	2
	Grade 2	3	5	7	16	10	7	13	11
	Grade 3	2	0	4	3	0	1	3	8
	Hyaline casts (total)	17	17	23	23	15	19	23	23
	Grade 1	12	12	11	3	5	11	7	4
	Grade 2	4	4	9	14	10	7	11	11
	Grade 3	1	1	3	6	0	1	5	8
	Mononuclear cell infiltrates	3	6	9	14	6	9	11	5
		Females				Females			
Eyes	Keratitis	0	0	6	24	0	0	6	19
	Epithelial hyperplasia	0	0	6	22	0	0	3	19
	Corneal vascularization	0	0	0	9	0	0	2	18
Thyroid	Follicular cell hypertrophy	2	1	0	4	0	1	2	0
	Colloid alteration (total)	9	11	23	24	2	11	21	19
	Grade 1	9	8	11	4	2	10	9	7
	Grade 2	0	3	9	8	0	0	5	4
	Grade 3	0	0	3	8	0	1	7	7
	Grade 4	0	0	0	4	0	0	0	1
	Pigment deposition	0	0	5	17	0	1	8	8

Source: Eiben, 2005

Offspring findings

Pyrasulfotole did not affect the number of implantations or prenatal loss. There were no effects at any dose level on the live birth index or on the ratio of males to females. There was a treatment-related decrease in viability, and consequently the mean litter size on postpartum (PP) day 4 in F2 pups at 3000 ppm. The slight decreases in viability indices seen at 30 and 300 ppm were generally within the historical control range and at 30 ppm were due to total litter loss of two dams before LD 4, therefore were not treatment-related.

There was no effect on anogenital distance in either males or females in the F2 generation.

The only treatment-related clinical sign in pups was the finding of animals cold to the touch, which was only increased in F1 pups at 3000 ppm where it occurred in 12 pups compared to five in the control. There were no treatment-related findings in F2 pups.

Ophthalmological examination of pups at the time of weaning on postnatal day (PND) 28 showed a dose-related increased incidence of corneal opacity and/or corneal neovascularization at 300 and 3000 ppm in both F1 and F2 pups.

Both litter body weight (mean body weight for males and females taken together) and pup body weight (mean body weight for males or females, taken separately) were decreased (with statistical significance) in the F1 and F2 pups at 3000 ppm at weaning on day 28. In F2 females at 3000 ppm body weight was also decreased to a statistically significant extent at LD 21.

Table 17. Litter parameters after dietary administration of pyrasulfotole to parental animals

	Dose group (ppm)							
	F1 pups				F2 pups			
Parameter	**0**	**30**	**300**	**3000**	**0**	**30**	**300**	**3000**
Mean implantations	12.42	12.09	12.05	12.27	11.58	12.83*	12.35	10.83
Mean prenatal loss	0.96	1.36	1.64	1.32	0.95	1.67	1.04	1.17
Number born	275	236	229	241	202	268	260	174
Number born dead	1	6	5	4	1	4	1	7
% males on day 0	48.86	51.98	52.33	50.39	50.99	52.48	51.52	49.21
Viability index	92.24	89.86	92.94	93.35	92.45	83.79	78.89	71.60
Lactation index	97.83	95.75	88.47	89.29	98.61	96.54	92.88	89.06
Mean litter size:								
Day 0	11.38	10.45	10.18	10.77	10.58	11.00	11.26	9.28
Day 4, pre culling	11.04	10.24	9.45	10.05	10.28	10.09	10.25	7.88
Day 4, post culling	7.74	7.71	7.45	7.91	8.00	7.45	7.40	6.56*
Day 21	7.61	7.48	7.10	7.09	7.89	7.14	6.95	6.40
Live birth index	99.31	97.34	97.97	98.47	99.56	98.46	99.60	96.59
Ophthalmological findings in pups								
Diffuse corneal opacity (%)	0	0	0	10.5	0	0	2.6	12.5
Reticulate corneal opacity (%)	0	0	4.9	30.2	0	0	0	8.9
Cornea neovascularization (%)	0	0	0	2.3	0	0	14.5	50.0
Mean litter weight, males and females combined (g)								
Lactation day 0	5.40	5.52	5.84*	5.60	4.41	5.69	5.66	5.79
Lactation day 4, post culling	8.63	8.93	9.24	8.71	8.84	9.02	8.36	8.26
Lactation day 7	14.06	14.09	14.16	13.28	13.85	14.40	12.95	12.82
Lactation day 28	71.79	75.43	73.67	66.13**	76.81	79.96	75.72	68.31**
Pup body weight, males (g)								
Lactation day 0	5.52	5.67	6.00*	5.78	5.59	5.85	5.85	5.96
Lactation day 28	74.93	78.09	76.07	68.46**	80.16	82.85	78.78	72.86**
Pup body weight, females (g)								
Lactation day 0	5.28	5.40	5.65	5.44	5.21	5.54	5.47	5.65
Lactation day 21	40.63	42.99	42.64	39.09	43.39	45.80	42.90	38.66**
Lactation day 28	68.72	72.15	70.89	64.23**	73.67	77.05	71.47	64.41**
Necropsy findings								
Autolysis, *n*	3	0	1	1	1	0	3	5
[affected pups per litter, %]	[4.63]	[0.0]	[0.79]	[0.51]	[0.48]	[0.0]	[2.38]	[5.56]
No milk in stomach, *n*	5	4	1	5	1	1	2	4
[affected pups per litter, %]	[3.92]	[5.41]	[0.79]	[5.56]	[5.26]	[2.08]	[1.19]	[10.29]
Dilated and/or enlarged kidney, *n*	1	1	1	3	1	1	1	3
[affected pups per litter, %]	[0.35]	[0.45]	[0.79]	[2.02]	[0.40]	[0.83]	[0.37]	[1.96]

* $p < 0.05$. ** $p < 0.01$; Source: Eiben, 2005

Investigations of developmental landmarks, only conducted for F1 pups, revealed a treatment-related, statistically significant delay in preputial separation at 300 and 3000 ppm the age for which was 44.2 and 46.3 days, respectively compared to 41.0 days for the control. A marginal delay in vaginal opening at 3000 ppm (34.2 days compared to 32.6 days for control) was also observed. The corresponding body weights for preputial separation were higher at 300 and 3000 ppm compared to controls, but given the lack of a dose–response relationship any correlation between the delay in these landmarks and body weight was unclear. However, there were clear effects on pup body weight gains at 3000 ppm as shown by the day 28 body weights.

There were no treatment-related effects on organ weights in either F1 or F2 weanlings. Absolute brain weight was decreased in male and female F1 and F2 weanlings (decrease of 4 to 7% compared to controls; statistically significant). However, relative brain weight was unaffected, thus the decrease in absolute brain weight was considered due to decreased body weight. Absolute spleen weights were decreased in F1 male and female weanlings at 3000 ppm (15% and 14% decrease compared to control; statistically significant), and in males the relative spleen weight was also slightly decreased (–10% compared to control; statistically significant). However, this effect was not observed in the F2 weanlings and thus was considered not to be treatment-related.

Macroscopic examinations revealed autolysis and no milk in the stomach corresponding with the increased mortality during lactation seen in the F2 pups at 3000 ppm. The incidence of dilated and/or enlarged kidneys was increased at 3000 ppm in F1 and F2 pups.

No NOAEL for parental systemic toxicity could be set in this study. In males at all dose levels tested, thyroid weight was increased and histopathological changes were seen (pigment deposition and colloid alteration). Therefore, the LOAEL was 30 ppm (equal to 2.5 mg/kg bw per day), although the toxicological significance of these changes was considered equivocal. The NOAEL for offspring toxicity was 30 ppm (equal to 2.0 mg/kg bw per day) based on a delay in balanopreputial separation in F1 weanlings at 300 ppm (equal to 22 mg/kg bw per day). The NOAEL for reproductive toxicity was 3000 ppm (equal to 272 mg/kg bw per day), the highest dose tested in the absence of treatment-related adverse effects (Eiben, 2005).

(b) Developmental toxicity

Rat

In a developmental toxicity study, pyrasulfotole (95.7% purity) was administered to groups of 25 Sprague Dawley rats daily by gavage from GDs 6 to 20 at the following doses: 0, 10, 100 or 300 mg/kg per day in 0.5% aqueous methylcellulose. Clinical signs were monitored daily, and morbidity and mortality were checked twice daily on weekdays and once daily on weekends. Body weights were measured on GDs 0, 6, 8, 10, 12, 14, 16, 18 and 21. Food consumption was measured for the periods GDs 1–6, 6–8, 8–10, 10–12, 12–14, 14–16, 16–18 and 18–21. On GD 21, the rats were sacrificed and subjected to macroscopic examination. The number of ribs was counted, and livers weighed. Gravid uterine weight was measured, and the number of corpora lutea, implantations, resorptions, and live and dead fetuses counted. The sex and individual body weights of live fetuses were also determined. Live fetuses were killed by subcutaneous injection of sodium pentobarbital and examined for external observations. Approximately half of the fetuses from each litter were preserved for freehand sectioning, while the other half were skinned, eviscerated, and stained in alizarin red S and alcian blue. Fetal observations were classified as common variants (changes occurring in more than approximately 5% of the control population), minor anomalies (slight, relatively rare structural changes that are not obviously detrimental), and malformations (very rare or obviously lethal changes).

There were no treatment-related mortalities during the study. One control female was sacrificed for humane reasons on GD 17 due to accidental trauma. Clinical signs related to treatment were observed at 100 and 300 mg/kg bw per day, and included increased salivation, anogenital soiling, intense yellow urine and vaginal discharge.

There was no effect of treatment on pregnancy rate in any group.

With gavage administration of pyrasulfotole at 300 mg/kg bw per day, maternal body weight was slightly decreased during the latter part of gestation. Body weight change was decreased at 100 and

300 mg/kg bw per day (statistically significant) in the interval GDs 6-8. Thereafter body weight change was similar across all groups. Maternal corrected body weight change was reduced to a statistically significant extent at 300 mg/kg bw per day. There was a treatment-related, biologically and/or statistically significant decrease in food consumption compared to controls at 300 mg/kg bw per day between GDs 6 and 16.

The only treatment-related finding at gross necropsy of the dams was in one animal at 300 mg/kg bw per day, which showed yellow sediment in the kidney and gritty content in the urinary bladder. Liver weight was increased in the 300 mg/kg bw per day group (+6%; statistically significant) and slightly increased at 100 mg/kg bw per day (+5%) compared to liver weight in control rats.

There were no treatment-related effects on the number of corpora lutea, implantation sites, either early or late resorptions, number of live fetuses, male/female ratio or percent pre- or post-implantation loss per litter. Fetal body weight was biologically and/or statistically decreased to a significant degree at 100 and 300 mg/kg bw per day.

Table 18. Maternal and fetal findings in developmental rat study with pyrasulfotole

		Dose group (mg/kg bw per day)			
		0	**10**	**100**	**300**
Clinical signs in dams					
Salivation		0	0	7	25
Anogenital soiling		0	0	0	4
Intense yellow urine		0	0	0	7
Vaginal discharge		0	0	0	5
Pregnancy rate					
		100%	96%	92%	100%
Maternal body weight and body weight gain (g)					
Body weight	GD0	256.0	257.8	259.2	259.4
	GD6	293.3	295.3	296.0	292.0
	GD8	299.7	301.3	299.0	293.0
	GD10	310.3	312.0	310.0	303.4
	GD21	431.6	439.2	428.4	420.4
Body weight gain	GD 0 – 6	37.3	37.5	36.9	32.6
	GD 6 – 8	6.4	6.0	3.0*	1.0**
	[% compared to control]		[–6%]	[–53%]	[–84%]
	GD 8 - 10	10.6	10.8	11.0	10.4
	Corrected body weight gain	72.8	74.9	70.0	61.3*
Food consumption (g/day)					
GD 1–6		26.2	26.3	26.5	26.0
GD 6–8		26.7	26.2	24.9	22.7**
GD 8–10		26.8	27.2	26.7	24.8*
GD 10–12		27.2	27.9	26.6	26.1
GD 12–14		27.5	27.5	26.5	25.9*
GD 14–16		28.0	28.1	27.5	26.8
GD 16–18		29.9	31.0	30.5	29.8
GD 18–21		30.1	30.6	29.9	29.1

	Dose group (mg/kg bw per day)			
	0	10	100	300
Uterine contents and fetal weights				
Number of corpora lutea per dam	17.2	17.0	16.6	16.9
Number of implantation sites per dam	16.0	15.2	14.5	15.1
Pre-implantation loss (%)	6.2	10.0	12.2	10.5
Post-implantation loss (%)	13.1	5.6	7.0	7.0
Number ofearly resorption per litter	2.0	0.8	1.0	0.9
Number of late resorption per litter	0.1	0.0	0.0	0.0
Number of live fetuses per litter	13.9	14.4	13.5	14.1
Number of of live fetuses	333	345	311	353
Number of of dead fetuses	1	0	0	1
Percent male fetuses	49.7	54.2	51.0	47.3
Fetal body weight (g)	5.44	5.48	5.32	5.14**
Male fetal bw (g)	5.58	5.60	5.45*	5.28**
Female fetal bw (g)	5.30	5.34	5.18	5.01**
Fetal skeletal observations: fetus (litter)				
Presence of 27 presacral vertebrae	0	0	1(1)	2(1)
7th cervical centrum, unossified	19 (10)	19 (14)	40 (18)	63 (18)
5th and/or 6th sternebrae: incomplete ossification or bipartite	21 (11)	29 (11)	44 (20)	66 (22)
14th thoraric rib: short	2 (1)	2 (1)	13 (7)	19 (10)
5th metacarpal incomplete ossification	1 (1)	0 (0)	2 (2)	9 (5)
1st metatarsal unossified	0 (0)	11 (6)	20 (9)	18 (9)

GD: Gestation day; * $p < 0.05$; ** $p < 0.01$ Source: Wason, 2006b

A total of four fetuses at 300 mg/kg bw per day from two different litters presented a malformation of hindpaw (polydactyly). The low incidence of this finding, the lack of other related findings in either forepaws or hindpaws, and the lack of ossification of the extra phalanges in the two fetuses (of the four involved) which were examined by skeletal staining, suggest that this malformation was a chance finding.

One fetus at 10 mg/kg bw per day had a malformation (umbilical hernia), which was considered not related to treatment. Three of the fetuses with hindpaw polydactyly (300 mg/kg bw per day), all of which were in the same litter, also showed an anomaly of forelimb hyperflexion, misshapen digits on the forepaws, and malpositioning of the digits on both the forepaws and the hindpaws. There were no other treatment-related anomalies or variants in any dose group.

Examination of the fetuses for visceral findings revealed no treatment-related findings.

Fetal skeletal examinations revealed two of the four fetuses with the external observation of hindpaw polydactyly also had malformations of short tibia and femur, supernumerary cartilaginous phalanges, fused cartilage for several metatarsals and phalanges, and an unossified fifth metatarsal. These findings were considered unrelated to treatment because of their low incidence. The only anomaly which was evaluated as related to treatment was an increase at 300 mg/kg bw per day of 27 presacral vertebrae. Treatment-related variants observed in this study were primarily linked to ossification; such incidences appeared to be increased by 100 and 300 mg/kg bw per day treatment with pyrasulfotole.

The NOAEL for maternal toxicity was 300 mg/kg per day, the highest dose tested. The embryo/fetal NOAEL was 10 mg/kg per day based on delayed ossification and reduced fetal weights observed at 100 mg/kg bw per day (Wason, 2006b).

Rabbit

Groups of 25 mated New Zealand White rabbits were exposed to pyrasulfotole (95.7% purity) by gavage from GD 6 to 28. The doses given were 0, 10, 75 or 250 mg/kg bw per day in suspension in aqueous solution of 0.5% methylcellulose. Clinical signs were monitored and animals were checked twice daily on weekdays and once daily on weekends and holidays for morbidity or mortality. Body weight was measured on GDs 3, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 29. Food consumption was measured on these days as well as for the periods GD 3–4, 4–5 and 5–6. On GD 29 the rabbits were sacrificed and subjected to macroscopic examination. Liver weight and the number of ribs were noted for each animal, and kidney and urinary bladder were examined for the presence of gritty material. Gravid uterine weight, number of corpora lutea, number of implantations, resorptions, and live and dead fetuses, and the individual weight of live fetuses, were recorded for each animal. Live fetuses were sacrificed and examined externally. The heads of approximately half the fetuses were fixed for later examination. All fetuses were dissected for visceral findings, fixed and stained for skeletal examination. Fetal observations were classified as common variants (changes occurring in more than approximately 5% of the control population), minor anomalies (slight, relatively rare structural changes that are not obviously detrimental) and malformations (very rare or obviously lethal changes)

There were no mortalities or abortions during the study. Clinical signs included yellow or beige sediments in the urine of females at 75 and 250 mg/kg bw per day noted on several occasions, with a greater incidence at 250 mg/kg bw per day. Since pyrasulfotole is excreted in the urine and forms a yellow sediment, the occurrence of such sediment was clearly treatment-related, however its presence was seen as an indication of compound excretion rather than as an adverse effect in itself.

Gavage administration of pyrasulfotole had no effect on pregnancy rate, with rates ranging from 88% to 96%. Maternal body weight gain at 250 mg/kg bw per day was reduced between days 8 and 10 to a statistically significant extent, with an overall reduction between days 6 and 29 of 31% compared to controls. Corrected maternal body weight at 250 mg/kg bw per day was slightly decreased (not statistically significant) compared to controls. At 250 mg/kg bw per day food consumption was consistently reduced compared to controls, with the reduction achieving statistical significance on several occasions. The liver weight of the dams was increased, with statistical significance, by 22% compared to controls at 250 mg/kg bw per day. Prominent lobulation of the liver was also noted at 250 mg/kg bw per day in two out of 25 females.

At 250 mg/kg bw per day, fetal body weight was decreased to a statitically significant degree both for males and females and combined sexes. Although combined-sexes fetal weight was reduced at both 10 and 75 mg/kg bw per day, these decreases did not show a dose–response relationship and were therefore considered not to be related to treatment. There was no effect of treatment on the number of corpora lutea, implant sites, early or late resorptions, number of live or dead fetuses or percent pre- or post-implantation losses per dam. Neither was there an effect with any group on the percentage of male fetuses.

Table 19. Maternal and fetal findings in developmental rabbit study with pyrasulfotole

	Dose group (mg/kg bw per day)			
	0	**10**	**75**	**250**
Clinical signs in dams				
Intense yellow sediment in urine	0	0	1	25
Few faeces	0	2	3	5
Beige sediment in urine	0	0	1	0
Pregnancy rate				
	96%	92%	92%	88%

		Dose group (mg/kg bw per day)			
		0	10	75	250
Maternal body weight and body weight gain (kg)					
Body weight	GD 3	3.46	3.47	3.50	3.49
	GD 6	3.49	3.52	3.52	3.51
	GD 8	3.50	3.53	3.53	3.51
	GD 10	3.53	3.56	3.55	3.51
	GD 29	3.84	3.85	3.88	3.75
Body weight gain	GDs 3–6	0.03	0.04	0.02	0.01
	GDs 6–8	0.01	0.02	0.02	0.00
	GDs 8–10	0.03	0.02	0.02	0.00**
	GDs 10–14	0.07	0.07	0.07	0.05
Maternal food consumption (g/day)					
GD 3–6		169.3	175.4	173.1	167.8
GD 6–8		169.5	168.1	165.7	144.0**
GD 8–10		171.5	165.7	169.5	143.9**
GDs 10–14		161.0	156.4	150.8	131.2**
GDs 14–18		158.0	146.0	147.7	120.5**
GDs 18–22		162.7	153.4	151.3	130.8**
GDs 22–26		139.0	131.9	134.2	117.6
GDs 26–29		124.9	117.8	127.7	114.2
Uterine findings and fetal weights					
Number of corpora lutea per dam		11.4	12.0	12.0	12.5
Number of implantation sites per dam		9.3	10.2	9.9	10.1
Pre-implantation loss (%)		19.7	14.8	17.2	18.0
Post-implantation loss (%)		10.4	15.7	8.7	7.1
Number of early resorption per litter		0.5	1.2	0.6	0.4
Number of late resorption per litter		0.2	0.0	0.1	0.0
Number of live fetuses per litter		8.3	8.6	9.0	9.4
Number of of live fetuses		198	197	208	206
Number of of dead fetuses		6	10	5	8
Percent of male fetuses		50.6	46.9	48.5	51.9
Fetal body weight (g)		38.2	36.4*	36.2**	32.5**
Male fetal body weight (g)		38.1	36.4	36.8	32.6**
Female fetal body weight (g)		38.3	36.4	35.6	32.3**

GD: Gestation day; Source: Wason, 2006c

* $p < 0.05$, ** $p < 0.01$

There was no effect of treatment on the incidence of external malformations. Two fetuses were observed to have malformations, one at 75 mg/kg bw per day with umbilical hernia and one in the control group one in the control group which was acaudate and showed gastroschisis median, spina bifida and hindlimb hyperflexion. As these findings showed no dose–effect relationship they were considered not to be treatment-related. There was a biologically significant increase in the incidence of runt fetuses at 250 mg/kg bw per day when compared to control animals: 45 fetuses in 13 litters in the 250 mg/kg bw per day group compared with 13 fetuses in six litters in the control group.

There were no findings from visceral examinations which indicated any effect of treatment in any treatment group.

Skeletal examinations revealed patterns of altered ossification and minor skeletal variants and anomalies. At 250 mg/kg bw per day these changes consisted of increased incidences of 13th thoracic rib (unilateral/bilateral) with the presence of 27 presacral vertebrae, atlas centrum unossified, an extra ossification site between the atlas and axis, 13th thoracic rib (unilateral or bilateral) or 13th thoracic rib (bilateral, short unilateral), 1st metacarpal incompletely ossified or unossified, insertion point(s) of pelvic girdle on second sacral vertebra, and bilateral incomplete ossification of the pubis. At 75 mg/kg bw per day these changes included an increased incidence of 13th thoracic rib (unilateral/bilateral) with the presence of 27 presacral vertebrae, extra ossification site between the atlas and axis, 13th thoracic rib (unilateral/bilateral) or 13th thoracic rib (bilateral, short unilateral), and insertion point(s) of pelvic girdle on second sacral vertebra. There were no treatment-related visceral or skeletal findings at 10 mg/kg bw per day.

The NOAEL for maternal toxicity was 75 mg/kg bw per day based on decreased food consumption and body weight gain. The embryo/fetal NOAEL was 10 mg/kg bw per day based on altered ossification patterns and minor skeletal variants and anomalies (Wason, 2006c).

2.6 Special studies

(a) Neurotoxicity

Rat

Study 1

The acute neurotoxicity of pyrasulfotole (95.7% purity) was investigated in Wistar rats (12/sex per dose) at doses of 0, 200, 500 or 2000 mg/kg bw, administered by oral gavage suspended in an aqueous solution of 0.5% methylcellulose containing 0.4% Tween 80.

In a preliminary ADME study (Totis, 2002) the time after oral gavage dosing of pyrasulfotole to the peak blood concentration of the test substance was found to be 30 minutes in both males and females. Accordingly, conduct of a functional observational battery (FOB) and motor activity studies began approximately, but no less than, 30 minutes after dosing, and was concluded approximately 2.5 hours after treatment. The full schedule of FOB and motor activity assessments comprised testing one week prior to dosing, approximately 30 minutes after dosing, and on days 7 and 14 after dosing. The FOB included standard parameters including home cage and open field observations, reflex testing, and determination of forelimb and hindlimb grip strength, and landing foot splay. Motor and locomotor activity were measured according to standard methods over a 60-minute period for each animal. On day 14 after dosing all animals were sacrificed. Half of the animals at each dose level were anaesthetized (50 mg/kg bw pentobarbital by intraperitoneal injection) and perfused via the left ventricle with a sodium nitrite flush followed by fixative in phosphate buffer. The brain was weighed, and the brain, spinal cord, eyes with optic nerve, sciatic, tibial, and sural nerves, Gasserian ganglia, gastrocnemius muscle and both forelimbs were removed and preserved for examination, as were any gross lesions. The remaining 50% animals at each dose level were not perfused following sacrifice. All animals were subjected to a gross necropsy. Histopathology was conducted on the nervous tissues from the six perfused animals in the control and high-dose groups only. Cageside observations were performed at least once daily for mortality and any clinical signs of moribundity. Animals were weighed weekly as a component of the FOB. Additionally, all animals were weighed on the day of sacrifice. Food consumption and cholinesterase activity were not measured in this study.

There were no mortalities at any dose level. Treatment-related clinical signs were limited to urine stains, nasal and oral stains, and stained forepaws. These stains were seen in all treatment groups, with a general dose-related incidence, and were attributed to the excretion of pyrasulfotole in the urine leading to intense yellow/orange coloration of the urine. The staining had reversed in almost all animals by the time of sacrifice. As the staining was due to compound excretion, it was considered not to be an adverse or toxicologically significant finding.

There were no effects on body weight in either males or females at any dose level.

There were no effects on any parameters of the functional observation battery, including grip strength and landing foot splay, in any dose group in either males or females.

Analysis of the pretreatment motor and locomotor activity measurements in groups which were later administered pyrasulfotole showed that a variation of ±20% in motor and locomotor activity were within the normal range and differences after treatment that fall within this range were considered not treatment-related. Motor and locomotor activity were decreased with biologically and/or statistical significance in both males and females at 2000 mg/kg bw on day 0 only. There were no other treatment-related effects on either parameter at any other dose level, or at any dose level on day 7 or day 14 after dosing.

Table 20. Motor and locomotor activity (total activity counts for session) and percent difference from control in male and female rats administered pyrasulfotole by oral gavage

	Dose group (mg/kg bw/day)							
	Male				Female			
Day	**0**	**200**	**500**	**2000**	**0**	**200**	**500**	**2000**
	Motor activity, total activity counts per session							
Pretest	424±195	531±180	383±129	529±246	631±207	538±239	697±165	617±226
Day 0	411±290	466±87	402±139	307±113	488±161	357±109	421±99	306±85
Day 7	434±131	436±89	416±138	448±118	538±176	393±135	405±136	414±119
Day 14	413±158	395±117	350±98	445±111	527±165	439±92	486±143	463±74
	Locomotor activity, total activity counts per session							
Pretest	219±101	291±100	216±83	289±123	265±69	303±107	303±56	290±95
Day 0	232±148	282±49	218±69	153±49	323±129	218*±81	229*±64	161*±53
Day 7	293±112	299±83	274±97	194±92	343±131	239±95	245±106	261±82
Day 14	269±116	264±88	244±84	307±102	335±102	265±70	301±94	299±83
	Motor activity, % change relative to control							
Pretest	-	+25	–10	+25	-	–15	+10	–2
Day 0	-	+13	–2	–25	-	–27	–14	–37
Day 7	-	+0.5	–4	+3	-	–27	–25	–23
Day 14	-	–4	–15	+8	-	–17	–8	–12
	Locomotor activity, % change relative to control							
Pretest	-	+33	–1	+32	-	–13	+14	+9
Day 0	-	+22	–6	–34	-	–33*	–29*	–50*
Day 7	-	+2	–6	+0.3	-	–30	–29	–24
Day 14	-	–2	–9	+14	-	–21	–10	–11

* $p < 0.05$, ** $p < 0.01$ Source: Gilmore & Sheets, 2005

There was no effect on brain weight (the only organ weighed) at any dose of pyrasulfotole in either males or females. There were no treatment-related macroscopic findings observed at any dose in either males or females. Nor were there any treatment-related effects observed at 2000 mg/kg bw when compared to controls, therefore histopathological sections were not made at either 200 or 500 mg/kg bw.

In this study the NOAEL was 500 mg/kg bw based on decreased motor activity and locomotor activity (both sexes) at 2000 mg/kg bw (Gilmore & Sheets, 2005).

Study2

In a sub-chronic assessment of the potential neurotoxicity of pyrasulfotole (95.7% purity), groups of 12 male and 12 female Wistar rats were given pyrasulfotole by dietary administration at levels of 0, 500, 2500 or 5000 ppm for 13 weeks (equal to 0, 32.3, 166 and 345 mg/kg bw per day for males, 0, 41.9, 206, and 416 mg/kg bw per day for females).

Clinical signs were assessed twice daily on weekdays and once daily on weekends and holidays. Body weight and food consumption were measured on a weekly basis.Motor activity and FOB assessments were made on five occasions, once in the week prior to the start of the feeding period, and once each during weeks 2, 4, 8 and 13. The FOB included standard parameters including home cage and open field observations, reflex testing, and determination of forelimb and hindlimb grip strength, and landing foot splay. Motor and locomotor activity were measured according to standard methods over a 60-minute period for each animal. Ophthalmological examinations were conducted in all animals prior to the start of the study and during week 12. After at least 90 days dietary administration of pyrasulfotole all animals were sacrificed. Half of the animals at each dose level were anaesthetized and perfused via the left ventricle with a sodium nitrite flush followed by fixative in phosphate buffer. The brain was weighed, and brain, spinal cord, eyes with optic nerve, sciatic, tibial and sural nerves, Gasserian ganglia, gastrocnemius muscle and both forelimbs were removed and preserved for examination, as were any gross lesions. The other 50% of the animals at each dose level were not perfused. All animals were subjected to a gross necropsy. Histopathology was conducted on the nervous tissues from the six perfused animals in the control and high-dose groups only.

There were no mortalities in either males or females during the study. Treatment-related clinical signs were limited to urine staining in both males and females, largely occurring in a dose-related manner. The yellow to brownish-orange stains observed were judged to be due to urinary excretion of pyrasulfotole and were considered not to be indicative of toxicity.

There was no treatment-related effect on body weights or body weight gain in either males or females at any dose level. There was no treatment-related effect on food consumption in either males or females at any dose level. Food consumption was increased to a statistically significant extent in males at 5000 ppm in the periods day 21–28 and 28–35, and the periods day 70–77 through to day 77–84. However, this was considered not to be indicative of a toxic effect as corresponding body weights were unaffected in these males and no comparable increase in food consumption was observed in females at 5000 ppm.

Ophthalmological examination revealed no treatment-related effects.

There was no effect on any parameter of the FOB, including grip strength and landing foot splay, in either males or females at any point during the study.

There were no treatment-related alterations in either motor or locomotor activity in either males or females at any dose. There were occasional (statistically nonsignificant) increases in motor or locomotor activity at various time points, but they followed no dose-related pattern, were generally limited to one sex and were opposite to the treatment-related change observed in the acute neurotoxicity study. Therefore, these increases were considered not to be treatment-related.

There was no effect on brain weight in either males or females at any dose. There were no treatment-related gross lesions or other macroscopic observations in either male or female rats. There were no histopathological findings relative to controls which would be indicative of a treatment-related effect in male or female rats at 5000 ppm. In the absence of any treatment-related effect at 5000 ppm, tissues were not examined at lower doses.

There were no indications of a neurotoxic effect due to pyrasulfotole after 90 days dietary administration, nor were there effects on body weight or food consumption, therefore the NOAEL for this study was the top dose of 5000 ppm (equal to 345 mg/kg bw per day) (Gilmore & Hoss, 2005).

Study 3

In a developmental neurotoxicity screening study pyrasulfotole (96.2% purity), was administered in the diet to groups of 30 sperm-positive female Wistar rats from GD 6 until LD 21 at concentrations of 0, 45, 450 or 4500 ppm. Dietary concentrations were adjusted during lactation to provide a constant dosage throughout the treatment period. These concentrations corresponded to an average daily intake of 0, 3.8, 37.1 and 354 mg/kg bw per day.

Dams were observed for clinical signs at least once daily. Body weight and food consumption were measured on a weekly basis, on GDs 6, 13 and 20 and LDs 0, 7, 14 and 21. Maternal body weight was also measured on LD 4. A functional observation battery was conducted on pregnant females on GDs 13 and 20, and for 10 dams/dietary level on LDs 11 and 21. Litters were culled to eight pups on LD 4, with four male and four female pups wherever possible. Dams were sacrificed on LD 21 following weaning of their litters, and a gross necropsy examination conducted.

As soon as possible after pup birth, anogenital distance was measured and pups were tattooed. Surviving pups were counted, sexed, and weighed individually on LDs 0, 4, 11, 17 and 21. Offspring were monitored daily throughout lactation for clinical signs or morbidity. After weaning on day 21 the pups were monitored twice daily for morbidity and mortality and were weighed on a weekly basis as well as on the day that vaginal patency or balanopreputial separation was achieved. Food consumption was not measured. Pups were examined daily starting from PND 29 (females) or PND 38 (males) for developmental landmarks. Pupil constriction was tested in all pups on PND 21. Detailed clinical observations and FOB were conducted on PNDs 4, 11, 21, 35, 45 and 60. Motor activity was assessed on PNDs 13, 17, 21 and 60. Auditory startle habituation was examined on PND 22. Learning and memory were assessed on PNDs 22, 29, 60 and 67. Brain weight and neuropathology were examined either on PND 21 or 75. All tests used at least 10 offspring/sex per dose, and with the exception of learning and memory, no animal was tested more than once in the same test.

At least 10 male and 10 female offspring per dose were selected for ophthalmological examination at approximately 50–60 days old. On PND 21, ten male and ten female offspring were sacrificed, and their brains collected whole for micropathological examination and morphometric analysis. On PND 75 a further 10 male and 10 female offspring were sacrificed and perfused; from these brain, spinal cord, both eyes with optic nerves, bilateral sciatic, tibial and sural nerves, Gasserian ganglia, gastrocnemius muscle and both forelimbs were collected and fixed. Brains were weighed. In both cases the anterior–posterior length of the cerebrum and of the cerebellum were measured with vernier callipers prior to sectioning. Other measurements made after histologic sectioning were the thickness of the frontal cortex, parietal cortex and hippocampal gyrus, horizontal width of the caudate putamen and height of the cerebellum. The offspring not selected for neuropathological examination were sacrificed without examination.

Maternal findings

There was no mortality amongst parental animals during either gestation or lactation, nor were there any treatment-related clinical signs observed during gestation. During lactation, ocular opacities were observed in five females at 450 ppm and in 14 females at 4500 ppm. During the FOB, ocular opacities were observed in three females at 450 ppm and seven females at 4500 ppm.

Body weight and body weight gain were not affected during gestation or lactation at any dose. Food consumption was increased at 4500 ppm during gestation, although statistically significant this finding was considered to be due to wastage due to palatability issues. Installation of grates to reduce wastage in week 2 of gestational treatment markedly reduced food consumption at 4500 ppm. During lactation food consumption was reduced to a statistically significant extent at both 450 ppm (LDs 0–7 and 7–14) and at 4500 ppm (all periods). These reductions were attributed to unpalatability as there was no effect on body weight at either dose.

A treatment-related (but not statistically significant) decrease in fertility index compared to controls was noted at 4500 ppm which rated 86.7 compared to 100 in the control group. There were no other effects on reproductive parameters.

Offspring findings

No effects due to treatment were observed on litter size, viability or other litter parameters. There were no treatment-related clinical signs observed during lactation in either males or females. In the postweaning phase, treatment-related findings were restricted to ocular opacities in six males and one female at 4500 ppm, and two females at 450 ppm. There was no effect due to dietary administration of pyrasulfotole on the incidence of moribund pups or pups found dead.

Body weight at birth was similar at all doses. On PND 4 (after culling) body weight was decreased in males at 4500 ppm (statistically significant), while females showed a statistically not significant decrease in body weight at 4500 ppm, as did both sexes at 450 ppm. Body weight and body weight gain were reduced to a statistically significant extent in males and females combined at 450 and 4500 ppm from PND 11 to PND 21. After weaning, decreased body weight continued in males, with statistical significance at 450 and 4500 ppm, until the end of the study. Similarly for females following weaning, statistically significant decreases in body weight were seen at 450 ppm for the first three weeks after weaning, and at 4500 ppm for the first two weeks after weaning. Differences from control body weights during lactation reached a maximum of 7% at 450 ppm and 11% at 4500 ppm. Body weight gain was also reduced, by 8% relative to controls at 450 ppm and by 12% at 4500 ppm. After weaning, body weight

reduction in males showed a maximum of 9% at 450 ppm and 13% at 4500 ppm. In females, body weight was reduced by a maximum of 8% at 450 ppm and 11% at 4500 ppm. These effects on body weight were considered to be treatment-related.

Preputial separation was delayed to a statistically significant extent at 4500 ppm (mean 46.7 days) and delayed but not significantly at 450 ppm (mean 46.0 days); mean for controls was 44.1 days. This increase in time to preputial separation was considered as related to treatment and a secondary effect of reduced body weight. There was no effect on time to vaginal patency. Pupillary constriction on day 21 in both males and females was not affected by treatment.

The only treatment-related changes at FOB assessment were ocular opacities in one female at 450 ppm and one female at 4500 ppm. These changes were first noted on PNDs 45 and 35, respectively, and persisted in both cases until PND 60. There were no treatment-related effects observed on either motor or locomotor activity in either males or females at any dose level during lactation or postweaning phases. There were no treatment-related effects on auditory startle at any time during the study at any dose level.

There was no effect of treatment during the acquisition phase of passive avoidance testing. However, during trial 1 of the retention phase, males at 4500 ppm showed a statistically significant decreased latency to crossing compared to controls. Females at 4500 ppm showed a similar, although statistically not significant, decrease in latency to crossing. This effect was considered to be related to treatment. Although there were other statistically significant differences observed during the passive avoidance testing, these were all considered unrelated to treatment as they were generally within values for historical control data, not related to treatment, and/or seen in one sex only.

Table 21. Passive avoidance performance, as measured on postnatal day 22, in male and female offspring of dams administered pyrasulfotole in the diet

		Dose group (ppm)			
		F0 animals			
Session	**Parameter**	**0**	**45**	**450**	**4500**
		Males			
1 (Learning)	Number tested	16	16	16	16
	Number included in analysis	16	16	16	16
	Trials to criterion	2.9	3.0	3.5*	3.4*
	Latency, trial 1 (s)	49.0	69.6	31.2	27.5
	Latency, trial 2 (s)	180	180	162.5	151.0*
	Failed to meet criterion	0	0	0	0
	Failed to cross during learning phase	2	0	0	0
2 (Retention)	Number tested	14	16	16	16
	Number included in analysis	14	16	16	16
	Trials to criterion	2.2	2.0	2.4	2.8*
	Latency, trial 1 (s)	169.4	180.0	175.4	137.7*
	Latency, trial 2 (s)	177.0	180.0	174.2	169.7
		Females			
1 (Learning)	Number tested	16	16	16	16
	Number included in analysis	16	16	16	16
	Trials to criterion	3.2	2.5	3.3	3.1
	Latency, trial 1 (s)	24.8	20.8	45.0	41.8
	Latency, trial 2 (s)	179.2	171.8	177.8	180.0
	Failed to meet criterion	0	0	0	0
	Failed to cross during learning phase	0	0	1	1

		Dose group (ppm)			
		F0 animals			
Session	**Parameter**	**0**	**45**	**450**	**4500**
2 (Retention)	Number tested	16	16	15	15
	Number included in analysis	16	16	14	15
	Trials to criterion	2.4	2.7	2.6	2.9
	Latency, trial 1 (s)	153.2	162.3	139.5	125.1
	Latency, trial 2 (s)	177.9	166.9	170.2	169.4

* $p < 0.05$, ** $p < 0.01$ Source: Gilmore, Sheets & Hoss, 2006

There were no treatment-related effects on acquisition and retention in either males or females during the water maze testing.

Ophthalmological examinations revealed a statistically significant increase in retinal atrophy at 4500 ppm, with four males and four females affected at this dose. This was considered to be a treatment-related finding. Retinal atrophy was also observed in three mid-dose females, but in none of the mid-dose males. There were no other findings at ophthalmological examination which were considered to be treatment-related.

Absolute brain weight was decreased with statistical significance for males and females at 4500 ppm , females at 450 ppm on day 21, and for females at 4500 ppm on day 75. Relative brain weight in these groups was no different from controls, indicating that the decreased absolute brain weight was related to the reduced body weight at these doses. At day 21, cerebellum length was decreased in males and females to a statistically significant degree at 4500 ppm, as was cerebrum length for females at 450 and 4500 ppm. At day 75 a statistically significant decrease in cerebellum length was seen in males at 4500 ppm; similarly in females at 4500 ppm, but in their case the decrease was not statistically significant. There was no effect on cerebrum length in either males or females at day 75. The decreases in cerebellum length observed on days 21 and 75 were considered treatment-related.

Micropathological brain measurements on day 21, showed a treatment-related, statistically significant decrease in cerebellum height in males and females at 450 and 4500 ppm. There were other, minor differences from the control in other micropathological brain measurements, but such minor findings were considered not to be treatment-related as there was no relationship to dose, the measurements were within historical control data values, and concurrent control data was either at the extreme end of the historical range or exceeded it. On day 75, cerebellar height was decreased to a statistically significant degree at 4500 ppm in males and females. There were no other treatment-related findings in either males or females.

Table 22. Brain measurements, from offspring sacrificed on postnatal day 21 or postnatal day 75

	Sex and dose level (ppm)							
	Male				Female pups			
Parameter	**0**	**45**	**450**	**4500**	**0**	**45**	**450**	**4500**
	Postnatal day 21 (measurements in mm)							
Anterior/posterior cerebrum: length	13.52	13.67	13.31	13.24	13.63	13.38	13.07*	13.00*
Anterior/posterior cerebellum: length	7.20	7.03	7.12	6.80*	7.13	4.06	6.83	6.65*
Frontal cortex	1.728	ND	1.769	1.805	1.823	ND	1.763	1.749
Parietal cortex	2.008	1.918	1.901	1.922	1.999	1.939	1.846	1.850*
Caudate putamen	2.982	ND	3.097	3.052	3.029	ND	2.974	2.915
Hippocampal gyrus	1.723	ND	1.673	1.451*	1.737	ND	1.696	1.575*
Cerebellum: height	4.367	4.165	4.057	4.005*	4.292	4.164	4.086	3.869

	Sex and dose level (ppm)							
	Male				Female pups			
Parameter	0	45	450	4500	0	45	450	4500
	Postnatal day 75 (measurements in mm)							
Anterior/posterior cerebrum: length	14.56	14.69	14.45	14.64	14.12	14.11	14.24	13.92
Anterior/posterior cerebellum: length	7.56	7.69	7.41	7.21*	7.57	7.52	7.47	7.25
Frontal cortex	1.760	ND	1.656	1.713	1.761	ND	1.662*	1.792
Parietal cortex	1.863	ND	1.808	1.880	1.855	ND	1.827	1.833
Caudate putamen	3.345	ND	3.302	3.424	3.386	ND	3.172*	3.330
Hippocampal gyrus	1.758	ND	1.790	1.684	1.631	ND	1.731	1.543
Cerebellum: height	3.831	ND	4.380*	3.618*	3.978	ND	4.488*	3.756*

ND: Not determined; * $p < 0.05$, ** $p < 0.01$ Source: Gilmore, Sheets & Hoss, 2006

On histopathological examination of the brains and other nervous tissues, the only treatment-related finding was retinal degeneration in one male at 4500 ppm.

The NOAEL for maternal animals administered pyrasulfotole by dietary incorporation was 45 ppm (equal to 3.8 mg/kg bw per day) based on a decreased fertility index and ocular opacity observed at 450 ppm (equal to 37.1 mg/kg bw per day). Please note that eye effects are further discussed in section 2.6(c) mechanistic studies. The embryo/fetal NOAEL was 45 ppm (equal to 3.8 mg/kg bw per day) based on decreased body weight, delayed preputial separation, decreased cerebrum and/or cerebellum length and decreased cerebellum thickness observed at 450 ppm (equal to 37.1 mg/kg bw per day) (Gilmore, Sheets & Hoss, 2006).

(b) Immunotoxicity

No specific studies regarding immunotoxicity were available for pyrasulfotole.

(c) Mechanistic studies

In vitro inhibition of 4-hydroxyphenylpyruvate dioxygenase in various species

The compound 2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione (NTBC) (purity 99.7%) was used as a model inhibitor of the 4-HPPDase enzyme and was dissolved in DMSO for incubation with liver cell preparations from the rat, mouse, dog, rabbit and human. The cell preparations were Liverbeads™, immobilized hepatocytes in an alginate matrix. The preparations were isolated from male Wistar rats, male CD-1 mice, male beagle dogs, male New Zealand White rabbits, and a liver sample from a female human. Liverbeads™ were incubated with buffer, buffer plus NTBC (30 µM), buffer plus tyrosine (550 µM), or buffer plus tyrosine (550 µM) plus NTBC (30 µM). After addition of NTBC or solvent to the incubation buffer, the plates were incubated for 0, 2, or 4 hours at 37°C. At the end of the incubation, the Liverbeads™ were dissolved, hepatocytes were sonicated, and the suspension was transferred to vials for storage at –80°C until analysed. Concentrations of *L*-tyrosine and of *p*-hydroxyphenyl lactic acid (HPLA) were determined by HPLC analysis. Protein concentration was measured on each hepatocyte sample using the Biorad method, with control Liverbeads (without cells) as a blank control value.

Tyrosine concentrations in the incubation medium did not change over the incubation time, nor was there any effect on tyrosine due to adding NTBC to the medium. Under basal conditions (no added tyrosine) the concentration of HPLA was below the limit of quantification (LOQ) in both absence and presence of NTBC in the rat, dog, and rabbit incubations. Concentrations of HPLA were below the LOQ in incubations with human Liverbeads™ under basal conditions, however incubation of the Liverbeads™ with NTBC for 2 or 4 hours increased HPLA concentration to quantifiable limits in a time-dependent manner. In the mouse incubation, low levels of HPLA were detected under basal conditions

but these did not increase with time of incubation. Addition of NTBC to the mouse incubation markedly increased HPLA concentrations and they proceeded in a time-dependent manner. When tyrosine was added to basal medium, HPLA concentrations were below the LOQ for rat, dog, rabbit, and human incubations. Low levels of HPLA , similar to those observed under basal conditions, were observed in mouse liverbead™ incubations. Addition of both tyrosine and NTBC to the incubation medium increased the production of HPLA in nearly all species. However, production of HPLA was still very low in the rat and rabbit, and no HPLA was observed at any time point in the dog incubations. By contrast, HPLA concentrations were markedly increased in both mouse and human incubations.

Table 23. Concentration, in µg/mg protein, of HPLA in Liverbeads™ incubations from rat, mouse, dog, rabbit, and human

		Species				
Conditions	**Time**	**Rat**	**Dog**	**Rabbit**	**Mouse**	**Human**
Basal	0	< LOQ	< LOQ	< LOQ	0.15	< LOQ
	2	< LOQ	< LOQ	< LOQ	0.24	< LOQ
	4	< LOQ	< LOQ	< LOQ	0.25	< LOQ
Basal + NTBC	0	< LOQ	< LOQ	< LOQ	0.18	< LOQ
	2	< LOQ	< LOQ	< LOQ	0.42	0.33
	4	< LOQ	< LOQ	< LOQ	0.69	0.54
Basal + tyrosine	0	< LOQ	< LOQ	< LOQ	0.17	< LOQ
	2	< LOQ	< LOQ	< LOQ	0.20	< LOQ
	4	< LOQ	< LOQ	< LOQ	0.26	< LOQ
Basal + tyrosine + NTBC	0	< LOQ	< LOQ	< LOQ	0.12	< LOQ
	2	0.19	< LOQ	< LOQ	0.73	0.54
	4	0.23	< LOQ	0.36	1.31	1.08

This study demonstrated that t in vitro there were marked differences among species in their ability to metabolise tyrosine both under basal conditions and in the presence of complete inhibition of the tyrosine catabolic pathway. The rat, dog, and rabbit preparations displayed a very low ability to produce HPLA, a diversionary metabolite that enables reduction of plasma tyrosine concentrations. By contrast, in mice and humans the production of HPLA was induced by addition of NTBC to the system. The production of HPLA under NTBC inhibition was also increased compared to basal conditions, when excess tyrosine was present (Totis, 2006).

The effect of tyrosinemia on pregnancy and embryo-fetal development in the rat

The compound 2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione (NTBC) (purity 99.7%) and *L*-tyrosine (purity > 99%) were administered singly or in combination to pregnant female Sprague Dawley rats (23 animals per group). NTBC was suspended in demineralized water and administered by oral gavage at 10 µg/kg bw per day on GDs 6 –20 inclusive, while *L*-tyrosine was incorporated into the diet at a concentration of 20 000 ppm, and a tyrosine-containing diet was provided to the relevant groups on GDs 6–21. The control group and the group receiving only *L*-tyrosine were administered demineralized water by oral gavage on GDs 6–20 as a control for NTBC administration.

Clinical signs, morbidity and mortality were monitored daily throughout gestation. Body weights and food consumption were measured on GDs 6, 8, l0, 12, 14, 16, 18 and 21. On GD 21, prior to sacrifice, blood samples were collected for determination of plasma tyrosine concentration. The rats were then sacrificed and subjected to gross necropsy, livers were weighed, and liver, thyroid, and pancreas were preserved for possible histopathological examination. The reproductive tract was weighed and the number of corpora lutea, implantations, resorptions, live and dead fetuses counted, and the sex and individual body weights of the live fetuses recorded. Live fetuses were then sacrificed by subcutaneous injection of sodium pentobarbital and examined externally. All fetuses were processed and double-stained with alizarin red S and alcian blue. A limited number of skeletal end-points were then evaluated from approximately half the fetuses in each litter. Fetal skeletal observations were classified as common

variants (changes occurring in more than approximately 5% of the control population), minor anomalies (slight, relatively rare structural changes that are not obviously detrimental), or malformations (very rare or obviously lethal changes).

One female in the NTBC group was sacrificed on GD 13, based on body weight loss of 21 g between GDs 10 and 12 as well as clinical signs. There were no macroscopic findings at autopsy, and there was no clear relationship between treatment and the clinical signs observed. There were no findings at clinical examination which could be related to treatment. There was no effect of treatment on pregnancy rate.

The administration of dietary *L*-tyrosine alone had no effect on maternal body weight or body weight gain. In the group administered NTBC by oral gavage there was a statistically nonsignificant reduction in body weight gain of 58% between GDs 6 and 8. Thereafter body weight gain in this group was comparable to the controls. In the NTBC+tyrosine group, reduction in body weight gain between GDs 6 and 8 was not statistically significant at 55%, with overall body weight gain between GDs 6 and 21 slightly reduced, but not with statistical significance, by 6%. Corrected body weight gain was similar for all groups.

There was a slight decrease in food consumption in the NTBC+tyrosine group, between GDs 18 and 21. Tyrosine intake was roughly similar in groups receiving diets containing tyrosine at 20 000 ppm

Necropsy of the dams revealed a very low incidence of mottled kidneys in all three treatment groups, but not in the controls. Four of the animals in the group receiving both tyrosine and NTBC showed minimal corneal opacity, which was not observed in any other treatment group. These findings were considered to be related to treatment. There was no effect of treatment on liver weight in any group.

Maternal plasma tyrosine concentrations were increased in all treatment groups when compared to controls, however the effect of either dietary tyrosine or NTBC by gavage alone was minimal compared to the effect of both tyrosine and NTBC together. Tyrosine concentrations were 46 nmol/mL in the control group, 216 nmol/mL in the tyrosine group, 389 nmol/mL in the NTBC group, and 2888 nmol/mL in the NTBC+tyrosine group.

There was no effect of treatment on the number of live fetuses, early or late resorptions, or dead fetuses. Mean fetal body weight (both for combined sexes and for each sex separately) was reduced (statistically significant) in the group administered both tyrosine and NTBC, and was slightly reduced in the group administered NTBC, but this was not statistically significant.

There were no findings observed at external examination which were considered to be related to treatment. Fetal skeletal examination revealed no treatment-related malformations or anomalies. An increased incidence of variant findings was only observed in the group tyrosine and NTBC together, mainly involving decreases in ossification.

This study demonstrates that induction of marked maternal tyrosinemia is necessary for observation of fetal skeletal variants. In the treatment groups (tyrosine or NTBC alone) where maternal tyrosine was only slightly increased relative to controls, there was no biologically significant effects on skeletal parameters. In the group administered both tyrosine and NTBC, where maternal plasma tyrosine was markedly increased, there was also a biologically significant increase in skeletal findings such as decreased ossification and increase of 14th rib(s) or ossification points on the 14th thoracic vertebra. (Kennel, 2006).

Exploratory 14-day (ocular toxicity) study with tyrosine in the rat and mouse

This study was conducted primarily to examine differences in the development of corneal opacity between rats and mice after administration of diets containing dietary tyrosine. A secondary objective was to determine whether there was a difference in sensitivity between strains of rats. *L*-Tyrosine (purity 98%) was incorporated into rodent diet at 0%, 2%, and 5% and fed to groups of five male and five female CD rats, Brown Norway rats, and CD-1 mice for a period of 14 days.

Clinical signs and moribundity were monitored daily. Body weights and food consumption were measured on a weekly basis. Ophthalmological examinations were conducted during acclimatization and

on study days 2, 3, 7, 8 and 14. Blood was collected on study day 15 prior to necropsy for determination of plasma tyrosine concentrations. Following sacrifice, animals were subjected to a gross necropsy. Histological sections of the eyes of selected animals were prepared.

There were no mortalities during the study. Treatment-related clinical signs were observed only in the CD rats receiving 5% tyrosine, with dark urine seen in all males in the second week of the study and in three females on day 14 only. There was no effect on body weights or food consumption in any group.

Corneal opacity was observed at clinical examination in one of the male CD rats receiving 5% dietary tyrosine.

On ophthalmological examination no treatment-related corneal opacities were observed in female rats or in either male or female mice. Corneal opacity was not seen in male rats of either strain at 2% dietary tyrosine. In CD rats, all male rats at 5% tyrosine showed corneal opacity by day 7, with progression of the opacity over time. One male Brown Norway rat at 5% tyrosine showed slight corneal opacity only visible on day 14. In all cases the corneal opacities were of the characteristic "snowflake" pattern.

Plasma tyrosine concentrations in male CD and male Brown Norway rats, showed a dose-related increase in plasma tyrosine concentration. In female rats, plasma tyrosine concentrations were only determined for CD rats at 0% and 5% dietary tyrosine. Male CD-1 mice showed no increase in plasma tyrosine concentrations after two weeks administration of 5% dietary tyrosine. Plasma tyrosine concentrations were not determined in male mice at 2% dietary tyrosine, or in female mice at any dose level. The male Brown Norway rat that was observed on day 14 to have a corneal opacity was found to have plasma tyrosine concentrations markedly higher than those in the other four male Brown Norway rats in its group.

There were no treatment-related findings at gross necropsy, other than the corneal opacity observed in one male Brown Norway rat. Microscopic examination of eyes from animals which did not show corneal opacities at ophthalmological examination did not reveal any treatment-related findings. In CD rats which had corneal opacities at ophthalmological examination, the findings included inflammatory reactions in the cornea, oedematous or swollen nuclear changes in the corneal epithelial cells and corneal vacuolation. In the Brown Norway rat the findings included inflammation of the anterior chamber.

Table 24. Plasma tyrosine concentrations and incidence of corneal opacities in rats and mice after 14 days administration of 0%, 2% or 5% dietary tyrosine

		Dose group (ppm)			
		Males		Females	
Species and strain	**Dietary tyrosine**	**Plasma tyrosine, (mg/L)**	**Corneal opacities**	**Plasma tyrosine, (mg/L)**	**Corneal opacities**
CD rat	0%	20.8	No	12.8	No
	2%	59.5	No	DNC	DNC
	5%	114.0	Yes	61.6	No
Brown Norway rat	0%	11.8	No	DNC	DNC
	2%	31.9	No	DNC	DNC
	5%	168.0	Yes	DNC	DNC
CD-1 mice	0%	13.0	No	DNC	DNC
	2%	DNC	DNC	DNC	DNC
	5%	17.7	No	DNC	DNC

DNC: Data not collected; This study demonstrates that male rats are more sensitive than female rats to the development of a plasma tyrosine concentration sufficient to lead to corneal opacities after feeding tyrosine in the diet. Likewise, male rats are more sensitive to the effects of dietary tyrosine on plasma tyrosine concentrations than are male mice (Esdaile, 1995).

14-day comparative toxicity feeding study in male beagle dogs with technical grade pyrasulfotole

The purpose of this study was to evaluate the potential cause of differential urinary system toxicity noted in two previous dietary dog studies conducted with pyrasulfotole. This study was conducted to determine whether the type of dietary regimen provided to dogs influenced either the absorption or the excretion of pyrasulfotole (AE 0317309)in the urine. Previously, a 28-day study was conducted in which the dogs were fed a mixture of 300 g of powdered diet, pyrasulfotole, and 450 mL of water mixed into a slurry. In that study administration of pyrasulfotole in the moistened diet at either 13 000 or 26 000 ppm resulted in the formation of urinary calculi, while no calculi were observed in animals fed diet containing pyrasulfotole at 5000 ppm. In an additional dietary study in which pyrasulfotole was suspended in corn oil and acetone, then added to dry feed, administration of 1500, 9000 and 18 000 ppm resulted in moribundity and calculus formation at 9000 and 18 000 ppm in males, as well as blood in the urine in males at all doses. The finding of blood in the urine at 1500 ppm in males fed pyrasulfotole as part of a dry diet, below the NOAEL of the 28-day study with moistened diet (5000 ppm), suggested that the amount of water in the diet was a major factor in the variation between the two studies.

Test compound pyrasulfotole is highly water soluble and excreted intact via the kidney. It was hypothesized that when the test compound was administered with a large volume of water, the kinetics might change, increasing the absorption and excretion from the body, thus minimizing the production of urinary calculi and the resulting urinary tract damage. Comparison of the effects of the two diets was conducted by examination of relevant clinical chemistry and urinalysis parameters in a 16-day feeding trial, along with measurement of pyrasulfotole concentration in plasma and urine from the wet-diet and dry-diet groups.

Pyrasulfotole (purity 95.7%) was administered at 18 000 ppm in the diet to two groups of five male beagle dogs. One group received 300 g of powdered diet mixed with pyrasulfotole and 450 mL of tap water ("wet feed"), while the other group received 300 g canine diet with pyrasulfotole added via suspension of the test compound in corn oil and acetone ("dry feed"). The diet was made available to the animals for 1.5 hours each day, and leftover food was removed at the end of that time and weighed.

Clinical signs and moribundity were monitored daily. Body weights were measured on study days 0, 7 and 16, and food consumption was measured on a daily basis. Blood and urine were collected during the prestudy acclimatization period and on study days 6 and 14. Samples for measurement of blood urea nitrogen (BUN), creatinine and standard urinalysis parameters were taken at these time points. Urine was also collected on days 15–16 for a 24-hour period following the last administration of treated diet, at 2, 4, 6, 8, 12 and 24 hours after the last feed. These samples were used for measurement of urinary pyrasulfotole concentration. Blood was collected at two, four and six hours after the last feed for measurement of plasma pyrasulfotole concentration. On study day 16, the animals were sacrificed by intravenous injection of a standard euthanasia solution and subjected to gross necropsy of the urinary tract along with kidney weight measurement.

There were no mortalities in either group in this study. Treatment-related clinical signs were limited to red urine observed in two dogs of the dry-feed group, one on study day 7 and one on study days 7, 8, 10 and 12, and a tan substance in the faeces of the wet-feed dogs. Body weight was biologically significantly reduced in both diet groups compared with controls. There was no treatment-related effect on food consumption in either the wet-feed or the dry-feed group.

There was a treatment-related increase in BUN and blood in the urine in both dietary groups. Urinary ketones were also increased in both groups, but as this was indicative of urinary excretion of pyrasulfotole (itself a ketone) it was not considered to be related to toxicity of the substance.

There was no effect on kidney weight in either group. Necropsy revealed two dogs in the dry-feed group with calculi in several locations in the urinary tract, and the urinary bladders of these animals showed moderate thickening. There were no findings in the other dogs in this group, and no findings in any of the dogs in the wet-feed group.

Dogs in the wet-feed group had slightly higher plasma concentrations of pyrasulfotole at two hours (111 µg/mL as against 73 µg/mL) and four hours (100 µg/mL as against 61 µg/mL) after the last feed than did the dogs in the dry group. However, by six hours after dosing, plasma concentrations were roughly equivalent: 48 µg/mL in the wet-feed group compared with 45 µg/mL in the dry feed

group. Urine concentrations of the test substance were higher in the dry-food group at nearly all urine collection time points, as shown in Table 25.

Table 25. Concentrations of pyrasulfotole, in µg/mL, in urine from dogs administered pyrasulfotole either with dry or wet food

Time after feeding (hours)	Treatment regime	
	Dry feed	Wet feed
2	6159	6662
4	6254	5059
6	5794	4048
8	6126	3988
10	5681	2871
12	4426	1659
24	301	306

Source: Eigenberg, 2006b

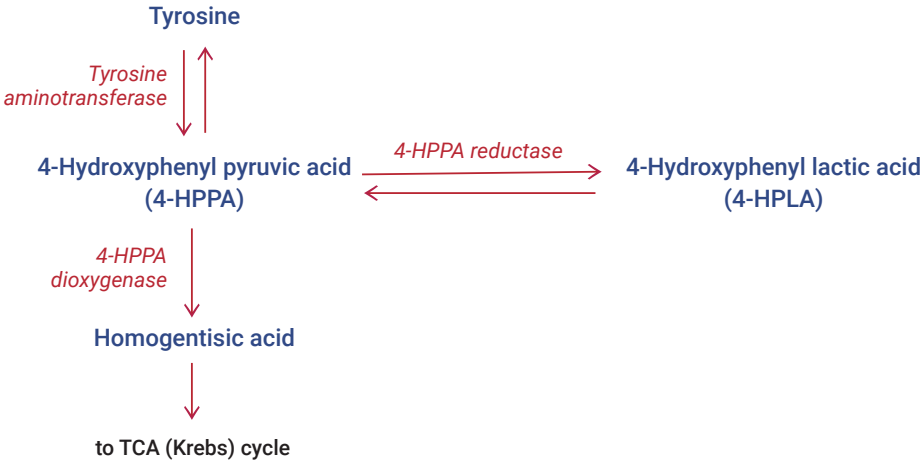
The results of this study indicated that the addition of water to the diet slightly increased plasma concentrations of pyrasulfotole and had a biologically significant effect on the rate of excretion of the test substance.

In the dry-feed group, urine pyrasulfotole concentrations remained elevated for longer, although both groups showed similar concentrations 24 hours after dosing. Additionally, urinary calculi were observed in two animals of the dry-feed group, but not in any of the wet-feed group. This further confirmed that, as these calculi have been previously demonstrated to be primarily pyrasulfotole, addition of water to the diet reduced formation of calculi in the urinary tract.

In conclusion, kidney function was impaired in both treatment groups, as indicated by an elevation in BUN and the presence of blood in the urine. Urinary tract calculi and thickening of the urinary bladder were observed in the dry-food group. Higher levels of pyrasulfotole were found in urine for a longer period of time in the dry-food group. Plasma clearance also appeared slower in the dry-food group. Data from this study indicates that the observed difference in kinetics potentiates urinary tract irritation and the formation of calculi in the urinary tract system. (Eigenberg, 2006b).

The sponsor did not provide information on why these four mechanistic studies were submitted, nor any discussion on the proposed mode of action. However, tyrosinemia leading to corneal effects is a known mechanism associated with 4-HPPD inhibitors. Rats might be especially sensitive to inhibition of this enzyme because they lack the capacity to produce *p*-hydroxyphenyl lactic acid (4-HPLA), that enables the reduction of plasma tyrosine levels when 4-HPPD is inhibited (See Fig. 3).

Figure 3. Metabolism of tyrosine



In the first mechanistic study, inhibition of 4-HPPD by the model inhibitor NTBC in various species was studied in vitro. Based on these results, the rat did indeed appear to have a low ability to produce 4-HPLA compared to other species. However, it was the dog that showed the lowest ability to produce 4-HPLA and in this respect the mouse was similar to the rat. In the repeat-dose studies with pyrasulfotole, eye effects were only observed in the rat, not the dog or rabbit. So this is not consistent with the hypothesis that corneal lesions are related to the inability to produce 4-HPLA.

Pyrasulfotole is a herbicide which exerts its mode of action via inhibition of the enzyme 4-HPPD, which occurs in plants and animals. However, no studies were submitted investigating the effect of pyrasulfotole on 4-HPPD, plasma tyrosine levels or tyrosine aminotransferase (TAT) activity in mammals, therefore direct evidence for this MOA is missing.

The key events involved in this mode of action are:

1. Inhibition of 4-HPPD
2. Increase in systemic tyrosine concentrations
3. Clearance of excess tyrosine via TAT
4. Tyrosine-related spectrum of toxicological effects.

As already indicated, no specific data is available for pyrasulfotole regarding the inhibition of 4-HPPD in animals. However it is the established herbicidal MOA in plants. Considering that 4-HPPD inhibitors have a common substructure which binds tightly to a single common active site in both plant and mammal 4-HPPD, and that this 4-HPPD active sequence is similar across plants and animals, it may be assumed that pyrasulfotole also inhibits animal 4-HPPD.

Regarding the increase in systemic tyrosine concentrations or clearance of excess tyrosine via TAT, no specific data for pyrasulfotole is available. It is known that the degree of tyrosinemia is species-specific and dependent upon the innate activity of TAT, the rate-limiting enzyme in the tyrosine catabolic pathway. In the metabolic pathway for tyrosine TAT is the first enzyme. If the second enzyme (HPPD) is inhibited, excess tyrosine is cleared as phenolic acids in the urine. The rate of clearance under these conditions depends on the inherent activity of TAT, which is species dependent, and higher in the mouse than in the rat.

Toxicological effects related to tyrosinemia include effects on body weight, liver, kidneys, eyes, thyroid and on reproduction/development in the rat. In the studies available for pyrasulfotole, eye effects (corneal opacity, neovascularization) were observed in the 90-day rat study, two-year rat study, two-generation rat study and the developmental neurotoxicity study in the rat. In studies with other species no eye effects were reported. Effects on body weight and kidney weight were seen in several species, however in rats these effects occurred at lower dose levels indicating a higher sensitivity. Ossification effects occurred both in the rat and rabbit developmental studies at similar dose levels.

Overall, considering that the herbicidal MOA of pyrasylfotole is inhibition of 4-HPPD, it seems likely that the eye effects observed in rats are linked to the postulated MOA of tyrosinemia. However, since direct evidence for this MOA for pyrasulfotole is missing, the effects found on eyes cannot be dismissed.

(d) Studies with metabolites – RPA 203328

Several studies are available for metabolite RPA 203328 (MTFM-BA; AE-B197555) including acute oral toxicity, short-term studies in rat, genotoxicity studies and a developmental rat study.

Acute oral toxicity

Table 26. Results of acute toxicity study with pyrasulfotole metabolite RPA 203328

Species	Strain	Sex	Route	Purity	LD_{50} (mg/kg bw)	Reference
Rat	Sprague Dawley	Male & Female	Oral	99.7%	> 5000 (male and female)[a]	Bigot, 1995

LD_{50}: median lethal dose.

[a] Clinical signs included dyspnea, piloerection, soiled fur or mucoid faeces or increased. No effect on body weight or pathological findings.

Short term toxicity

In a 28-day rat toxicity study RPA 203328 (purity 99.7%) was administered via dietary incorporation to groups of 10 male and 10 female Sprague Dawley rats at dietary concentrations of 0, 150, 500, 5000 or 15 000 ppm (equal to 0, 11.1, 37.6, 377 and 1118 mg/kg bw per day in males, 0, 12.7, 42.7, 421 and 1269 mg/kg bw per day in females). Clinical signs, moribundity, and mortality were checked twice daily on weekdays and once daily on weekends and holidays. Body weight and food consumption were measured weekly throughout the treatment period. Ophthalmological examinations were conducted during the acclimatization period on all animals, and on rats in the control and 15 000 ppm groups during weeks 2 and 4. In the last week of the study, animals were fasted overnight on one night prior to blood collection the following morning by puncture of the retro-orbital venous plexus, the samples used for haematology and clinical chemistry examinations. Urine was collected overnight a few days later from fasted animals. Following sacrifice, the rats were subjected to gross necropsy and selected organs were weighed. Samples of organs and tissues were preserved for histopathological examination.

There were no mortalities or clinical signs of toxicity during the study in either males or females. There was no effect of RPA 203328 administration in the diet on body weight or food consumption. Rats in the top-dose group showed no ophthalmological abnormalities compared to control rats at any time during the study. There were no treatment-related effects on any haematological or clinical chemical parameters. Urine pH was reduced in males at 15 000 ppm (–11% compared to control; statistically significant), and refractive index was increased in males at 500 and 15 000 ppm (+0.3% and +0.4% compared to control, respectively; statistically significant). However, the toxicological relevance of these findings was considered limited. There were no effects on organ weights in any treatment group. No treatment-related effects were observed in any animals at gross necropsy nor were there any treatment-related histopathological findings.

The NOAEL in this study was 15 000 ppm (equal to 1118 mg/kg bw per day), the highest dose tested (Dange, 1995).

In a sub-chronic rat toxicity study RPA 203328 (purity 99%) was incorporated into rodent diet at concentrations of 0, 1200, 4800 or 12 000 ppm (equal to 0, 73.2, 306, and 769 mg/kg bw per day for males, 0, 93.1, 371, and 952 mg/kg bw per day for females) and administered to groups of 10 male and 10 female Sprague Dawley rats for 90 days. Clinical signs, and mortality were checked twice daily on weekdays and once daily on weekends and holidays. Prior to the start of the study and in week 12, the grasping, righting, corneal, pupillary, auditory startle and head shaking reflexes were tested for each animal. Body weight and food consumption were measured on a weekly basis. Ophthalmological examinations were conducted on all animals prior to the start of the study and on the control and high-dose animals in week 12. Also in week 12, blood was collected for haematology and clinical chemistry. Urine was collected overnight on the night prior to sacrifice. Following sacrifice gross examination was conducted, selected organs were weighed, and samples of organs and tissues were preserved for microscopic examination.

There were no mortalities or clinical signs of toxicity during the study in either males or females. There were no effects of treatment on reflexes. There was no effect of RPA 203328 administration in the diet on body weight or food consumption. Rats in the top-dose group showed no ophthalmological abnormalities compared to control rats at any time during the study. There were no treatment-related effects on any haematological or clinical chemical parameter. Urinary pH was decreased to a statistically significant degree at 4800 and 12 000 ppm in females without a dose–response relationship (–11% compared to control for both treatment groups). However, there were no other findings so this decrease in pH was considered not to be toxicologically relevant. There were no treatment-related effects on organ weights at any dose in either males or females. Macroscopic findings were noted at necropsy, included dark or yellowish colour of the liver, dark kidneys, and marked lobular liver pattern. However, these were not dose-related and there were no histopathological correlates to any of these findings and thus they were considered not to be treatment related. There were no observations at histopathology which were considered to be related to dietary administration of RPA 203328.

The NOAEL for this study was 12 000 ppm (equal to 769 mg/kg bw per day), the highest dose tested (Bigot, 1998).

Genotoxicity

Table 27. Overview of genotoxicity tests with pyrasulfotole metabolite RPA 203328

End-point	Test object	Concentrations/doses tested	Purity	Results	Reference
		In vitro			
Bacterial gene mutation	*Salmonella typhimurium* TA1535, TA1537, TA98 and TA100[a]	Plate incorporation, (± S9): 100, 250, 500 1000, 2500 and 5000 µg/plate in DMSO (two experiments)	99.7%	Negative	Percy, 1994
Chromosome aberration[b]	Chinese hamster ovary cells	**With and without S9 activation:** 3 h exposure/20 h harvest: 931–2710 µg/mL in DMSO **Without S9 activation:** 17.8 h exposure 20 h harvest: 924, 1320, 1690, 2700 µg/mL in DMSO **With S9 activation:** 3 h exposure/20 hour harvest: 924–2700 µg/mL in DMSO	99.0%	Negative	Murli, 1998
Mammalian gene mutation	Chinese hamster ovary cells (HPRT locus)	**Without S9 activation:** 84.5 to 2700 µg/mL in DMSO **With S9 activation:** 338 to 2700 µg/mL in DMSO	99.0%	Negative	Cifone, 1998
		In vivo			
Micronucleus induction in mice	CD-1 mice (6 males/dose per harcest time)	500, 1000, and 2000 mg/kg bw in 0.5% aqueous methyl cellulose, (gavage)	95.7%	Negative[c]	Curry, 1998

DMSO: Dimethyl sulfoxide.

[a] Study did not include strain TA102 or *E. coli* WP2 *uvrA* to detect oxidative and DNA cross-linking mutagens

[b] The current OECD 473 guideline (2016) indicates that at least 300 metaphases should be scored. In this study only 200 metaphases were scored, however, this was in line with the version of the guideline in place at time the study was conducted (OED 473, 1997).

[c] No clinical signs of systemic exposure were observed; there was no effect of treatment on the ratio of polychromatic to normochromatic erythrocytes

Developmental toxicity

In a developmental toxicity study, RPA 203328 (99.0% purity) was administered to groups of 25 Sprague Dawley rats daily by gavage from GD 6 to 20 at the following doses: 0, 75, 250 or 750 mg/kg bw per day in 0.5% aqueous methylcellulose. Clinical signs were monitored daily, and morbidity and mortality were checked twice daily on weekdays and once daily on weekends. Body weights were measured on GDs 0, 3, 6, 8, 10, 12, 14, 16, 18 and 21. Food consumption was measured for the periods of GDs 1–3, 3–6, 6, 6–8, 8–10, 0–12, 12–14, 14–16, 16–18 and 18–21. On GD 21 animals were sacrificed by inhalation of carbon dioxide and subjected to a gross necropsy. The reproductive tract was weighed, and the number of corpora lutea, implantation sites, resorption sites, live and dead fetuses, and the sex and individual weights of viable fetuses were recorded. Viable fetuses were sacrificed by subcutaneous injection of sodium pentobarbital and examined externally. Approximately half the fetuses were then used for internal examination by freehand serial sectioning, while the other half were used for skeletal staining.

There were no treatment-related mortalities during the study. There was a dose-related incidence of transient salivation at treatment, with six animals affected at 250 mg/kg bw per day and 18 affected at 750 mg/kg bw per day. In some animals at the top dose there was also a red nasal discharge observed shortly after treatment. Both salivation and nasal discharge resolved within approximately one hour

after treatment. These findings were evaluated to be due to the acidity of the test substance, and were not indicative of toxicity. There was no effect of treatment on pregnancy in any group. There were statistically significant reductions in body weight changes at 250 and 750 mg/kg bw per day, which were considered to be related to administration of RPA 203328. There was also a treatment-related effect on food consumption at 250 and 750 mg/kg bw per day. The decreased food consumption and decrease in body weight were considered to be secondary to a local irritating effect, considered likely since the test substance is a benzoic acid derivative of pyrasulfotole.

Table 28. Selected findings for the developmental study in rat with RPA 203328

		Dose group (mg/kg bw per day)			
		0	**75**	**250**	**750**
		Pregnancy rate			
		96%	100%	100%	100%
		Body weight (g)			
Body weight	Day 6	306.8	305.7	300.4	301.0
	Day 8	314.9	311.4	306.1	305.6
	Day 10	325.3	321.5	313.4	309.2
	Day 12	335.5	330.6	319.1	317.4
	Day 14	346.8	342.0	329.4	325.2
	Day 21	445.8	439.9	423.7	415.7
Body weight gain	Days 6–8	8.0	5.7	5.7	4.6
	Days 8–10	10.4	10.1	7.3	3.6**
	Days 10–14	21.5	20.5	16 .0**	16 .0
	Days 14–1 8	43.9	43.1	42.6	42.1
	Days 18–21	55.1	54.8	51.7	48.4
	Corrected body weight gain	68.2	63.9	46.8**	43.1**
		Food consumption (g/day)			
Days 6–8		29.5	28.3	27.6	25.9**
Days 8–10		29.5	29.0	26.4	24.7**
Days 10–12		30.2	28.6	25.7**	25.4**
Days 12–14		28.9	27.6	25.2**	24.2**
Days 14–16		28.9	28.1	25.2**	25.4**
Days 16–18		29.4	28.4	26.3*	26.2*
Days 18–21		28.9	28.1	25.2**	25.4**
		Uterine findings			
Number of corpora lutea		18.0	17.3	17.8	16.7
Number of implantations		15.9	15.2	16.4	15.8
Pre-implantation loss (%)		8.9	11.1	7.0	4.9
Early resorption		1.1	0.5	0.6	0.7
Late resorption		0.1	0.0	0.1	0.1
Post-implantation loss (%)		7.1	3.4	4.4	4.9
		Fetal observation			
Male sex ratio		0.49	0.46	0.52	0.52
Fetal body weight (sexes combined)		5.39	5.34	5.26	5.27

* $p < 0.05$, ** $p < 0.01$ Source: Repetto-Larsay, 1999

There were no findings at gross necropsy for the dams that were related to treatment. There were no effects of administration of RPA 203328 on the number of corpora lutea, implantations, litter size, live fetuses, resorptions, pre- or post-implantation losses, on sex ratio, or on fetal body weight. There were no treatment-related findings from external, visceral, or skeletal examinations that were related to treatment. There were three fetuses with external malformations, but these findings were unrelated to each other and showed no dose relationship. Occasional skeletal findings were noted in all groups, however, they were generally within historical control data and did not show any relationship to dose.

In the absence of any adverse systemic effects the NOAEL for maternal toxicity of metabolite RPA 203328 was 750 mg/kg bw per day, the highest dose tested. The NOAEL for developmental toxicity was 750 mg/kg bw per day, the highest dose tested (Repetto-Larsay, 1999).

3. Observations in humans

Occupational medical surveillance of workers exposed to pyrasulfotole in production and formulation, has been performed on a routine basis since start of production in 2010 and has revealed no medical problems related to the handling of pyrasulfotole. To the present date no accidents with pyrasulfotole have occurred with workers and no consultations of the Medical departments due to work or contact with pyrasulfotole have been required.

No reports are available on any symptoms for researchers handling the product in field trials.

No human poisoning cases or epidemiological studies have been published. Direct reports sentto the manufacturer have not been linked to pyrasulfotole.

4. Microbial aspects

No information for pyrasylfotole was available concerning mechanism and type of antimicrobial action, effects on the microbiome of the human gastrointestinal tract, antimicrobial spectrum of activity or antimicrobial resistance mechanisms and genetics.

Comments

Biochemical aspects

The toxicokinetics and metabolism of pyrasulfotole have been investigated in the rat, following oral dosing. Although only a preliminary study, the toxicokinetics of pyrasulfotole were investigated following a single oral dose via gavage of 100 mg/kg bw [^{14}C]pyrasulfotole administered to male and female rats. Excretion via urine was greater than via the faeces for both sexes. The C_{max} value for radioactivity was higher for males (75.48 µg equiv./g) than for females (56.84 µg equiv./g). The absorption of the radioactivity was very rapid with mean T_{max} interpolated values of 0.5 hours for both sexes. The elimination half-life values based upon the terminal data points also indicated a slight sex difference and the values obtained were found to be around four hours for males and around six hours for females. Pyrasulfotole was found not to be extensively metabolized in the rat, with only five radioactive fractions in urine and four radioactive fractions in faecal extracts.

In the main study, absorption, distribution, metabolism and excretion (ADME) were studied in male rats following single dosing with 10 mg/kg bw [^{14}C]pyrasulfotole, labelled either at the phenyl or pyrazole positions. At time of termination (48 to 52 hours post dosing) oral absorption was 74–76% based on urine, tissues and carcass. Distribution showed limited amounts of pyrasulfotole in tissues, as less than 2% of the administered dose (AD) remained in the carcass and tissues. Highest residue levels were found in the liver and kidney. Excretion following a single oral or intravenous dose at 10 mg/kg body weight (bw) was rapid; 96–111% of the AD excreted within 24 hours. Irrespective of route or label, the majority of the radioactivity was excreted in the urine (73–91%), with less being excreted in the faeces (8–32%). Much of the administered dose was rapidly excreted in the urine within six hours of dosing (57–84%). Regarding metabolism, 87–95% of AD was excreted unchanged as

pyrasulfotole. Minor metabolites in urine and faeces were hydroxymethyl pyrasulfotole (up to 2.3% found), desmethyl pyrasulfotole (up to 8.1% found), and AE-B197555 (RPA 203328; up to 1.4% found). The major metabolic pathway occurred via *N*-demethylation of pyrasulfotole.

Toxicological data

The acute oral median lethal dose (LD_{50}) of pyrasulfotole was > 5000 mg/kg bw (Schüngel, 2004). No data on acute dermal or inhalation toxicity, skin or eye irritation or skin sensitization were provided.

In repeated-dose toxicity studies on mice, rats and dogs, the main effects were on urinary tract, bladder, kidney and eyes.

In an exploratory 14-day oral gavage study in mice, pyrasulfotole was administered at doses of 0, 100, 300 or 1000 mg/kg bw per day. In this study, no NOAEL could be identified as kidney effects (multifocal cortical tubuloepithelial degeneration and/or multifocal basophilic tubules) were seen at all dose levels tested (Langrand-Lerche, 2001a).

In a 28-day dietary toxicity study in mice, pyrasulfotole was administered at dietary concentrations of 0, 200, 1000 or 5000 ppm (equal to 0, 35.8, 192 and 961 mg/kg bw per day in males, 0, 45.0, 233, and 1082 mg/kg bw per day in females). The NOAEL was 1000 ppm (equal to 192 mg/kg bw per day) based on gritty content in the urinary bladder and histopathological findings in the bladder seen in males only, at 5000 ppm (equal to 961 mg/kg bw per day) (McElligott, 2002).

In a 90-day dietary toxicity study in mice, pyrasulfotole was administered at dietary concentrations of 0, 100, 750, 1500 or 3000 ppm (equal to 0, 16.5, 124, 259 and 500 mg/kg bw per day for males, 0, 19.7, 152, 326 and 617 mg/kg bw per day for females). The NOAEL was 3000 ppm (equal to 500 mg/kg bw per day), the highest dose tested (Steiblen, 2003).

In a 14-day exploratory dietary study in rats, pyrasulfotole was administered at dietary concentrations of 0, 400, 2000 or 7500 ppm (equal to 0, 27.4, 135 and 497 mg/kg bw per day for males, 0, 32.0, 157, and 547 mg/kg bw per day for females). The NOAEL was 2000 ppm (equal to 135 mg/kg bw per day) based on decreased body weight gain seen in males at 7500 ppm (equal to 497 mg/kg bw per day) (Langrand-Lerche, 2001a).

In a 90-day toxicity study in rats, pyrasulfotole was administered at dietary concentrations of 0, 2, 30, 1000, 7000 or 12 000 ppm (equal to 0.0, 0.13, 1.96, 66, 454, and 830 mg/kg bw per day for males, 0, 0.15, 2.32, 77, 537 and 956 mg/kg bw per day for females). The NOAEL was 1000 ppm (equal to 66 mg/kg bw per day) based on mortality, clinical signs, increased liver and kidney weight, effects on the eyes, macroscopic findings in the bladder and ureter, and histopathological findings in kidney, bladder and ureter at 7000 ppm (equal to 454 mg/kg bw per day) (Langrand-Lerche, 2003).

In a 28-day toxicity study in dogs, pyrasulfotole was administered at dietary concentrations of 0, 5000, 13 000 or 26 000 ppm (equal to 0, 174, 469, and 860 mg/kg bw per day for males, 0, 171, 440 and 782 mg/kg bw per day for females). No NOAEL could be identified in this study; the low dose of 5000 ppm (equal to 171 mg/kg bw per day) was the LOAEL based on clinical chemistry findings (Kennel, 2002).

In a 90-day toxicity study in dogs, pyrasulfotole was administered at dietary concentrations of 0, 100, 5000 or 1000 ppm (equal to 0, 3, 17, and 40 mg/kg bw per day for males, 0, 3, 17 and 33 mg/kg bw per day for females). The NOAEL was 1000 ppm (equal to 33 mg/kg bw per day), the highest dose tested (Eigenberg, 2005).

In a one-year toxicity study in dogs, pyrasulfotole was administered at dietary concentrations of 0, 250, 1000 or 3000 ppm (equal to 0, 7, 34, and 101 mg/kg bw per day for males, 0, 9, 33 and 93 mg/kg bw per day for females). The NOAEL was 250 ppm (equal to 7 mg/kg bw per day) based on increased incidence and severity of tubular dilatation in the kidneys accompanied by increased kidney weight seen at 1000 ppm (equal to 34 mg/kg bw per day) (Eigenberg, 2006a).

In a 78-week chronic toxicity/carcinogenicity study in mice, pyrasulfotole was administered at dietary concentrations of 0, 100, 1000 or 4000 ppm (equal to 0, 13.6, 137 and 560 mg/kg bw per day for males, 0, 16.7, 168, and 713 mg/kg bw per day for females). A NOAEL for systemic toxicity could not

be identified. The lowest dose of 100 ppm (equal to 13.6 mg/kg bw per day) was the LOAEL based on gall bladder stones seen at all dose levels tested. Regarding carcinogenicity, the NOAEL was 1000 ppm (equal to 137 mg/kg bw per day) based on urinary tract carcinoma and papilloma observed at 4000 ppm (equal to 560 mg/kg bw per day). These tumours were seen in the presence of urinary stones/calculi (Steiblen, 2006).

In a two-year chronic toxicity/carcinogenicity study in rats, pyrasulfotole was administered at dietary concentrations of 0, 25, 250, 1000 or 2500 ppm (equal to 0, 1.0, 10, 41 and 104 mg/kg bw per day for males, 0, 1.4, 14, 57 and 140 mg/kg bw per day for females). The NOAEL for systemic toxicity was 25 ppm (equal to 1.0 mg/kg bw per day) based on the effect on the eyes and increased plasma cholesterol at 250 ppm (equal to 10 mg/kg bw per day). In males one squamous cell papilloma and one squamous cell carcinoma were found at the high dose of 2500 ppm (equal to 104 mg/kg bw per day), and these were considered to be treatment-related. The findings were considered to be related to a chronic stimulation of the cornea, and thus caused by a nongenotoxic mechanism (Wason, 2006a).

The Meeting concluded that pyrasulfotole is carcinogenic in mice and rats.

Pyrasulfotole was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found.

The Meeting concluded that pyrasulfotole is unlikely to be genotoxic.

In view of the lack of genotoxicity, the fact that only urinary tract tumours were observed in mice at the highest dose tested in the presence of urinary stones/calculi, and that the corneal effects observed in rats at the highest dose tested are most likely related to pyrosulfotole's pesticidal mode of action to which rats are more sensitive, the Meeting concluded that pyrasulfotole is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation reproductive toxicity study in rats, pyrasulfotole was administered at dietary concentrations of 0, 30, 300 or 3000 ppm (equal to 0, 2.5–3.7, 26–34 and 272–354 mg/kg bw per day for males, 0, 2.0–4.2, 22–39 and 229–393 mg/kg bw per day for females). No NOAEL for parental toxicity could be identified. In males at all dose levels tested, thyroid weight was increased and histopathological changes were seen (pigment deposition and colloid alteration), although the toxicological significance of these changes was considered equivocal. Therefore, the parental LOAEL was 30 ppm in males (equal to 2.5 mg/kg bw per day). The reproductive NOAEL was 3000 ppm (equal to 272 mg/kg bw per day) the highest dose tested. The offspring NOAEL was 30 ppm (equal to 2.0 mg/kg bw per day) based on a delay in balanopreputial separation in F1 weanlings seen at 300 ppm (equal to 22 mg/kg bw per day) (Eiben, 2005).

In a developmental toxicity study in rats, pyrasulfotole was administered by gavage at dose levels of 0, 10, 100 or 300 mg/kg bw per day. The maternal NOAEL was 300 mg/kg bw per day, the highest dose tested. The embryo/fetal NOAEL was 10 mg/kg bw per day based on delayed ossification and reduced fetal weights observed at 100 mg/kg bw per day (Wason, 2006b).

In a developmental toxicity study in rabbits, pyrasulfotole was administered by gavage at dose levels of 0, 10, 75 or 250 mg/kg bw per day. The maternal NOAEL was 75 mg/kg bw per day based on decreased food consumption and body weight gain observed at 250 mg/kg bw per day. The embryo/fetal NOAEL was 10 mg/kg bw per day, based on altered ossification patterns and minor skeletal variants observed at 75 mg/kg bw per day (Wason, 2006c).

The Meeting concluded that pyrasulfotole is not teratogenic.

In an acute neurotoxicity study in rats, pyrasulfotole was administered by gavage at dose levels of 0, 200, 500 or 2000 mg/kg bw. The NOAEL was 500 mg/kg bw based on decreased motor and locomotor activity seen at 2000 mg/kg bw (Gilmore & Sheets, 2005).

In a 90-day neurotoxicity study in rats, pyrasulfotole was administered at dietary concentrations of 0, 500, 2500 or 5000 ppm (equal to 0, 32.3, 166 and 345 mg/kg bw per day for males, 0, 41.9, 206 and 416 mg/kg bw per day for females). The systemic and neurotoxicity NOAELs were both 5000 ppm (equal to 345 mg/kg bw per day) the highest dose tested (Gilmore & Hoss, 2005).

In a developmental neurotoxicity study in rats, pyrasulfotole was administered at dietary

concentrations of 0, 45, 450 or 4500 ppm (equal to averaged values of 0, 3.8, 37.1 and 354 mg/kg bw per day). The NOAEL for maternal toxicity was 45 ppm (equal to 3.8 mg/kg bw per day) based on a decreased fertility index and ocular opacity observed at 450 ppm (equal to 37.1 mg/kg bw per day). The embryo/fetal NOAEL was 45 ppm (equal to 3.8 mg/kg bw per day) based on decreased body weight, delayed preputial separation, decreased cerebrum and/or cerebellum length and decreased cerebellum thickness observed at 450 ppm (equal to 37.1 mg/kg bw per day) (Gilmore, Sheets & Hoss, 2005).

The Meeting concluded that pyrasulfotole showed potential developmental neurotoxicity in rat pups at the highest dose level tested, however these effects were observed in the presence of maternal toxicity.

No evidence of immunotoxicity was reported in routine toxicological studies with pyrasulfotole.

The Meeting concluded that pyrasulfotole is unlikely to be immunotoxic.

Toxicological data on metabolites and/or degradates

Metabolite RPA 203328

RPA 203328 (MTFM-BA; AE-B197555; 2-methylsulfonyl-4-trifluoromethylbenzoic acid) is a minor rat metabolite and a plant metabolite. The acute oral LD_{50} of this metabolite was >5000 mg/kg bw (Bigot, 1995).

In a 28-day dietary toxicity study in rats, RPA 203328 was administered at dietary concentrations of 0, 150, 500, 5000 or 15 000 ppm (equal to 0, 11.1, 37.6, 377 and 1118 mg/kg bw per day for males, 0, 12.7, 42.7, 421 and 1269 mg/kg bw per day for females). The NOAEL was 15 000 ppm (equal to 1118 mg/kg bw per day) the highest dose tested (Dange, 1995).

In a 90-day dietary toxicity study in rats, RPA 203328 was administered at dietary concentrations of 0, 1200, 4800 or 12 000 ppm (equal to 0, 73.2, 306 and 769 mg/kg bw per day for males, 0, 93.1, 371 and 952 mg/kg bw per day for females). The NOAEL was 12 000 ppm (equal to 769 mg/kg bw per day) the highest dose tested (Bigot, 1998).

Metabolite RPA 203328 was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found.

In a developmental toxicity study in rats RPA 203328 was administered by gavage at dose levels of 0, 75, 250 or 750 mg/kg bw per day. The maternal NOAEL and embryo/fetal NOAEL were 750 mg/kg bw per day, the highest dose tested (Repetto-Larsay, 1999).

The Meeting concluded that metabolite RPA 203328 is not of any toxicological concern.

Pyrasulfotole-desmethyl (AE 1073910)

Desmethyl pyrasulfotole is a metabolite that was found in the rat ADME study at levels up to 8.1%. Taking ionto account the oral absorption of pyrasulfotole at about 75%, total level for this metabolite in the rat will be around 10% of the absorbed dose. No specific toxicity studies with this metabolite were available. Considering the amount found in the rat and its structural similarity to pyrasulfotole, it can be assumed that desmethyl pyrasulfotole is of similar toxicity to the parent compound. The reference values of pyrasulfotole can therefore be applied.

Pyrasulfotole-desmethyl-*O*-glucoside

Pyrasulfotole-desmethyl-*O*-glucoside was not found as such in the rat ADME study and no specific toxicity studies are available. However, considering this metabolite is the conjugate of desmethyl-pyrasulfotole, the same conclusion applies. The reference values of pyrasulfotole can be applied.

Pyrasulfotole-hydroxymethyl (AE 0317309)

Hydroxymethyl-pyrasulfotole is a minor metabolite in the rat (up to 2.3% found). No specific toxicity studies with this metabolite were available. Considering the structural similarity to the parent compound pyrasulfotole, the reference values of pyrasulfotole can be applied.

Microbiological data

In the case of pyrasulfotole no information was available concerning type of antimicrobial action and its mechanism, effects on the microbiome of the human gastrointestinal tract, antimicrobial spectrum of activity or antimicrobial resistance mechanisms and genetics.

Human data

In reports on manufacturing plant personnel no adverse health effects were noted.

No information on accidental or intentional poisoning in humans was available.

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

Toxicological evaluation

The Meeting established an ADI of 0–0.01 mg/kg bw, based on the NOAEL of 1 mg/kg bw per day in the two-year study in rats and using a safety factor of 100. The margin between the upper bound ADI and the LOAEL for urinary tract carcinoma and papilloma in mice is 56 000. It is noted that in the two-generation rat study a LOAEL of 2.5 mg/kg bw per day (lowest dose tested) was identified. However, the effects seen at this LOAEL were considered of equivocal toxicological significance, therefore the Meeting decided to base the ADI on the NOAEL from the two-year rat study.

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

Levels relevant to risk assessment of pyrasulfotole

Species	Study	Effect	NOAEL	LOAEL
Mouse	78-week study of toxicity and carcinogenicity[a]	Toxicity	-	100 ppm, equal to 13.6 mg/kg bw per day[d]
		Carcinogenicity	1000 ppm, equal to 137 mg/kg bw per day	4000 ppm, equal to 560 mg/kg bw per day
Rat	Acute neurotoxicity study[b]	Neurotoxicity	500 mg/kg bw	2000 mg/kg bw
	Two-year studies of toxicity and carcinogenicity[a,d]	Toxicity	25 ppm, equal to 1.0 mg/kg bw per day	250 ppm, equal to 10 mg/kg bw per day
		Carcinogenicity	1000 ppm, equal to 41 mg/kg bw per day[c]	2500 ppm, equal to 104 mg/kg bw per day
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	3000 ppm, equal to 272 mg/kg bw per day[c]	-
		Parental toxicity	-	30 ppm, equal to 2.5 mg/kg bw per day[d]
		Offspring toxicity	30 ppm, equal to 2.0 mg/kg bw per day	300 ppm, equal to 22 mg/kg bw per day
	Developmental toxicity study[b]	Maternal toxicity	300 mg/kg bw per day[c]	-
		Embryo and fetal toxicity	10 mg/kg bw per day	100 mg/kg bw per day
	Developmental neurotoxicity[a] study[a]	Maternal toxicity	45 ppm, equal to 3.8 mg/kg bw per day	450 ppm, equal to 37.1 mg/kg bw per day
		Embryo and fetal toxicity	45 ppm, equal to 3.8 mg/kg bw per day	450 ppm, equal to 37.1 mg/kg bw per day

Species	Study	Effect	NOAEL	LOAEL
Rabbit	Developmental toxicity study[b]	Maternal toxicity	75 mg/kg bw per day	250 mg/kg bw per day
		Embryo and fetal toxicity	10 mg/kg bw per day	75 mg/kg bw per day
Dog	One-year study of toxicity[a]	Toxicity	250 ppm, equal to 7 mg/kg bw per day	1000 ppm, equal to 34 mg/kg bw per day
Metabolite RPA 203328 (MTFM-BA; AE B197555)				
Rat	Four-week study of toxicity[a]	Toxicity	15 000 ppm, equal to 1118 mg/kg bw per day[c]	-
	Ninety-day study of toxicity[a]	Toxicity	12 000 ppm, equal to 769 mg/kg bw per day[c]	-
	Developmental toxicity study[b]	Maternal toxicity	750 mg/kg bw per day[c]	-
		Embryo and fetal toxicity	750 mg/kg bw per day[c]	-

[a] Dietary administration [b] Gavage administration [c] Highest dose tested [d] Lowest dose tested

Acceptable daily intake (ADI) applies to pyrasulfotole, desmethyl-pyrasulfotole (AE 1073910) and pyrasulfotole-desmethyl-O-glucoside, expressed as pyrasulfotole

0–0.01 mg/kg bw

Acute reference dose (ARfD) applies to pyrasulfotole, desmethyl-pyrasulfotole (AE 1073910) and pyrasulfotole-desmethyl-O-glucoside, expressed as pyrasulfotole

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 pyrasulfotole

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	74–76% at 10 mg/kg bw within 48–52 hours based on urine, tissues and carcass
Dermal absorption	No data
Distribution	Limited; less than 2% remaining in carcass and tissues; highest concentrations in liver and kidney
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	Rapid excretion (96–111% excreted within 24 hours), predominantly in urine (73–91%), less in faeces (8–32%)
Metabolism in animals	Limited, as 87–95% was excreted as unchanged pyrasulfotole; hydroxymethyl-pyrasulfotole, desmethyl-pyrasulfotole and AE-B197555 were minor metabolites in urine and faeces
Toxicologically significant compounds in animals and plants	Pyrasulfotole
Acute toxicity	
Rat, LD_{50}, oral	> 5000 mg/kg bw
Rat, LD_{50}, dermal	No data
Rat, LC_{50}, inhalation	No data
Rabbit, dermal irritation	No data
Rabbit, ocular irritation	No data
Mouse, dermal sensitization	No data
Guinea pig, dermal sensitization	No data
Short-term studies of toxicity	
Target/critical effect	Urinary bladder (mouse); kidney (rat, dog); eyes (rat); urinary tract (rat, dog)
Lowest relevant oral NOAEL	7 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	Gall bladder stones (mouse); eyes, cholesterol (rat)
Lowest relevant NOAEL	1.0 mg/kg bw per day (rat)
Carcinogenicity	Carcinogenic in mice (urinary tract carcinoma and papilloma) and rats (corneal carcinoma and papilloma)[a]
Genotoxicity	Unlikely to be genotoxic[a]
Reproductive toxicity	
Target/critical effect	Parental: thyroid Offspring: delay in balanopreputial separation
Lowest relevant parental NOAEL	< 2.5 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	2.0 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	272 mg/kg bw per day (rat)
Developmental toxicity	
Target/critical effect	Delayed ossification, fetal weight (rat) Food consumption, body weight gain, ossification and skeletal variations (rabbit)
Lowest relevant maternal NOAEL	75 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	10 mg/kg bw per day (rat, rabbit)

Neurotoxicity	
Acute neurotoxicity NOAEL	50 mg/kg bw (rat)
Subchronic neurotoxicity NOAEL	345 mg/kg bw per day, highest dose tested (rat)
Developmental neurotoxicity NOAEL	3.8 mg/kg bw per day (rat)
Immunotoxicity	No data

Studies on toxicologically relevant metabolites

RPA 203328 (MTFM-BA; AE B197555)	
Acute oral LD50	> 5000 mg/kg bw (rat)
28-day NOAEL	1118 mg/kg bw per day, highest dose tested (rat)
90-day NOAEL	769 mg/kg bw per day, highest dose tested (rat)
Developmental toxicity: maternal and embryo/fetal NOAEL	750 mg/kg bw per day, highest dose tested (rat)
Genotoxicity	Not genotoxic: Ames, in vitro chromosome aberration, in vitro mammalian cell gene mutation, micronucleus in vivo
Microbiological data	No data.
Human data	No clinical cases or poisoning incidents had been recorded

[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)	100
ARfD	Unnecesary	-	-

[a] Applies to pyrasulfotole, desmethyl-pyrasulfotole (AE 1073910) and pyrasulfotole-desmethyl-*O*-glucoside, expressed as pyrasulfotole

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PYRAZIFLUMID

First draft prepared by
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Explanation

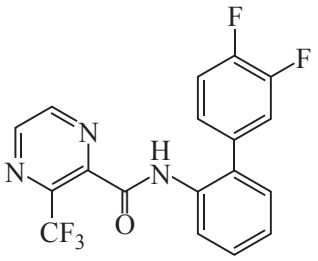
Pyraziflumid is the International Organization for Standardization (ISO)-approved common name for *N*-(3′,4′-difluorobiphenyl)-2-yl)-3-(trifluoromethyl)pyrazine-2-carboxamide (IUPAC), which has the Chemical Abstracts Service number 942515-63-1.

Pyraziflumid is a pyrazine-biphenyl type carboxamide SDHI (succinate dehydrogenase inhibitor) fungicide, for use on fruits such as pome, stone and citrus, as well as persimmon and grape. Its mode of action (MOA) is suppression of spore germination, mycelium elongation and sporogenesis by inhibiting the activity of mitochondrial electron transport chain complex II (succinate dehydrogenase complex) in filamentous fungi.

Pyraziflumid 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 the 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.

Figure 1. Chemical structure of pyraziflumid

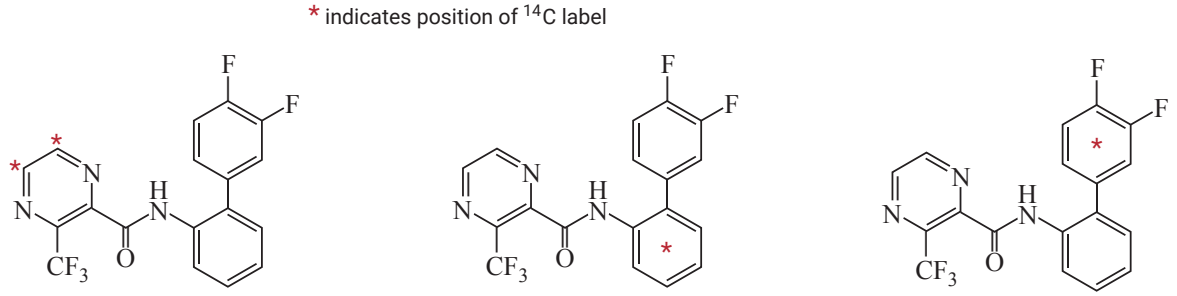


Evaluation for acceptable intake

1. Biochemical aspects

Absorption, distribution, metabolism and excretion (ADME) studies were conducted in rats using pyrazyflumid radiolabelled with ^{14}C in the pyrazinyl, aniline or the difluorophenyl rings (Fig. 2).

Figure 2. Position of ^{14}C label in pyraziflumid used in rat ADME studies



[*Pyrazinyl-5,6-*^{14}C]pyraziflumid

[*Aniline-U-*^{14}C]pyraziflumid

[*Difluorophenyl-U-*^{14}C]pyraziflumid

1.1 Absorption, distribution and excretion

Rat

The toxicokinetics and metabolism of [*pyrazinyl-5(6)-*^{14}C]pyraziflumid were investigated in Wistar Hannover (RccHan: WIST) rats (Table 1). Animals received either a low oral dose (1 mg/kg bw) or a high oral dose (100 mg/kg bw) of [*pyrazinyl-5(6)-*^{14}C]pyraziflumid (radiochemical purity: > 98%). The labelled pyraziflumid at a concentration of 0.5% weight for volume (w/v) was suspended in an aqueous vehicle of sodium carboxymethyl cellulose (CMC) containing 0.1% w/v Tween 80. Animals received the oral dose after 15–16 hours of fasting. The rats were observed for their moribundity, mortality and signs of toxicity just after administration, one hour post dose, and at the time of sample collection.

Table 1. Summary of experiments performed to investigate the toxicokinetics and metabolism of [pyrazinyl-5(6)-^{14}C]pyraziflumid in rats

Dose (mg/kg bw)	*N*	Sex	Source: sampling time (hours after dosing)
1	4	Male	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
1	4	Female	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
100	4	Male	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
100	4	Female	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
1	4	Male	Organs/tissues: 3
1	4	Male	Organs/tissues: 48
1	4	Male	Expired air: 24, 48, 72, 96 Urine: 24, 48, 72, 96, 120, 144, 168 Faeces: 24, 48, 72, 96, 120, 144, 168 Organs/tissues: 168
1	4	Female	Organs/tissues: 3
1	4	Female	Organs/tissues: 48
1	4	Female	Expired air: 24, 48, 72, 96 Urine: 24, 48, 72, 96, 120, 144, 168 Faeces: 24, 48, 72, 96, 120, 144, 168 Organs/tissues: 168
100	4	Male	Organs/tissues: 9
100	4	Male	Organs/tissues 48
100	4	Male	Expired air: 24, 48, 72 Urine: 24, 48, 72, 96, 120, 144, 168 Faeces: 24, 48, 72, 96, 120, 144, 168 Organs/tissues: 168
100	4	Female	Organs/tissues: 9
100	4	Female	Organs/tissues: 48
100	4	Female	Expired air: 24, 48, 72 Urine: 24, 48, 72, 96, 120, 144, 168 Faeces: 24, 48, 72, 96, 120, 144, 168 Organs/tissues: 168

The plasma maximum concentration (C_{max}), time to reach C_{max} (T_{max}), half-life ($t_{1/2}$) and total area under the concentration–time curve ($AUC_{0-\infty}$) values for radioactivity in males and females were almost identical (Table 2). Although T_{max} was reached at nine hours after a 1 mg/kg bw dose, it was noted that the blood and plasma levels at 1 mg/kg bw in males were already close to peak levels at 3 h and 6 h after dosing, and in females at 6 h after dosing. The $AUC_{0-\infty}$ values at the 100 mg/kg bw dose were about 80 times higher than those at 1 mg/kg bw, indicating that fractional absorption is only slightly lower following the high dose.

Table 2. Pharmacokinetics of pyraziflumid equivalents in rats following oral administration of [pyrazinyl-5(6)-^{14}C]pyraziflumid (

	Males		Females	
	1 mg/kg bw	100 mg/kg bw	1 mg/kg bw	100 mg/kg bw
$AUC_{0-168\,h}$ (mg × h/g)	4.2	332	4.9	376
$t_{½}$ (h) T_{max}–72 h	24	20	33	22
C_{max} (mg/g)	0.097	6.7	0.092	7.2
T_{max} (h)	9	24	9	24

C_{max}: Maximum concentration; T_{max}: Time to reach maximum concentration Source: Yoshizane, 2014a

$AUC_{0-\infty}$: Estimated area under the concentration–time curve from the time of dosing to infinity; bw: Body weight;

Radioactivity was excreted in faeces (81–93%), urine (6–16%) and expired air (3–5%) as shown in Table 3. Females tended to excrete almost twice as much radioactivity in urine as males. At 72 hours, 90% or more of the radioactive dose had been excreted.

Table 3. Recovery of [pyrazinyl-5(6)-^{14}C]pyraziflumid in excreta of rats over 168 hours following oral administration

Time period after dosing (hours)	Cumulative excretion (% of dose)					
	1 mg/kg bw dose			100 mg/kg bw dose		
	Urine	Faeces	Expired air	Urine	Faeces	Expired air
			Males			
0–24	4.0	39.9	3.4	2.5	57.8	1.9
0–48	6.9	62.2	4.2	4.8	78.8	2.4
0–72	8.3	77.1	4.5	5.6	88.0	2.5
0–168	9.4	87.3	NE	6.2	92.8	NE
Total		101.4			101.6	
			Females			
0–24	6.0	22.6	3.6	3.6	48.1	1.9
0–48	11.2	49.1	4.9	7.5	71.1	2.6
0–72	14.2	71.2	5.3	9.8	82.3	2.7
0–168	16.2	80.8	NE	11.1	88.5	NE
Total		102.5			102.3	

NE: Not examined

Source: Yoshizane, 2014a

The concentration of radioactivity in tissues at about T_{max} (3 h and 9 h after doses of 1 mg/kg bw and 100 mg/kg bw respectively) was highest in the gastrointestinal (GI) tract, liver, fat, kidney, thyroid, adrenal and ovaries (see Table 4). The levels of radioactivity in organs and tissues at 48 h and 168 h showed that the radiolabel was rapidly eliminated. No sex- or dose-related differences in tissue distribution were found (Yoshizane, 2014a).

Table 4. Distribution of radioactivity following oral doses of [pyrazinyl-5(6)-^{14}C]pyraziflumid

	Concentration of radioactivity in male/female (µg equiv./g)					
	1 mg/kg bw			100 mg/kg bw		
Time post dose	**3 hours**	**48 hours**	**168 hours**	**9 hours**	**48 hours**	**168 hours**
Blood	0.086/0.083	0.026/0.027	0.011/0.014	2.5/2.1	0.9/1.1	0.4/0.5
Plasma	0.114/0.103	0.038/0.028	0.003/0.005	4.6/3.6	1.1/1.4	< 0.1/< 0.1
Thyroid	0.461/0.443	0.032/0.027	0.010/0.008	22.7/9.2	1.1/1.3	0.3/0.2
Liver	1.703/1.950	0.252/0.142	0.043/0.044	35.1/26.4	9.6/9.2	2.7/2.9
Kidney	0.412/0.488	0.055/0.035	0.015/ 0.018	10.6/7.7	2.0/2.0	0.6/0.8
Adrenal	1.408/1.413	0.066/0.052	0.012/0.012	30.3/21.8	1.4/1.4	0.5/0.6
Stomach	1.605/1.304	0.055/0.038	0.010/0.011	20.1/15.6	1.5/1.9	0.4/0.4
Small intestine	2.623/3.346	0.668/0.808	0.018/0.011	98.8/94.9	20.6/42.7	0.6/0.6
Large intestine	0.734/0.737	0.353/0.476	0.016/0.011	63.8/81.0	30.3/22.7	0.7/0.7
Fat	2.382/3.705	0.718/0.480	0.020/0.011	133.3/111.3	10.8/13.7	0.6/0.6
Ovaries	0.890	0.062	0.010	13.5	1.6	0.4

Source: Yoshizane, 2014a

A number of experiments were conducted in Wistar Hannover (RccHan:WIST) rats to investigate the toxicokinetics and metabolism of [*aniline-U*-^{14}C]pyraziflumid (Table 5). The rats received either a low oral dose (1 mg/kg bw) or a high oral dose (100 mg/kg bw) of [*aniline-U*-^{14}C] pyraziflumid (radiochemical purity > 98%) suspended in an aqueous vehicle (0.5% w/v) of sodium carboxymethyl cellulose containing 0.1% w/v Tween 80. Animals received the oral dose after 15–16 hours of fasting. The rats were observed just after administration, one hour post dose and at the time of sample collection, for their moribundity, mortality and any signs of toxicity.

Table 5. Summary of experiments performed to investigate the toxicokinetics and metabolism of [aniline-U-^{14}C]pyraziflumid in rats

Dose (mg/kg bw)	*N*	Sex	Sampling time (hours after dosing)
1	4	Male	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
1	4	Female	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
100	4	Male	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
100	4	Female	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
1	4	Male	Organs/tissues: 3
1	4	Male	Organs/tissues: 48
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

The plasma $C_{max,}$ T_{max}, $t_{1/2}$ and $AUC_{0–\infty}$ values of radioactivity in males and females were almost identical (Table 6). The $AUC_{0–\infty}$ values at the 100 mg/kg bw dose were about 80 times higher than the values at 1 mg/kg bw, indicating that fractional absorption is only slightly lower following the high dose.

Table 6. Pharmacokinetics of [aniline-U-^{14}C]pyraziflumid equivalents in rats following oral administration

	Males		Females	
	1 mg/kg bw dose	100 mg/kg bw dose	1 mg/kg bw	100 mg/kg bw
$AUC_{0–168\,h}$ (mg × h/g)	3.6	253	3.6	266
$t_{½}$ (h) T_{max}–72 h	23	20	27	22
C_{max} (µg/g)	0.094	5.9	0.081	6.6
T_{max} (h)	6	12	6	9

$AUC_{0–\infty}$: estimated area under the concentration–time curve from the time of dosing to infinity; bw: body weight; C_{max}: maximum concentration; $t_{½}$: half-life: T_{max}: time to reach maximum concentration

Source: Yoshizane, 2014b

Radioactivity was excreted in faeces (88%) and urine (13%, see Table 7). No radiolabel was detected in the expired air. At 72 hours, 92% of the radioactive dose had been excreted.

Table 7. Recovery of radioactivity in excreta of male rats over 168 hours following oral administration of [aniline-U-^{14}C]pyraziflumid

	Cumulative excretion (% of dose)		
Dose	1 mg/kg bw		
Time period after dosing (h)	Urine	Faeces	Expired air
0–24	6.0	38.0	ND
0–48	10.0	65.6	NE
0–72	11.9	80.3	NE
0–168	13.2	88.1	NE
Total		101.4	

NE: Not examined; ND: Not detected; Source: Yoshizane, 2014b

The concentration of radioactivity in tissues at about T_{max} (three hours after a 1 mg/kg bw dose) was highest in the GI tract, liver, fat, kidney, thyroid and adrenals (Table 8). The levels of radioactivity in organs and tissues at 48 h and 168 h showed that radiolabel was rapidly eliminated. (Yoshizane, 2014b).

Table 8. Distribution of radioactivity following oral doses of [aniline-U-^{14}C]pyraziflumid

	Radioactivity concentration (µg equiv./g)		
	1 mg/kg bw		
Time post dose	3 hours	48 hours	168 hours
Blood	0.078	0.019	0.007
Plasma	0.115	0.022	0.001
Thyroid	0.429	0.027	0.003
Liver	1.568	0.238	0.027
Kidney	0.406	0.041	0.006
Adrenal	0.870	0.050	< 0.001
Stomach	1.241	0.030	0.002
Small intestine	2.063	0.460	0.009
Large intestine	0.227	0.394	0.007
Fat	2.504	0.581	0.012

Source: Yoshizane, 2014b

The toxicokinetics and metabolism of [*difluorophenyl-U-*^{14}C]pyraziflumid (radiochemical purity: > 98%) suspended in an aqueous vehicle (0.5% w/v of sodium CMC containing 0.1% w/v Tween 80) were studied in Wistar Hannover (RccHan:WIST) rats. An overview of the experimental designs is presented in Table 9. The rats received either a low oral dose (1 mg/kg bw) or a high oral dose (100 mg/kg bw) of [*difluorophenyl-U-*^{14}C]pyraziflumid. Animals received the oral dose after 15–16 hours of fasting. The rats were observed just after administration, one hour post dose and at the time of sample collection, for their moribundity, mortality and signs of toxicity.

Table 9. Summary of experiments performed to investigate the toxicokinetics and metabolism of [difluorophenyl-U-^{14}C]pyraziflumid in rats

Dose (mg/kg bw)	*N*	Sex	Sampling time (hours after dosing)
1	4	Male	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
1	4	Female	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
100	4	Male	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
100	4	Female	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
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

The plasma $C_{max,}$ and $AUC_{0-\infty}$ radioactivity values in males and females were almost identical (Table 10). Values of T_{max} were two-fold higher in females compared to males. The $AUC_{0-\infty}$ values at the higher, 100 mg/kg bw dose were 42–56 times higher than those at 1 mg/kg bw, indicating that fractional absorption is lower for the high dose.

Table 10. Pharmacokinetics of [difluorophenyl-U-^{14}C]pyraziflumid equivalents in rats following oral administration

	Males		Females	
	1 mg/kg bw dose	100 mg/kg bw dose	1 mg/kg bw dose	100 mg/kg bw dose
$AUC_{0-168\,h}$ (mg × h/g)	3.6	150	4.6	256
$t_{½}$ (h) T_{max}–72 h	22	18	26	17
C_{max} (µg/g)	0.107	3.9	0.116	5.5
T_{max} (h)	3	12	6	24

$t_{½}$: Half-life; bw: Body weight; Source: Yoshizane, 2015

$AUC_{0-\infty}$: Estimated area under the concentration–time curve from the time of dosing to infinity;
C_{max}: Maximum concentration; T_{max}: Time to reach maximum concentration;

Radioactivity was excreted in faeces (84%) and urine (13%, see Table 11). No radioactivity was detected in expired air. At 72 hours, 84% of the radioactive dose had been excreted.

Table 11. Recovery of radioactivity in excreta of male rats over 168 hours after oral administration of [difluorophenyl-U-^{14}C]pyraziflumid

	Cumulative excretion (% of dose)		
Dose	**1 mg/kg bw**		
Time after dosing (hours)	**Urine**	**Faeces**	**Expired air**
0–24	6.4	36.4	ND
0–48	10.2	58.4	NE
0–72	11.7	71.8	NE
0–168	12.9	83.7	NE
Total		96.6	

NE: Not examined; ND: Not detected; Source: Yoshizane, 2015

At 168 hours after dosing only very low levels of radioactivity (0.21 µg equiv. pyraziflumid/g or less) were detectable in organs and tissues (Yoshizane, 2015).

Biliary excretion following a single oral 1 mg/kg bw dose of [*pyrazinyl-5(6)*-^{14}C]pyraziflumid or [*aniline-U*-^{14}C]pyraziflumid (radiochemical purity > 98% for both) suspended in an aqueous vehicle (0.5% w/v of sodium carboxymethyl cellulose containing 0.1% w/v Tween 80) was studied in groups of four bile duct-cannulated male Wistar Hannover (RccHan:WIST) rats. Bile, urine and faeces were collected over 0–24, 24–48 and 48–72 hours. After 72 hours the rats were killed and radioactivity in excreta, GI tract, GI contents and liver was measured. The rats were not fasted at the time of dosing. Animals were observed just after administration, three hours post dose and at the time of sample collection, for their moribundity, mortality and signs of toxicity.

The excretion of radioactivity following oral dosing with radiolabelled pyraziflumid is presented in Table 12.

Table 12. Recovery of [pyrazinyl-5(6)-^{14}C]pyraziflumid and [aniline-U-^{14}C]pyraziflumid in bile duct-cannulated rats over 72 hours following oral administration

	Cumulative excretion (% of dosed radioactivity)					
	[pyrazinyl-5(6)-^{14}C]pyraziflumid			[aniline-U-^{14}C]pyraziflumid		
Time after dosing (hours)	Bile	Urine	Faeces	Bile	Urine	Faeces
0–24	62.8	5.0	7.1	57.7	4.9	4.9
0–48	79.3	6.4	8.3	79.2	6.5	6.1
0–72	83.2	6.8	8.5	85.3	7.0	6.3
Gastrointestinal contents [a]		0.09			0.05	
Cage wash [a]		0.12			0.20	
Sum of gastrointestinal tract and liver [a]		0.46			0.39	
Absorption [b]		90.5			92.7	
Total		99.2			99.2	

[a] Obtained at 72 hours postdose;

[b] Sum of radioactivity in bile, urine, liver and gastrointestinal tracts; Source: Yasunaga, 2014

The data show that radioactivity is excreted mainly in bile. Total absorption was 91–93%, based on the sum of radioactivity in bile, urine, liver and GI tract (Yasunaga, 2014).

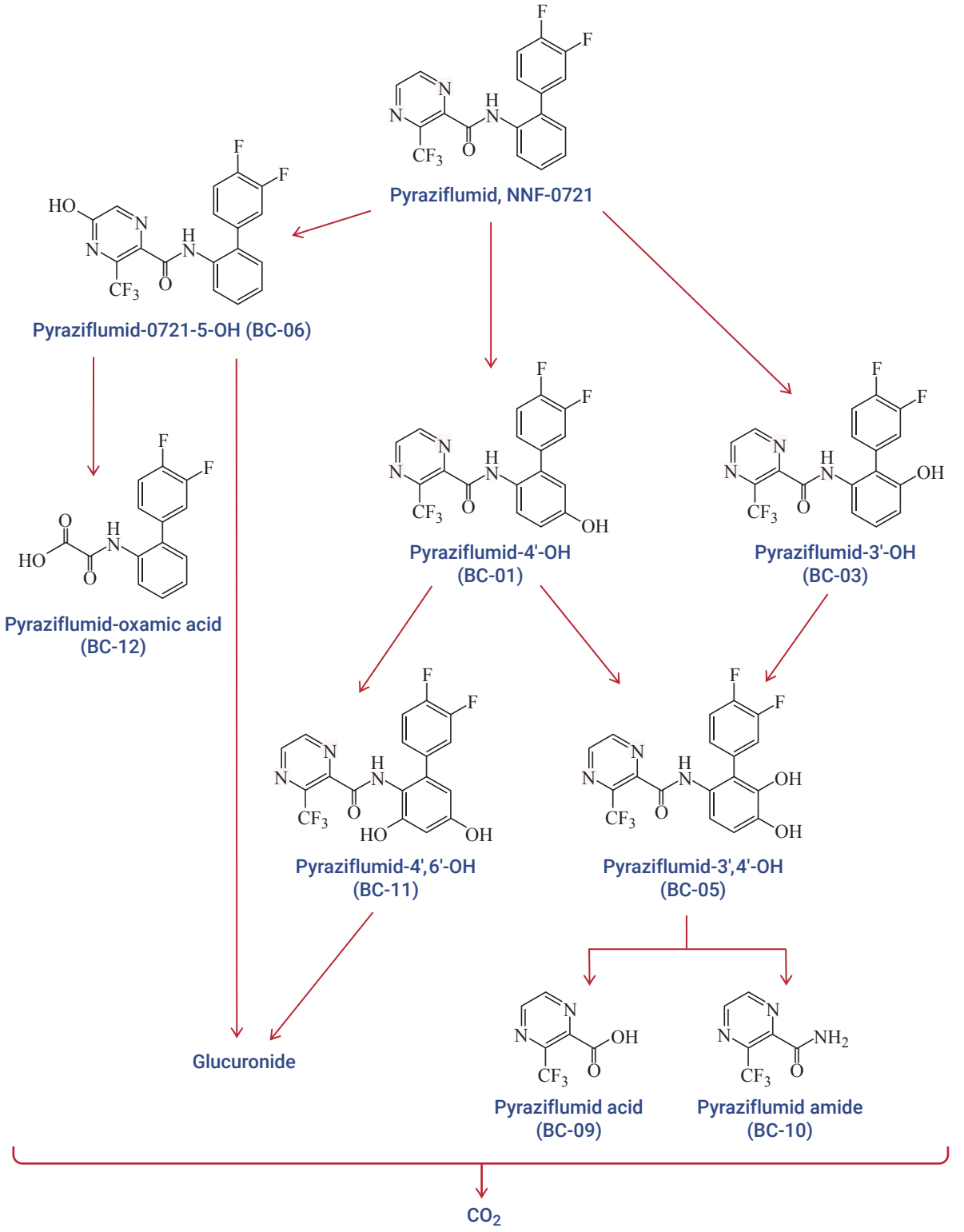
1.2 Biotransformation

The metabolism of [*pyrazinyl-5(6)*-^{14}C]pyraziflumid was studied in groups of Wistar Hannover (RccHan:WIST) rats treated with 1 or 100 mg/kg bw oral doses. The study design and toxicokinetics are described in section 1.1, (Yoshizane, 2014a). In the studies described above, urine and faeces were collected over 96 hours, plasma and liver samples collected at three hours (1 mg/kg bw group) or nine hours (100 mg/kg bw group). The collected samples were profiled for metabolites using thin layer chromatography (TLC) radioluminograms and by high-performance liquid chromatography (HPLC).

As shown in Table 13, unchanged pyraziflumid, pyraziflumid-4′-OH (BC-01) and pyraziflumid-3′-OH (BC-03) were the major metabolites in faeces. At 1 mg/kg bw, pyraziflumid-4′-OH (BC-01) was the predominant metabolite in faeces, while at 100 mg/kg bw, pyraziflumid was the major compound present. Minor metabolites found in faeces were pyraziflumid-5-OH (BC-06), pyraziflumid-3′,4′-OH (BC-05) and pyraziflumid-4′,6′-OH (BC-11). In urine, pyraziflumid-4′-OH (BC-01) and its glucuronide, pyraziflumid-3′-OH (BC-03) and its glucuronide, and pyraziflumid-3′,4′-OH glucuronide (that is, BC-05 glucuronide) were identified, but these accounted for less than 5% of dosed radioactivity. Pyraziflumid-acid (BC-09) and pyraziflumid-amide (BC-10) were also found in urine; these two are metabolites based on a pyrazinyl ring, produced by cleavage at the amide bond of pyraziflumid. The molecular structures of the metabolites are shown opposite in Fig. 3.

Pyraziflumid and, to a lesser extent, pyraziflumid-4′-OH (BC-01) were the major metabolites in plasma and liver. No significant qualitative difference in metabolite profile was found between dose groups or between sexes.

Figure 3. Proposed pathways of metabolism of pyraziflumid (NNF-0721) in rats



Source: Yoshizane, 2014a, 2014b, 2015 and Yasunaga, 2014

Table 13. Excretion of metabolites following a single oral administration of 1 or 100 mg/kg bw [pyrazinyl -5(6)-^{14}C] pyraziflumid to male and female rats

Metabolites	Metabolites excreted (% of dosed radioactivity)							
	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
Pyraziflumid	ND	12.14	ND	53.80	ND	7.84	ND	41.07
Pyraziflumid-4′-OH (BC-01)	1.80	42.38	1.43	19.29	4.00	43.20	2.97	26.31
Pyraziflumid-3′-OH (BC-03)	ND	7.39	ND	3.43	0.63	7.94	0.47	4.10
Pyraziflumid-3,4′-OH (BC-05)	ND	2.38	ND	2.20	ND	2.52	ND	1.88
Pyraziflumid-5-OH (BC-06)	ND	2.57	ND	0.78	0.14	1.56	ND	0.52
Pyraziflumid-acid (BC-09)	0.86	ND	0.68	ND	0.96	ND	0.61	ND
Pyraziflumid-amide (BC-10)	0.17	ND	0.15	ND	0.33	ND	0.21	ND
Pyraziflumid-4′,6-OH (BC-11)	ND	1.92	ND	1.06	ND	2.43	ND	1.88
Pyraziflumid-3′-OH glucuronide	ND	NE	ND	NE	0.49	NE	0.98	NE
Pyraziflumid-4′-OH glucuronide	2.47	NE	1.81	NE	2.59	NE	3.22	NE
Pyraziflumid-3,4′-OH glucuronide	0.09	NE	0.56	NE	0.05	NE	0.59	NE
Polar metabolites	0.65	2.16	0.32	1.59	1.22	2.20	0.70	1.99
Others	2.83	4.42	1.01	2.59	5.03[a]	2.90	0.82	1.64
Unextractable	0.01	8.06	<0.01	6.10	0.01	7.31	<0.01	6.81
Total	8.89	83.42	5.96	90.83	15.45	77.91	10.59	86.20

[a] No individual component exceeded 1.98% of dose;
NE: Not examined, since in faeces, radioactivity retained at TLC origin was <5%, so enzyme digestion was not conducted;
ND: Not detected; Source: Yoshizane (2014a)

The metabolite profile indicates that pyraziflumid is metabolized mainly via hydroxylation of the pyrazinyl and aniline rings, followed by conjugation with glucuronic acid. There is limited cleavage of the molecular backbone of pyraziflumid. A small fraction of the pyrazinyl ring metabolites is further metabolized to carbon dioxide. No marked sex- or dose-related differences in metabolism were seen (Yoshizane, 2014a).

The metabolism of [*aniline-U*-^{14}C]pyraziflumid was studied in groups of male Wistar Hannover (RccHan:WIST) rats treated with a 1 mg/kg bw oral dose. The study design and toxicokinetics are described in section 1.1. (Yoshizane, 2014b). Urine and faeces were collected over 96 hours, plasma and liver samples collected at three hours in the above studies. These were profiled for metabolites using TLC radioluminograms and HPLC.

As shown in Table 14, unchanged pyraziflumid, pyraziflumid-4′-OH (BC-01) and pyraziflumid-3′-OH (BC-03) were the major metabolites found in faeces. Minor metabolites in faeces were pyraziflumid-5-OH (BC-06), pyraziflumid-3′,4′-OH (BC-05) and pyraziflumid-4′,6′-OH (BC-11). In urine, pyraziflumid-4′-OH (BC-01) and its glucuronide and pyraziflumid-3′,4′-OH glucuronide (that is, BC-05 glucuronide) were identified, but these accounted for only 5% or less of dosed radioactivity. Pyraziflumid-oxamic acid (BC-12) was also found in urine; this metabolite is based on the aniline ring produced by cleavage at the pyrazinyl ring, . The molecular structures of the metabolites are shown in Fig. 3.

Unchanged pyraziflumid and, to a lesser extent, pyraziflumid-4′-OH (BC-01) were the major metabolites in plasma and liver.

Table 14. Excretion of metabolites following a single oral administration of 1 mg/kg bw [aniline-U-^{14}C]pyraziflumid to male rats

Metabolite	Metabolites excreted (% of administered dose)	
	Urine	Faeces
Pyraziflumid	ND	13.81
Pyraziflumid-4′-OH (BC-01)	1.21	36.72
Pyraziflumid-3′-OH (BC-03)	ND	6.91
Pyraziflumid-3,4′-OH (BC-05)	ND	2.78
Pyraziflumid-5-OH (BC-06)	ND	3.78
Pyraziflumid-4′,6-OH (BC-11)	ND	3.11
Pyraziflumid-oxamic acid (BC-12)	1.37	ND
Pyraziflumid-3′-OH glucuronide	ND	NE
Pyraziflumid-4′-OH glucuronide	5.01	NE
Pyraziflumid-3,4′-OH glucuronide	0.56	NE
Polar metabolites	0.78	3.53
Others	3.59	5.70 [a]
Unextractable	0.02	7.62
Total	12.53	83.96

[a] No individual component exceeded 3.59% of the dose;

NE: Not examinaed, since in faeces, radioactivity retained at TLC origin was < 5%, so enzyme-digestion was not conducted;

ND: Not detected; Source: Yoshizane (2014b)

The metabolite profile indicates that pyraziflumid is hydroxylated at the aniline and pyrazinyl rings followed by the conjugation of these products with glucuronic acid. There is limited cleavage of the molecular backbone of pyraziflumid (Yoshizane, 2014b).

The metabolism of [*difluorophenyl-U*-^{14}C]pyraziflumid was studied in groups of male Wistar Hannover (RccHan:WIST) rats treated with a 1 mg/kg bw oral dose. The study design and toxicokinetics are described in section 1.1. (Yoshizane, 2015). Urine and faeces collected over 96 hours in the above studies were profiled for metabolites using TLC radioluminograms and HPLC.

As shown in Table 15, pyraziflumid-4′-OH (BC-01), pyraziflumid-3′-OH (BC-03) and unchanged pyraziflumid were the major metabolites in faeces. Minor metabolites in faeces were pyraziflumid-5-OH (BC-06), pyraziflumid-3′,4′-OH (BC-05) and pyraziflumid-4′,6′-OH (BC-11). In urine, pyraziflumid-4′-OH (BC-01) and its glucuronide and pyraziflumid-3′,4’-OH (BC-05) glucuronide were identified, but these accounted for less than 3.5% of dosed radioactivity. Pyraziflumid-oxamic acid (BC-12) was also found in urine; this metabolite is based on the aniline ring produced by cleavage at the pyrazinyl ring. The molecular structures of the metabolites are shown in Fig. 3.

Table 15. Excretion of metabolites following a single oral administration of 1 mg/kg bw [difluorophenyl -U-^{14}C]pyraziflumid to male rats

Metabolite	Metabolites excreted (% of dosed radioactivity) [a]	
	Urine	Faeces
Pyraziflumid	ND	5.75
Pyraziflumid-4′-OH (BC-01)	2.16	37.49
Pyraziflumid-3′-OH (BC-03)	ND	8.75
Pyraziflumid-3,4′-OH (BC-05)	ND	3.61
Pyraziflumid-5-OH (BC-06)	ND	3.98
Pyraziflumid-4′,6-OH (BC-11)	ND	3.39
Pyraziflumid-oxamic acid (BC12)	1.33	ND
Pyraziflumid-3′-OH glucuronide	ND	NE
Pyraziflumid-4′-OH glucuronide	3.49	NE
Pyraziflumid-3,4′-OH glucuronide	0.18	NE
Polar metabolites	1.31	2.19
Others [b]	3.85	6.78
Unextractable	0.01	8.22
Total	12.33	80.16

Source: Yoshizane, 2014c

[a] All values are mean of four individuals; urine and faeces obtained until 96 h post dose were analyzed;

[b] No individual component exceeded 3.81% of dose;

ND: Not detected.

NE: Not examined, since in faeces, radioactivity retained at polar metabolites was < 5%, so enzyme-digestion was not conducted.

The metabolite profile indicates that pyraziflumid is hydroxylated at the aniline and pyrazinyl rings following which the products are conjugated with glucuronic acid. There is limited cleavage of the molecular backbone of pyraziflumid (Yoshizane, 2014c).

The metabolite profile in bile, urine and faeces following an oral dose of 1 mg/kg bw [*pyrazinyl-5(6)*-^{14}C]pyraziflumid or [*aniline-U*-^{14}C]pyraziflumid was studied in groups of male bile duct-cannulated Wistar Hannover (RccHan:WIST) rats. The study design and toxicokinetics are described in section 1.1, (Yasunaga, 2014). Bile, urine and faeces collected over 72 hours during the above studies were profiled for metabolites using TLC radioluminograms and HPLC.

As shown in Table 16, regardless of the radiolabel employed, pyraziflumid-4′-OH glucuronide (that is, BC-01 glucuronide), pyraziflumid-3′-OH glucuronide (that is, BC-03 glucuronide) and pyraziflumid-4′-OH (BC-01) were the major metabolites in bile. No parent compound was detected in bile. In addition, pyraziflumid-3′-OH (BC-03), pyraziflumid-5-OH (BC-06), pyraziflumid-acid (BC-09), pyraziflumid-amide (BC-10), pyraziflumid-oxamic acid (BC-12) and pyraziflumid-3′,4′-OH glucuronide (that is, BC-05-glucuronide) were also identified in bile as minor metabolites.

Unchanged pyraziflumid was the major compound in faeces, with pyraziflumid-4′-OH (BC-01) present as a minor metabolite.

In urine, low levels of pyraziflumid-4′-OH (BC-01) and its glucuronide, pyraziflumid-oxamic acid (BC-12), pyraziflumid-3′-OH (BC-03) and its glucuronide, pyraziflumid-5-OH (BC-06) and its glucuronide and pyraziflumid-3′,4′-OH glucuronide (that is, BC-05 glucuronide) were identified, but these accounted for less than 2% of dosed radioactivity. The molecular structures of the metabolites are shown in Fig. 3 (Yasunaga, 2014).

Table 16. Excretion of metabolites in bile, urine and faeces following a single oral dose of 1 mg/kg bw [pyrazinyl-5(6)-^{14}C]pyraziflumid or [aniline-U-^{14}C]pyraziflumid to bile duct-cannulated male rats

Metabolite	Metabolites excreted (% of dosed radioactivity)					
	[pyrazinyl-5(6)-^{14}C] pyraziflumid			[aniline-U-^{14}C] pyraziflumid		
	Bile	Urine	Faeces	Bile	Urine	Faeces
Pyraziflumid	ND [a]	ND	8.16	ND	ND	5.92
Pyraziflumid-4′-OH (BC-01)	6.01	0.37	0.12	4.46	0.35	0.12
Pyraziflumid-3′-OH (BC-03)	0.18	0.06	ND	0.18	0.02	ND
Pyraziflumid-3,4′-OH (BC-05)	ND	ND	ND	ND	ND	ND
Pyraziflumid-5-OH (BC-06)	3.07	0.07	ND	3.70	0.05	ND
Pyraziflumid-amine (BC-08)	-	-	-	ND	ND	ND
Pyraziflumid-acid (BC-09)	0.62	1.08	ND	-	-	-
Pyraziflumid-amide (BC-10)	0.77	0.12	ND	-	-	-
Pyraziflumid-4′,6-OH (BC-11)	ND	ND	ND	ND	ND	ND
Pyraziflumid-oxamic acid (BC-12)	-	-	-	0.56	1.30	ND
Pyraziflumid-4′-OH glucuronide	47.84	2.45	ND	46.91	1.71	ND
Pyraziflumid-3′-OH glucuronide	9.98	0.64	ND	8.70	0.19	ND
Pyraziflumid-3,4′-OH glucuronide	1.00	ND	ND	1.00	0.07	ND
Pyraziflumid-5-OH glucuronide	ND	0.11	ND	ND	0.08	ND
Polar metabolites	7.22 [a]	0.74	ND	7.15 [b]	0.38	ND
Others	6.16 [a]	1.15	ND	12.26 [b]	2.82	ND
Unextractable	0.36	0.01	0.23	0.40	0.02	0.21
Total	83.22	6.80	8.51	85.32	7.00	6.25

[a] No individual component exceeded 2.16% and 1.44% of dose in polar metabolites and others, respectively;

[b] No individual component exceeded 1.31% and 3.57% of dose in polar metabolites and others, respectively;

ND: Not detected; Source: Yasunaga, 2014

In vitro

An in vitro study was performed to investigate the metabolism of pyraziflumid in liver microsomes from rat, mouse, rabbit, goat, dog and human.

Radiolabelled [*pyrazinyl-5(6)*-^{14}C] pyraziflumid and [*aniline-U*-^{14}C] pyraziflumid were added to liver microsome suspensions at final concentrations of 0.3 µM. After the reaction was initiated by the addition of 100 µL of 10 mM nicotinamide adenine dinucleotide phosphate (NADPH) solution, the reaction mixture was incubated for 60 minutes at 37 ± 1°C. Resulting metabolite profiles are shown in Tables 17 and 18.

Pyraziflumid and pyraziflumid-4′-OH (BC-01) were identified as the major metabolites with all types of liver microsome. Whereas in rat the parent compound was found to be the major compound present (80–91%) with respect to administered dose (AD), in all other species the metabolite BC-01 predominated. In addition, pyraziflumid-3′-OH (BC-03), pyraziflumid-3′,4′-OH (BC-05), pyraziflumid-5-OH (BC-06) and pyraziflumid-amide (BC-10) were present as minor metabolites. Pyraziflumid-5-OH (BC-06) production was greater with dog liver microsomes than with liver microsomes from other mammals. In the case of mouse, goat, dog and human liver microsomes, relatively large percentages of radiolabel were unextractable or remained at the TLC origin.

Identification of the metabolites showed revealed the same compounds in males and females and in the different test species. Only in their relative proportions did metabolites differ between sexes and species. It was noted however, that in mouse, goat, dog and human, relatively large percentages of radiolabel were unextractable or remained at the TLC origin. Of the species studied, the rat was relatively slow at metabolizing pyraziflumid. Pyraziflumid was not metabolized in the absence of NADPH in any of the microsome suspensions (Yasunaga, 2015).

Table 17. In vitro metabolism of [pyrazinyl-5(6)-^{14}C]pyraziflumid by liver microsomes from various mammals in the presence of NADPH

Metabolites	% of application									
	Buffer control	Rat (Wistar)		Mouse		Rabbit	Goat	Dog		Human
		Male	Female	Male	Female	Female	Female	Male	Female	Mixed gender
Pyraziflumid	100.0	80.32	91.21	ND	1.47	44.30	ND	22.83	9.20	11.14
Pyraziflumid-4'-OH (BC-01)	ND	9.47	6.65	45.41	36.62	47.06	47.71	43.14	32.48	38.57
Pyraziflumid-3'-OH (BC-03)	ND	0.64	ND	ND	ND	ND	ND	ND	ND	ND
Pyraziflumid-3,4'-OH (BC-05)	ND	ND	ND	ND	1.05	ND	1.90	ND	4.58	6.82
Pyraziflumid-5-OH (BC-06)	ND	1.04	0.73	ND	1.16	ND	ND	3.91	5.66	ND
Pyraziflumid-amide (BC-10)	ND	ND	ND	3.71	1.74	ND	3.40	ND	ND	2.75
Origin	ND	6.18	0.79	27.25	39.12	6.11	23.48	22.80	36.02	23.91
Others	ND	0.47	ND	ND	1.05	ND	8.22	2.47	3.08	5.76
Unextractable	ND	1.89	0.62	23.63	17.79	2.54	15.30	4.85	8.98	11.05
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

ND: Not detected; Source: Yasunaga, 2015

Table 18. In vitro metabolism of [aniline-U-^{14}C]]pyraziflumid by liver microsomes from various mammals with NADPH

Metabolites	% of application									
	Buffer control	Rat (Wistar)		Mouse		Rabbit	Goat	Dog		Human
		Male	Female	Male	Female	Female	Female	Male	Female	Mixed gender
Pyraziflumid	99.97	82.54	90.94	ND	1.25	42.95	1.52	19.97	5.94	10.22
Pyraziflumid-4'-OH (BC-01)	ND	8.59	6.51	36.80	40.79	47.38	47.98	44.82	29.55	37.89
Pyraziflumid-3'-OH (BC-03)	ND	0.57	ND	ND	ND	ND	ND	ND	ND	ND
Pyraziflumid-3,4'-OH (BC-05)	ND	ND	ND	0.66	1.22	ND	1.03	ND	6.09	9.45
Pyraziflumid-5-OH (BC-06)	ND	0.97	0.71	ND	1.35	ND	ND	4.91	6.94	ND
Origin	ND	4.62	0.65	37.57	35.57	7.31	24.26	19.38	32.16	28.02
Others	ND	0.95	0.61	0.42	2.08	ND	8.63	5.30	9.14	3.93
Unextractable	0.03	1.76	0.58	24.56	17.74	2.35	16.58	5.61	10.18	10.40
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

ND: Not detected; Source: Yasunaga, 2015

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

The results of studies on acute toxicity with pyraziflumid are summarized in Table 19.

Table 19. Summary of acute toxicity studies with pyraziflumid

Species	Strain	Sex	Route	Vehicle	Purity (%)	LD_{50}/LC_{50}	Reference
Rat	Wistar (RccHan):WIST	F	Oral	0.5% Na-CMC in 0.1 v/v Tween 80	94.8	> 2000 mg/kg bw (M/F)	Toga (2014) [a]
Rat	Sprague Dawley (Slc:SD)	M/F	Dermal	0.5% Na-CMC in 0.2 v/v Tween 80	94.8	> 2000 mg/kg bw (M/F)	Munechika (2013a) [b]
Rat	Wistar (RccHan:WIST)	M/F	Inhalation	-	94.8	> 2.1 mg/L (M/F)	Sieber (2013) [c]

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

bw: Body weight; v/v: volume for volume

[a] Six female rats received pyraziflumid by gavage at 2000 mg/kg bw. No deaths, clinical signs or gross pathological findings were observed during, or at the end of, the 14-day observation period. Batch no.2JB0013P.

[b] No deaths, clinical signs, local skin reaction or gross pathological findings were observed during, or at the end of, the 14-day observation period. Batch no. 2JB0013P.

[c] Rats were exposed nose only to pyraziflumid at a gravimetric chamber concentration of 2.1 mg/L (highest feasible concentration). No deaths were observed. Following exposure, all animals showed ruffled fur on day 1. No other clinical signs were observed. All rats showed slight body weight loss from day 1 to day 2. Thereafter normal body weight gain was observed. Macroscopic examination at the end of the 14-day observation period showed no treatment-related effects. Mass median aerodynamic diameter was 2.64 ± 1.93 µm. Batch no. 2JB0013P.

(b) Dermal irritation

In an acute dermal irritation study, the intact skin of three male Japanese White rabbits (Kbl:JW) was exposed for four hours under semi-occlusion to 0.5 g pyraziflumid (batch no. 2JB0013P; purity 94.8%; moistened with distilled water). Dermal irritation was scored at 1, 24, 48 and 72 hours after patch removal.

No skin irritation was observed at any time point. No treatment-related clinical signs or body weight effects were observed (Munechika, 2013b).

(c) Ocular irritation

In an acute eye irritation study, 78 mg of pyraziflumid (equivalent to 0.1 mL; batch no. 2JB0013P; purity 94.8%) was instilled into the conjunctival sac of the left eye of each of three male Japanese White rabbits (Kbl:JW). The untreated eye served as a control. The eyes were examined macroscopically according to the Draize method for signs of irritation at 1, 24, 48 and 72 hours post instillation.

No corneal opacity or iritis was observed. Minimal conjunctivitis was observed at one hour post instillation. No treatment-related clinical signs were observed. The pattern of body weight gain was normal. In this study, pyraziflumid was not irritating to the eye in rabbits (Munechika, 2013c).

(d) Dermal sensitization

In a dermal sensitization study using the local lymph node assay (LLNA), pyraziflumid (batch no. 2JB0013P; purity 94.8%) was applied at concentrations of 10, 25 and 50% w/v; the vehicle was dimethyl formamide (DMF). These test concentrations, together with a positive control of 25% v/v α-hexylcinnamaldehyde (HCA), and a vehicle control (acetone/olive oil mixture at 4:1) were applied daily to the dorsal skin of both ears of groups of five female CBA/J mice at 25 µL per ear for three

consecutive days. Five days after the commencement of the application, 5-bromo-2′-deoxyuridine (BrdU) was injected intraperitoneally into the animals at a rate of 5 mg/mouse. Twenty-four hours after BrdU injection, incorporation of BrdU into the cells prepared from draining the animals' auricular lymph nodes was measured by enzyme-linked immunosorbent assay (ELISA) for each treatment group. The mean absorbance per animal and the stimulation index (SI) were calculated. Additionally, clinical signs, ear thickness and body weight changes were recorded.

No local skin irritation and no increase greater than 25% in ear thickness or ear weight was observed. Incorporation of BrdU was not increased by pyraziflumid treatment. The calculated SI values in 10, 25 and 50% pyraziflumid treatment groups were 1.0, 1.5 and 1.2 respectively. In the HCA positive control group a significant increase of BrdU incorporation was observed and the SI was calculated as 4.5. No treatment-related clinical signs or effects on body weight gain were observed.

Under the conditions of this study, pyraziflumid showed no skin sensitization potential in mice (Munechika, 2015).

2.2 Short-term studies of toxicity

(a) Oral administration

Mouse

In a 28-day dietary range-finding toxicity study, pyraziflumid (batch no. 8; purity 99.86%) was administered to groups of five male and five female ICR [Crlj:CD1] mice at concentrations of 0, 300, 1000, 3000 or 10 000 ppm (equal to 0, 49, 164, 520 and 1744 mg/kg bw per day for males, 0, 66, 195, 759 and 2475 mg/kg bw per day for females). The animals were checked daily for mortality and clinical signs of toxicity. Body weights and feed consumption were measured weekly. Prior to termination, blood samples were taken for haematology and blood biochemistry. All the mice underwent complete necropsy. Brain, thymus, heart, liver, spleen, kidneys, adrenals, testes, epididymides, ovaries, and uterus were weighed. In all animals, lymph nodes (cervix), bone and bone marrow (sternum and femur), thymus, trachea, heart, thyroids (including parathyroids), liver, spleen, kidneys, adrenals, brain (including the medulla, cerebellum and cerebrum, excluding olfactory brain), pituitary, testes, epididymides, ovaries and uterus were examined microscopically.

No mortality and no treatment-related clinical signs were observed. Females at 10 000 ppm showed an 8% reduction in body weight gain. Feed consumption was not affected by treatment. A reduction (up to 70%) in stab neutrophils was observed in females of all dose groups. As the reduction was not dependent on dose, was not observed in males, and no effects on other leukocytes were found this observation was considered to be incidental. No other haematological changes were found. Blood biochemistry showed significant decreases in albumin (11–12%) in males and females, total cholesterol (22%) and protein (12%) in males and total bilirubin (37%) in females at 10 000 ppm. There were no other significant changes in biochemistry. Relative liver weight was significantly increased (12%) in males at 3000 and 10 000 ppm. Increases in relative weights of heart and kidneys were observed in some treatment groups in females, however the effects showed no dose-dependency. Necropsy showed enlargement and an accentuated lobular pattern of liver in one male at 3000 ppm. Histopathology showed hepatocyte hypertrophy around the periportal region in all males and females at 10 000 ppm.

The NOAEL was 3000 ppm (equal to 520 mg/kg bw per day), based on increased relative liver weight in males and changes in blood biochemistry in both sexes at 10 000 ppm (equal to 1744 mg/kg bw per day) (Nagai, 2015a).

In a 90-day dietary toxicity study, pyraziflumid (batch no. 9JB0003P, purity 96.8%) was administered to groups of 12 male and 12 female CD-1 (Crl:CD-1(ICR)) mice at 0, 800, 3000 or 8000 ppm (equal to 0, 119, 433 and 1183 mg/kg bw per day for males, 0, 146, 514 and 1456 mg/kg bw per day for females). The animals were checked daily for mortality and clinical signs of toxicity. Body weights and feed consumption were measured weekly. During week 13, blood samples were taken from all animals for haematology and blood biochemistry. All mice underwent complete necropsy.

Brain, kidneys, adrenals, spleen, heart, liver, ovaries, testes, epididymides and uterus with cervix were weighed. Microscopic examination was performed for all animals on the kidneys, liver, thyroid with parathyroids and any other tissues considered abnormal at macroscopic examination.

No treatment-related mortalities or clinical signs were observed. One female at 8000 ppm, was killed in extremis in week 12. Macroscopic and histopathological examination indicated that the poor condition of this animal was not related to treatment. Although body weight gain in high-dose females was slightly lower than in controls, no significant effects on body weight gains or feed consumption were observed. An increase in white blood cell count was observed in males at 3000 (46%) and 8000 ppm (79%). The significance of this is not clear, as no such effect was observed in females or in the 28-day study in mice. Blood biochemistry showed statistically significant reductions in cholesterol levels in males at 3000 (31%) and 8000 ppm (46%), and a slight reduction (15%; non-significant) in females at 8000 ppm. The decrease in triglyceride levels was statistically significant in males at 8000 ppm (39%). Further, in high-dose males reductions in total protein (13%) and albumin (16%) were observed, along with slight reductions (< 10%) in these parameters in males at 3000 ppm and females at 3000 and 8000 ppm. Alkaline phosphatase (ALP) levels were increased (73%) in high-dose males. Absolute and relative liver weights were increased in males at 3000 ppm (11% and 12%, respectively) and 8000 ppm (15% and 19%, respectively) and in females at 8000 ppm (15% and 18%, respectively). Histopathology showed generalized hepatocyte hypertrophy in males at 3000 and 8000 ppm, periportal hepatocyte hypertrophy in females at 3000 and 8000 ppm, and periportal hepatocyte fat in some males and females at 3000 and 8000 ppm.

The NOAEL was 800 ppm (equal to 119 mg/kg bw per day), based on reductions in cholesterol and increases in liver weight in males and on hepatocyte hypertrophy and periportal hepatocyte fat in both sexes at 3000 ppm (equal to 433 mg/kg bw per day) (Coleman, 2013).

Rat

In a 28-day dietary range-finding toxicity study, pyraziflumid (batch no. 9JB0004P; purity 97.2%) was administered to groups of five male and five female Wistar Hannover (RccHan:WIST) rats at 0, 500, 2500 or 10 000 ppm for 14 or 28 days. Dietary doses for the rats treated for 28 days were equal to 0, 38, 181 and 727 mg/kg bw per day for males and 0, 40, 186 and 696 mg/kg bw per day for females. The animals were checked daily for mortality and clinical signs of toxicity. Body weights and feed consumption were measured weekly. For the 28-day group, blood was sampled on the day of necropsy for haematological and clinical biochemistry examinations. At termination (14 or 28 days) all the rats were examined macroscopically. For animals terminated at 14 days pituitary, thyroids (including parathyroids), thymus, liver and spleen were weighed. In addition to this, for animals terminated at 28 days the brain, heart, kidneys, adrenals, testes, epididymides, ovaries and uterus were weighed. For the control and high-dose animals terminated after 28 days, all these organs were examined microscopically. For the mid-dose groups and the animals terminated after 14 days the thyroids and liver were microscopically examined.

No deaths were observed. Observed after 14 days of treatment at 10 000 ppm were stained fur around the anogenital region (three males, two females), soft feces (one male) and piloerection (one female). Reduced body weight gain was observed in high-dose males (up to 14%) and females (up to 20%) at all time points during treatment and in mid-dose females (up to 14%) at termination after 28 days of treatment. Haematology showed small but statistically significant decreases in haemoglobin in high dose males (6%) and in mean corpuscular volume in high-dose females (5%). The effects of 28 day treatment with pyraziflumid on blood biochemistry are shown in Table 20.

Table 20. Blood chemistry results for rats treated orally with pyraziflumid for 20 days

	Dose level (ppm)							
	Male				Female			
	0	**500**	**2500**	**10 000**	**0**	**500**	**2500**	**10 000**
GGTP (IU/L)	1	1	2#	12#	1	1	3#	13#
Total cholesterol (mg/dL)	48	63#	66#	138#	38	43	53#	124#
Urea nitrogen (mg/dL)	20.5	19.8	21.5	24.8	23.3	25.3	29.3	32.0*

	Dose level (ppm)							
	Male				Female			
	0	500	2500	10 000	0	500	2500	10 000
Albumin (g/dL)	3.5	3.4	3.3	3.4	4.0	3.7*	3.6**	3.4***
Albumin/globulin (ratio)	1.2	1.1#	1.1#	1.1#	1.3	1.2	1.2	1.2 *
Glucose (mg/dL)	111	120	101	84	95	85	90	90

# $p < 0.05$ (Steel's multiple comparison test); GGTP: γ-Glutamyl transpeptidase; Source: Nagai, 2015b

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Dunnett's multiple comparison test);

Total cholesterol was increased at all doses in males and at the mid and high dose in females. Activity of γ-glutamyl transpeptidase (GGTP) was increased in both sexes at the mid and high doses. Small decreases in albumin levels were seen in females at all doses and in albumin/globulin ratio in both sexes at the high dose and also in males at the low and mid doses, though there was no trend with dose. Blood glucose was decreased in high-dose males and blood urea nitrogen was increased in high-dose females.

Necropsy of the liver revealed dark discolouration, enlargement and an accentuated lobular pattern in both sexes at the high dose after 14 and 28 days of treatment. Some of these changes were also observed in a few mid-dose females. Dose-dependent increases in absolute and relative weights of liver (13–87%) were observed in both sexes at all doses, and of thyroid in both sexes (>20%) at mid and high dose . Absolute and relative thymus weights were reduced in high-dose females (>16%) and absolute and relative spleen weights were reduced in females at the high dose (>24%). Histopathology showed centrilobular and midzonal hepatocytic hypertrophy and follicular cell hypertrophy in the thyroids of both sexes at mid and high dose (Nagai, 2015b).

In a 13-week dietary toxicity study, pyraziflumid (batch no. 9JB0003P; purity 96.8%) was administered to groups of ten male and ten female Wistar Hannover (RccHan: WIST) rats at 0, 100, 500 or 5000 ppm (reduced to 2000 ppm from week 9 due to excessive toxicity) for males and 0, 100, 500 or 2000 ppm for females. Dietary doses for the rats treated at 0, 100 and 500 ppm were equal to 0, 7.1 and 36 mg/kg bw/day for males and 0, 8.6 and 42 mg/kg bw/day for females. For the high-dose males the dietary doses were equal to 435 mg/kg bw per day at 5000 ppm (weeks 1–8) and 151 mg/kg bw per day at 2000 ppm (weeks 9–13) and 172 mg/kg bw/day for females receiving 2000 ppm. The animals were checked daily for mortality and clinical signs of toxicity. A detailed physical examination and arena observation were carried out once per week. Assessments of motor activity, sensory reactivity and grip strength were performed on all animals during week 12 of treatment. Body weights and feed consumption were measured weekly. Ophthalmoscopy was performed on all animals before treatment, and on control and high-dose rats during week 13. During week 13 urine and blood were sampled for urine analysis and haematological and clinical biochemistry examinations. At termination all the rats were necropsied and adrenals, brain, epididymides, heart, kidneys, liver, ovaries, spleen, testes, thymus, thyroids (including parathyroids) and uterus with cervix were weighed. An extensive range of organs and tissues of the control and high dose animals were examined histologically. In addition, thyroid, kidneys, liver and lungs of the low- and mid-dose animals were examined histologically.

At 5000 ppm one male showing poor condition (hunched posture, thin build, abnormal tooth colour, dull eyes and piloerection) and marked weight loss was killed in week 8 of treatment. The severe effects were considered treatment-related. Two other males at 5000 ppm that showed similar clinical signs recovered when the dietary dose was reduced to 2000 ppm in week 9. One male at 100 ppm died following blood sampling on day 88 of treatment. This death was considered accidental.

Other observed clinical signs were abnormal brown staining of the tail between weeks 8 and 10 in males at 5000 ppm, pale lower incisors observed from week 6 in males at 500 or 5000↓2000 ppm and between weeks 8 and 10 in two females at 500 ppm, and from week 6 in females at 2000 ppm. Increased sacral muscle/body tone was observed from week 8 in females at 2000 ppm. No treatment-related findings were reported in detailed physical and arena observations, sensory reactivity, grip strength, motor activity, or in ophthalmic examination.

Bodyweight gain was reduced in females at 2000 ppm (up to 20%) and in males at 5000 ppm (up to 33%). In males a statistically significant reduction in body weight gain (11%) was already observed

in the first week of treatment. Following the reduction of the dietary dose to 2000 ppm the high-dose males showed a partial recovery of body weight gain, with final body weights 23% lower than controls. Average feed consumption was reduced by 14% throughout the treatment period in females at 2000 ppm and by 17 % in males at 5000 ppm. Following reduction of the dietary dose of pyraziflumid to 2000 ppm the feed consumption in males was comparable to controls.

At the end of the treatment period slight reductions in haematocrit and haemoglobin concentrations were observed in high-dose males and females, and slight reductions in mean cell haemoglobin and mean cell volume were found in high-dose females. Mean prothrombin times were shorter (ca 1–2 s) than controls in high-dose males and females. Although the effects were statistically significant, the magnitudes were small (6% or less) and they were considered not toxicologically relevant.

In week 13 in both sexes a decrease of total triglyceride concentration was observed at 500 ppm; 49% in males, 27% in females (not significant) and at the high dose 80% in males, 35% in females. In high-dose females total cholesterol was increased by 97%. The changes in triglyceride and cholesterol were considered due to effects on the triglyceride cycle. In both sexes at the high dose GGTP activity was markedly increased (9 and 19 IU/L for males and females respectively, versus 0 IU/L in controls) and in high-dose females urea was increased by 27%, while blood glucose was decreased by 11%. In females at 500 and 2000 ppm the serum albumin concentration was decreased by 10% and 19%, respectively. The clinical chemistry findings were attributed to an effect on the liver. At 2000 ppm, females showed a slight increase in potassium (13%) and a slight decrease in calcium (4%), which may be related to effects on the kidney.

Urinalysis showed a reduced urinary volume in high-dose females (36%) and males (35%) and in mid-dose males (25%), which was associated with an increase in specific gravity. In high-dose males a low urinary pH was observed (6.7 versus 7.2 in controls) and the presence of bilirubin was reported in seven high-dose females.

Relative liver weight in males and females at 500 ppm was increased by 19–22% and at the high dose by 48–52%. At the high dose, relative thyroid weight was increased in males (82%) and females (30%). Relative kidney weight was increased in females at 500 and 2000 ppm (7% and 10% respectively) and in males at 5000↓2000 ppm (15%).

Macroscopy revealed a dark appearance to the liver at 500 ppm or above in both sexes and an enlargement of the liver in two females given 2000 ppm. A darkening of the kidneys was observed in two females given 500 ppm, in the majority of males given 5000↓2000 ppm and in all females given 2000 ppm. Uteri of two females at 2000 ppm appeared thin. The thyroids of one male at 500 ppm, one male at 5000↓2000 ppm and two females at 2000 ppm appeared darker than normal.

Histopathological investigation revealed hepatocellular hypertrophy at 500 ppm and above, which was centrilobular in all animals, but extended into the midzonal region in males (mainly at the high dose), and in two high-dose males there were associated myeloid figures. Six high-dose females also displayed periportal fatty vacuolation. A fine brown pigment in the hepatocytes was observed in two females at 500 ppm and all high-dose males and females; this was often accompanied by pigment in the Kupffer cells. In the thyroid glands, there was follicular cell hypertrophy at 500 ppm and above, often accompanied by pale colloid with aggregations, and at the high dose six males and two females also showed some follicular hyperplasia. There was a fine brown pigment in the renal cortical tubules in four high-dose males, one mid-dose female and in all high-dose females.

The NOAEL was 100 ppm (equal to 7.1 mg/kg bw per day) based on changes in clinical signs, clinical chemistry and effects on liver and thyroid in both sexes, and on effects on kidney in females, at 500 ppm (equal to 36 mg/kg bw per day) (Stamp, 2010).

Dog

In a 28-day range-finding oral toxicity study, pyraziflumid (batch no. 2JB0013P; purity 94.8%) was administered in the diet to one male and one female beagle dog at a dose of 0, 250, 1000, 10 000, or 32 000 ppm (equal to 0, 8.1, 30, 283 and 721 mg/kg bw per day for males, 0, 7.8, 33, 309 and 761 mg/kg bw per day for females). The high dose was reduced to 20 000 ppm on day 6 due to excessive toxicity, and discontinued after two weeks of treatment. The animals were checked daily for mortality

and clinical signs of toxicity. A detailed clinical examination was performed pretreatment started and weekly thereafter. Body weights were measured pretreatment and weekly thereafter. Feed consumption was recorded daily and calculated on a weekly basis. Ophthalmology was performed pretreatment and during week 4. Blood and urine for haematology, clinical biochemistry and urinalysis were sampled pretreatment and during weeks 2 and 4; during week 3 clinical biochemistry alone. All dogs were necropsied and weights of brain, pituitary, thyroids with parathyroids (bilateral), heart, thymus, liver with gallbladder, kidneys (bilateral), spleen, adrenals (bilateral), testes (bilateral), epididymides (bilateral), prostate, ovaries (bilateral), and uterus were determined. Microscopic examination was performed on the liver.

There were no mortalities. At 32 000 ppm vomiting and mucous stool or no faeces was observed during the first week of treatment. At 10 000 ppm occasionally vomiting was observed in the male. At 0, 250 and 10 000 ppm mucous stool was occasionally observed. Body weight loss was observed at 32 000 ppm in the male (1.2 kg) and female (1.0 kg) during the first two weeks of treatment. Feed consumption was reduced at the high dose from day 4 of treatment and was noted up to one week after discontinuation of the treatment.

Urine analysis showed a marked excretion of bilirubin in both high dose animals at week 2 just before discontinuation of treatment. In these animals, decreases in platelet count and reticulocyte count, prolongation of prothrombin time and activated partial thromboplastin time (APTT) were observed at week 2. In addition, hematocrit (Ht), haemoglobin (Hb) concentration and erythrocyte count (RBC) were increased in the male dog at week 2. At the high dose, increases in alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), GGTP and total bilirubin, and a decrease in total cholesterol at week 2 of treatment were observed in both sexes. The female dog showed a decrease in BUN at week 2. Histopathology showed slight to moderate centrilobular hepatocellular degeneration in the liver at the high dose.

At 10 000 ppm the female dog showed increases in ALT from week 2 onwards and in ALP, AST, GGTP and total bilirubin from week 3 onwards. At necropsy, the female dog exhibited a coarse surface to the liver. Histopathology showed moderate centrilobular hepatocellular degeneration, moderate hepatocellular single cell necrosis and slight granuloma in the liver of the female dog.

No effects were observed at 250 or 1000 ppm (Kuwahara, 2012).

Study 1

In a 90-day oral toxicity study, pyraziflumid (batch no. 2JB0013P; purity 94.8%) was administered in the diet to groups of four male and four female beagle dogs at doses of 0, 200, 1000 or 10 000 ppm. In males the high dose was reduced to 5000 ppm from week 3 due to severe toxicity in these animals. Subsequently, in one high-dose male treatment was discontinued during week 7 due to persistent decrease in body weight and feed consumption despite reduction in the dose. The dietary doses were equal to 0, 6.0, 29 and 167 mg/kg bw per day for males and 0, 6.2, 31 and 320 mg/kg bw per day for females. The animals were checked daily for mortality and clinical signs of toxicity. A detailed clinical examination was performed pretreatment started and weekly thereafter. Body weights were measured pretreatment and weekly thereafter. Feed consumption was recorded daily. Ophthalmology was performed pretreatment and during week 13. Blood samples for haematology and clinical biochemistry were collected pretreatment and during weeks 2, 5, 9 and 13. Urine analysis was performed pretreatment and during weeks 7 and 13. All dogs were necropsied and weights of brain, pituitary, thyroids with parathyroids (bilateral), heart, thymus, liver with gallbladder, kidneys (bilateral), spleen, adrenals (bilateral), testes (bilateral), epididymides (bilateral), prostate, ovaries (bilateral), and uterus were determined. Microscopic examination was performed on an extensive range of organs and tissues.

There were no mortalities or treatment-related clinical signs. At the high dose, two males showed body weight loss (0.3 kg and 1.2 kg) during the first three weeks of treatment with pyraziflumid at 10 000 ppm. Feed consumption in these animals began to decrease from week 2 onward. After the reduction in dose at week 3 to 5000 ppm, one male slowly improved in body weight gain and feed consumption and slowly recovered while the other male continued to lose body weight (2.2 kg) and feed consumption remained at about one-third of the provided amount until treatment of this animal was discontinued during week 7. Ophthalmology did not show any effect due to treatment. Some significant changes in haematology were noted but were considered not to be treatment-related because they occurred temporarily or without a relationship to dose. Urine analysis showed increased bilirubin levels

in the two high-dose male dogs with body weight loss. This was considered related to the increased plasma bilirubin levels in these animals. Data from clinical chemistry are presented in Table 21.

Table 21. Selected clinical chemistry parameters (values in % of control) of dogs treated orally with pyraziflumid for 90 days

Parameter	Treatment week	Sex and dose level (ppm)					
		Male			Female		
		200	1000	10 000↓ 5000	200	1000	10 000
Alkaline phosphatase, ALP (IU/L)	2	86	91	168	100	104	10
	5	84	90	289	108	115	144**
	9	84	90	173	105	116	190*
	13	81	85	157	105	139	234**
Aspartate aminotransferase, AST (IU/L)	2	97	90	213	104	107	118
	5	100	90	155	111	96	119
	9	97	91	128	107	104	122
	13	91	88	103	104	104	125
Alanine aminotransferase, ALT (IU/L)	2	103	97	1372	84	103	84
	5	116	100	668	92	97	84
	9	103	103	571	80	93	80
	13	103	103	344	94	103	100
GGTP (IU/L)	2	84	76	135	88	94	106
	5	87	74	223	96	100	104
	9	89	82	142	94	100	97
	13	85	75*	118	103	106	110
Total protein (g/dL)	BDL	101	99	101	106**	99	104
	5	103	102	105	109*	100	106
	9	103	102	101	108*	102	111**
	13	101	99	99	110*	105	110*
Albumin (g/dL)	9	105	104	108**	103	102	103
	13	105	103	111**	104	104	103
Globulin (g/dL)	BDL	97	96	97	112*	99	108
	5	102	101	99	116*	96	111
	9	103	99	91	118*	104	127**
	13	102	95	90	119	106	125*
Albumin/globulin (ratio)	9	102	106	119	86	97	77**
	13	99	108	119	86	100	78*
Total bilirubin (mg/dL)	2	100	100	150	86	71	86
	5	100	100	340	117	100	100
	9	86	100	143	88	88	88
	13	86	114	129	100	117	117

BDL: Below detection limit

Source: Kuwahara (2013)

* $p < 0.05$; ** $p < 0.01$ (by Dunnett's test or another Dunnett's-type test)

High dose males showed non-significant increases in ALP, AST and ALT which peaked at weeks 2–5 and GGTP and total bilirubin which peaked at week 5. Data for individual animals showed that these changes were more marked in some animals than in others. In females a statistically significant increase

in ALP was seen which progressed with duration of treatment. In addition, significant increases in globulin and a significant decrease in the albumin/globulin ratio (A/G) were observed in the later phases of the treatment period.

Relative liver weights were increased by 17% at the high dose in males and 21% in females. The increase in relative liver weight (16%) in males at the mid dose was not accompanied by any changes in histopathology or clinical chemistry. Changes in absolute and relative spleen weight in males were not considered treatment-related as they lacked dose–response relationship and were not accompanied by histopathological changes or changes in haematological parameters. Necropsy showed no effect of treatment. Histopathology of the high-dose male that lost body weight during the first weeks of treatment revealed moderate brown pigment deposition in Kupffer cell, hepatocellular single cell necrosis, hepatocellular degeneration and slight granuloma. In the cytoplasm of degenerated hepatocytes, irregular shaped spaces were observed and sometimes cells were expanded with the spaces. The distribution of degenerated hepatocytes in the liver lobe architecture did not show any specific pattern in periportal, midzonal or centrilobular zones. In the high-dose male for which treatment was discontinued in week 7 only a moderate level of brown pigment deposition in liver Kupffer cells was found. In the liver of a high-dose female, slight brown pigment deposition (likely hemosiderin and lipofuscin) was noted in Kupffer cells along with hepatocellular single cell necrosis. In the low- and mid-dose groups no treatment-related histopathological changes were noted.

The NOAEL was 1000 ppm, equal to 29 mg/kg bw per day, based on effects on body weight and feed consumption in males and liver toxicity in both sexes at 10 000↓5000 ppm, equal to 167 mg/kg bw per day (Kuwahara, 2013).

Study 2

In a one-year oral toxicity study, pyraziflumid (batch no. 2JB0013P; purity 94.8%) was administered in the diet to groups of four male and four female beagle dogs at a dose of 0, 200, 1000 or 5000 ppm. One male in the 5000 ppm group was found dead on day 8, and blood biochemistry conducted on day 8 on all surviving males in the 5000 ppm group and all females on day 5, showed marked increases in liver-related parameters in two of three surviving males and two of four females treated at 5000 ppm. As a result of these observations treatment of the high-dose animals was discontinued from day 9 for males and from day 4 for females. After a withdrawal and recovery period the dose level was reduced from 5000 ppm to 2000 ppm. Treatment was restarted at day 21 (week 4) for males and day 28 (week 5) or day 75 (week 11) for females. The dietary doses were equal to 0, 5.4, 28 and 51 mg/kg bw per day for males and 0, 5.5, 28, and 48 mg/kg bw per day for females. The animals were checked daily for mortality and clinical signs of toxicity. A detailed clinical examination was performed before treatment started and weekly thereafter. Body weights were measured pretreatment and weekly from weeks 1 to 13 and every four weeks thereafter. Feed consumption was recorded daily and intakes were calculated on a weekly basis. Ophthalmology was performed pretreatment and during week 52. Haematology and urine analysis were performed pretreatment and during weeks 26 and 52. Clinical biochemistry was performed pretreatment and during weeks 2, 4, 7, 13, 26 and 52. All dogs were necropsied, and weights of brain, pituitary, thyroids with parathyroids (bilateral), heart, liver with gallbladder, kidneys (bilateral), spleen, adrenals (bilateral), testes (bilateral), epididymides (bilateral), prostate, ovaries (bilateral), and uterus were determined. Microscopic examination was performed on an extensive range of organs and tissues.

No treatment-related effects were observed on ophthalmology, haematology, urine analysis or organ weights. At the high dose, two males were found dead within the first two weeks of treatment. In addition, another male and two females were killed in extremis 5–31 weeks after the restart of treatment at 2000 ppm. In these dogs clinical signs indicating debility (lateral position, decreased spontaneous motor activity) and/or jaundice were observed. No mortalities or treatment-related clinical signs were observed in animals from any of the other treatment groups. The males that were found dead showed a body weight loss of about 1 kg. The animals that were killed in a moribund state showed a body weight loss of 2.4–2.6 kg and a marked reduction in feed consumption throughout the treatment period. No marked effects on body weight or feed consumption were found in the animals that survived until the end of the study.

Clinical biochemistry data are presented in Table 22.

Table 22. Data on selected clinical biochemistry parameters (% of control) of dogs treated orally with pyraziflumid for one year

Parameter	Treatment week	Sex and dose level (ppm)					
		Male			Female		
		200	1000	5000↓ 2000	200	1000	5000↓ 2000
Alkaline phosphatase, ALP (IU/L)	BT	111	106	94	114	94	137**
	2	110	101	<3 dogs [a]	125	100	663*
	4	122	111	<3 dogs [a]	123	105	192*
	7	107	114	<3 dogs [a]	121	114	169**
	13	102	112	<3 dogs [a]	107	116	426
Aspartate amino-transferase, AST (IU/L)	BT	108	96	100	100	119*	108
	2	104	104	<3 dogs [a]	112	116	424**
Blood urea nitrogen, BUN (mg/dL)	13	102	104	<3 dogs [a]	97	116	129*
Albumin (g/dL)	2	106	107*	<3 dogs [a]	100	105	103
	4	106	107*	<3 dogs [a]	99	103	99
	13	107*	106	<3 dogs [a]	101	104	108
Total cholesterol (mg/dL)	2	104	133*	<3 dogs [a]	98	115	88
Total bilirubin (mg/dL)	7	100	140**	<3 dogs [a]	120	120	180

BT: Before initiation of treatment; <3 dogs: The number of dogs surviving was less than three;

* $p < 0.05$; ** $p < 0.01$ (Dunnett's test or another Dunnett's-type test) Source: Kuwahara, 2015

[a] The number of surviving dogs in the high-dose groups was less than 3 in males after week 2 and in females after week 13.

Clinical biochemistry examination revealed a marked elevation in ALP, AST, BUN and total bilirubin (not statistically significant) in the high-dose females. These increases could be attributed to the 5000 ppm treatment during the first week and were particularly high in dogs killed in extremis. All dogs found dead or killed in extremis showed markedly high values of these parameters, as well as in ALT and GGTP. At necropsy, findings indicative of liver injury, retarded body weight, malnutrition, and/or renal lesions were observed. Histopathology of the high-dose dogs showed severe hepatocellular degeneration, haemorrhage and slight oval cell hyperplasia in the liver of the two males that died during the first weeks of treatment. In the liver of the other three dogs killed in extremis, regeneration of hepatocytes, oval cell hyperplasia and fibrosis were observed, sometimes accompanied by single cell necrosis and degeneration of hepatocytes. Lipofuscin and hemosiderin deposition was observed in sinusoidal macrophages including Kupffer cells, likely the result of phagocytosis of erythrocytes and dead hepatocytes. These pigments were also found in regenerating hepatocytes. Further, brown pigment in bile canaliculi indicated cholestasis in the two females showing clinical jaundice. In the kidney of four out of five dogs found dead or killed in extremis, degenerative changes were observed, mainly in proximal tubular cells. In dogs that survived to the termination of treatment, treatment-related changes were noted in blood biochemical examination only; these amounted to apparent increases in ALP in two females, and AST in one female. The remaining high-dose male did not show any treatment-related changes in any of the parameters examined. No treatment-related histopathologic effects were observed in animals from the low- and mid-dose groups.

The NOAEL was 1000 ppm (equal to 28 mg/kg bw per day), based on mortality, clinical signs, body weight loss and reduced feed consumption in males, and on clinical biochemistry and histopathological signs of liver and kidney toxicity displayed in both sexes at 5000↓2000 ppm (equal to 48 mg/kg bw per day (Kuwahara, 2015).

(b) Dermal application

In a five-day range-finding dermal toxicity study, pyraziflumid (batch no. 2JB0013P; purity 94.8%) was applied under occlusion for at least six hours per day to the dorsal skin of groups of three female Sprague Dawley (Crl:CD(SD)) rats at 0 or 1000 mg/kg bw per day. The animals were checked daily for mortality, clinical signs of toxicity and for the condition of the application site. Body weights and feed consumption were measured daily. On day 6 all the rats were necropsied, and their livers weighed.

No effects of treatment were observed on any of the parameters investigated (Namiki, 2017a).

In a 90-day dermal toxicity study, pyraziflumid (batch no. 2JB0013P; purity 94.8%) was applied under occlusion for at least six hours per day to the dorsal skin of groups of 10 male and 10 female Sprague Dawley (Crl:CD(SD)) rats at 0, 40, 200 or 1000 mg/kg bw per day. The animals were checked daily for mortality, clinical signs of toxicity and for the condition of the application site. A detailed clinical examination was performed weekly. Body weights and feed consumption were measured weekly. All rats were subjected to ophthalmoscopy prior to treatment and rats from the control and high dose groups also examined during week 13. During week 13 urine was sampled for analysis. At termination blood was collected for haematology and clinical biochemistry. At termination all the rats were necropsied, and weights of brain, heart, liver, kidneys, testes, epididymides, seminal vesicles (with coagulating glands), thyroids (with parathyroids), spleen, thymus, adrenals, prostate, ovaries, and uterus were recorded. A wide range of organs and tissues of the control and 1000 mg/kg bw per day animals were examined histopathologically.

No effects of treatment on any of the parameters investigated were observed.

The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Namiki, 2017b).

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

In an 18-month dietary carcinogenicity study, pyraziflumid (batch no. 2JB0013P; purity 94.8%) was administered to groups of 51 male and 51 female CD-1 (Crl:CD-1(ICR)) mice at 0, 200, 2000 or 8000 ppm (equal to 0, 21, 227 and 905 mg/kg bw per day for males, 0, 25, 251 and 1030 mg/kg bw per day for females). The mice were checked daily for mortality and clinical signs. A detailed clinical examination, including palpation for masses, was carried out weekly. Body weight and feed consumption were recorded weekly during the first 14 weeks and every four weeks thereafter. Blood was sampled for haematological examinations including blood smears at weeks 52 and 78, and from all animals killed in extremis. Animals found dead or euthanized before the end of the treatment period and all others at the end of the treatment period, underwent necropsy. Adrenals, brain, heart, liver, kidneys, spleen, ovaries, uterus with cervix, testes and epididymides were weighed. A wide range of tissues was examined microscopically from all animals killed or dying prematurely, and all control and high-dose animals killed at the end of the treatment period. In addition, liver and abnormalities in tissues of the low- and mid-dose groups were examined histopathologically.

There was no effect of treatment on mortality, clinical signs or the incidence or distribution of masses. Body weight gain was reduced during the first week of treatment in all treatment groups. Body weight gains during the first week were 3.7, 2.8, 2.2 and 1.8 g in control, low-, mid- and high-dose males respectively, and 1.5, 1.1, 1.2 and 1.0 g in control, low-, mid- and high-dose females respectively. Body weights were lower than those of controls for males of the mid-dose (up to 6%) and high-dose (up to 7%) groups, occasionally reaching statistical significance. Body weights of females were not affected by treatment. Feed consumption was not affected by treatment. There was no effect of treatment on haematological parameters. Macroscopic evaluation showed enlarged livers in males at the mid and high dose.

Histopathology showed an increase in the incidence and severity of generalized hepatocyte hypertrophy in males at 2000 and 8000 ppm. A similar increase, but at a much lower incidence, was observed in females receiving 2000 and 8000 ppm. An increase in the incidence and severity of diffuse hepatocyte vacuolation was observed in high-dose males 8000 ppm and to a lesser extent in males at 2000 ppm. A similar increase was not seen in treated females. The incidence of periportal hepatocyte

vacuolation was increased in mid- and high-dose females. No treatment-related neoplastic lesions were reported.

The NOAEL was 200 ppm (equal to 21 mg/kg bw per day) based on liver toxicity observed in both sexes accompanied by slight reductions in body weight gain in males, particularly over the first week of treatment at 2000 ppm (equal to 227 mg/kg bw per day) (Coleman, 2015a, b).

Rat

In a two-year combined toxicity and carcinogenicity study, pyraziflumid (batch no. 2JB0013P; purity 94.8%) was administered in the diet to groups of Han Wistar (RccHan:WIST) rats. For the chronic toxicity phase of the study groups of 20 male and 20 female rats received pyraziflumid for 52 weeks at dietary concentrations of 0, 50, 100, 300 or 1000 ppm (equal to 0.0, 2.7, 5.3, 16 and 55 mg/kg bw per day for males, 0.0, 3.4, 7.4, 22 and 78.0 mg/kg bw per day for females. In the carcinogenicity phase, groups of 50 male and 50 female rats were fed for 104 weeks with dietary concentrations of 0, 50, 100, 300 or 1000 ppm (equal to 0.0, 2.2, 4.3, 13 and 46 mg/kg bw per day for males, 0.0, 2.9, 5.7, 18 and 66 mg/kg bw per day for females). The rats were checked daily for mortality and clinical signs, and weekly for palpable masses. A detailed clinical examination including palpation for masses was carried out weekly. Body weights and feed consumption were recorded weekly during the first 16 weeks and every four weeks thereafter. Ophthalmology was performed pretreatment and during week 52 on control and high-dose animals from the toxicity phase of the study. Blood was sampled for haematological and clinical biochemistry examinations at weeks 13, 26 and 52 from 10 males and 10 females from each group of the animals in the toxicity phase. Blood smears were prepared during weeks 52, 78 and 104 from all animals in the carcinogenicity phase of the study. Urine analysis was performed on 10 males and 10 females of the groups from the toxicity phase at weeks 13, 26, 30 (females only) and 52. Animals found dead or euthanized before or at the end of the treatment period underwent necropsy. Adrenals, brain, epididymides, heart, liver, kidneys, spleen, thymus, thyroid with parathyroids, ovaries, uterus with cervix, testes and epididymides were weighed from all animals from the toxicity and carcinogenicity phase. A wide range of tissues was examined microscopically from all animals killed or dying prematurely and all control and high-dose animals killed at the end of the treatment period. In addition, liver, kidney, thyroid (with parathyroids) and any abnormalities in tissues of the 50, 100 and 300 ppm groups were examined histopathologically.

There was no effect of treatment on mortality, clinical signs, incidence or distribution of masses or ophthalmology. In the toxicity phase group body weight was reduced when compared with controls to a statistically significant extent from week 7 in females receiving 1000 ppm, and from week 36 in females receiving 300 ppm. Final body weights at week 52 in the mid- and high-dose females were 9% and 21% lower than those of controls. Lower body weight when compared to controls was observed in males receiving 1000 ppm from week 1 to 7, but was not evident after week 8. High-dose males gained 25 g during the first week, compared to a 38 g weight gain in control males. Feed consumption was not affected in animals of the toxicity phase group. In the carcinogenicity phase groups, females at 100, 300 and 1000 ppm showed a 4%, 5% and 7% reduction in feed consumption over the treatment period. Males at 300 and 1000 ppm showed a 5% and 11% reduction in feed consumption compared to control males but only at week 1.

Haematological investigation of the toxicity phase animals in weeks 26 and 52 showed a statistically significant reduction in haemoglobin concentration in both males (4–6%) and in females (3–4%) receiving 1000 ppm which, in week 52, was also associated with a reduction of haematocrit, (up to 5%, statistically significant only in males). These effects were associated with a reduced mean cell haemoglobin (6–9%) and mean cell volume (4–7%) in females. There was no effect on the numbers of erythrocytes in the peripheral blood. The reduction of haemoglobin was not associated with a compensatory increase of reticulocyte numbers in the peripheral blood. At week 13 there was a small reduction of haematocrit and a small increase in mean cell haemoglobin concentration in high-dose males, and a small increase of erythrocyte count in high-dose females. However, except for the reduction in haematocrit (4%) in males, these findings were not observed at weeks 26 and 52. Females receiving 1000 ppm showed a small increase in the number of lymphocytes (33%) and monocytes (57%) at week 13. However, these effects were not observed at weeks 26 and 52, nor in males. All other effects on haematology parameters (neutrophil, leukocyte and monocyte cell counts, prothrombin and

activated partial thromboplastin times) were minor, lacked a dose–effect relationship, were inconsistent or confined to one sex and were therefore considered due to normal biological variation. Examination of blood smears of animals in the carcinogenicity phase of the study showed no effect due to treatment.

Clinical biochemistry showed a consistent increase in GGTP activity in males and females receiving 1000 ppm (2–6 IU/L compared with 0–1 IU/L in controls). Glucose concentration was consistently reduced in females at 300 ppm (12–18%) and 1000 ppm (17–9%) and in males at 1000 ppm (20–25%). Reductions in glucose were also observed at weeks 13 and 52 in males at 300 ppm (19–25%), at week 26 in females at 100 ppm (13%), and at week 52 in males at 100 ppm (12%). At 1000 ppm blood urea was consistently increased in females (20–24%) and at week 26 in males (16%). At 1000 ppm cholesterol concentrations were consistently high in males (25–62%) and females (44–99%), which were associated in both sexes with reduced triglyceride concentrations (43–47%) at week 52.

Total plasma protein concentrations were higher than controls (statistically significant) in the high-dose males throughout the treatment period (4–6%), in high dose females at week 13 (7%) and in males at 300 ppm at weeks 13 and 52 (3%). These increases in total protein concentrations were attributed to an increase in the globulin fraction.

Urine analysis showed no toxicologically significant effects.

Necropsy of the animals in the toxicity phase showed a dose-dependent increase in incidences of animals with dark-coloured kidneys, liver and thyroid at 300 and 1000 ppm. Dark-coloured kidney was also observed in one female at 100 ppm. Necropsy of the animals from the carcinogenicity phase also showed a dose-dependent increase in dark colouration of the kidney in females at 300 and 1000 ppm. Abnormal liver colour (pale or dark) were seen in males and females at 1000 ppm. In addition, in high dose females increased incidence of masses and depressions on the liver were observed. In males given 1000 ppm, cysts and masses in the thyroid glands occurred at higher incidences than in controls.

After 52 weeks there was a dose-dependent increase in relative liver and thyroid weight in both sexes at 300 and 1000 ppm (see Table 23). In addition, there was a small increase in the body weight-related kidney weights of females given 1000 ppm.

After 104 weeks a dose-dependent increase in relative liver weight was observed in males at 100 ppm and above, and in females at 300 ppm and above. Relative thyroid weight was increased in females at 100 ppm and above and in males at 300 ppm and above.

At 1000 ppm males and females showed a small but statistically significant increases in relative kidney weight in females and relative adrenal weight in both sexes.

Table 23. Effect on relative organ weights (expressed as % of body weight) in rats treated orally with pyraziflumid for two years

	Dose (ppm)				
	0	**50**	**100**	**300**	**1000**
	Toxicity phase (52 weeks)				
Males					
Liver weight	2.92 ± 0.16	3.06 ± 0.22	3.04 ± 0.26	3.46 ± 0.28**	3.77 ± 0.44**
Thyroids and parathyroids	0.0044 ± 0.0009	0.0050 ± 0.0011	0.0049 ± 0.0009	0.0057 ± 0.0012**	0.0067 ± 0.0011**
Females					
Liver weight	2.96 ± 0.44	3.06 ± 0.31	3.04 ± 0.27	3.37 ± 0.28**	4.77 ± 0.61**
Thyroids and parathyroids	0.0061 ± 0.0015	0.0071 ± 0.0013	0.0066 ± 0.0013	0.0074 ± 0.0021*	0.0085 ± 0.0015**
Kidney	0.561 ± 0.078	0.562 ± 0.072	0.569 ± 0.051	0.589 ± 0.047	0.621 ± 0.050**

	Dose (ppm)				
	0	50	100	300	1000
	Carcinogenicity phase (104 weeks)				
Males					
Liver weight	2.80 ± 0.26	2.92 ± 0.28	3.01 ± 0.37*	3.22 ± 0.45*	3.74 ± 0.45**
Thyroids and parathyroids	0.0058 ± 0.0033	0.0 059 ± 0.0020	0.0058 ± 0.0014	0.0063 ± 0.0013**	0.0104 ± 0.0067**
Adrenals	0.0098 ± 0.0027	0.0093 ± 0.0015	0.0101 ± 0.0019	0.0103 ± 0.0018	0.111 ± 0.0020*
Females					
Liver weight	2.80 ± 0.45	2.68 ± 0.29	2.87 ± 0.4	3.14 ± 0.38**	4.68 ± 0.73**
Thyroids and parathyroids	0.0062 ± 0.0016	0.0078 ± 0.0041	0.0071 ± 0.0016*	0.0075 ± 0.0022*	0.0084 ± 0.0019**
Adrenals	0.0176 ± 0.0033	0.0162 ± 0.0042	0.0168 ± 0.0034	0.0179 ± 0.0030	0.0200 ± 0.0043**
Kidneys	0.519 ± 0.073	0.493 ± 0.066	0.509 ± 0.071	0.533 ± 0.064	0.601 ± 0.064**

* $p < 0.05$; ** $p < 0.01$ Source: Stamp (2015b)

At 52 weeks, histopathological examination showed an increased incidence of cortical tubular pigmentation in kidneys of males at 300 and 1000 ppm and of females at 100 ppm or above, with a clear dose–effect relationship apparent. An increased incidence and/or severity of diffuse follicular cell hypertrophy was seen in males and females given 300 or 1000 ppm with a clear dose–effect relationship. Additionally, follicular cell hyperplasia was seen in two males given 1000 ppm. In the liver, centrilobular hypertrophy and centrilobular vacuolation were observed in males given 100 ppm or above with a clear dose–effect relationship. Additionally, dose-related increases in the incidence of single cell necrosis and of hepatocellular pigmentmentation were observed in males given 300 and 1000 ppm. In the females, dose-related centrilobular hypertrophy was observed in the liver of animals given 300 and 1000 ppm, while centrilobular vacuolation was seen in animals given 1000 ppm. In addition, an increased incidence and/or severity of single cell necrosis and hepatocellular pigmentation were also observed in females given 300 and 1000 ppm exhibiting a clear dose–effect relationship.

After 52 weeks of treatment a hepatocellular adenoma was seen in the liver of one female given 1000 ppm and follicular cell adenomas were seen in the thyroid glands of two males given 1000 ppm.

At 104 weeks histopathology showed a dose-dependent increase in the incidence and/or severity of centrilobular hypertrophy in the liver of both males and females at 300 and 1000 ppm, with a clear dose–effect relationship. A similar but marginal increase in incidence was also seen in males at 100 ppm. There was a dose-dependent increase in the incidence and/or severity of centrilobular vacuolation in males at 100, 300 and 1000 ppm.

A dose-dependent increase in the incidence and/or severity of hepatocellular pigmentation was seen in males at 300 ppm and above and in females at 100 ppm and above. An increase in the incidence of foci of cellular alteration was seen in males and females at 1000 ppm. The observed foci were predominantly basophilic or clear cell/pale in sub-classification. There was an increase in the incidence of angiectasis and multinucleated hepatocytes in females at 1000 ppm.

At 104 weeks a dose-dependent increase in the incidence and/or severity of diffuse follicular cell hypertrophy in the thyroid was seen in females at 300 and 1000 ppm, and in males at 1000 ppm. Similar but smaller increases in the incidence of diffuse follicular cell hypertrophy was also seen in males at 100 and 300 ppm and in females at 100 ppm. In males at 300 and 1000 ppm an increase in the incidence and/or severity of follicular cell hyperplasia was observed.

A summary of neoplastic findings is presented in Table 24. A statistically significant increase in the incidence of hepatocellular adenomas (12%), which was outside the historical control range (0–2% in both males and females), was seen in females at 1000 ppm.

In two males at 1000 ppm follicular cell carcinomas in the thyroid (outside the historical control range of 0–3.6%) were seen. There were increased incidences of follicular cell adenomas in

the thyroid glands of males given 100 ppm and in males and females given 300 and 1000 ppm. The incidences of follicular cell adenoma in males given 100, 300 or 1000 ppm and in females given 300 or 1000 ppm were above the range of the historical background data (males 0–10.9% and females 0–7.1%). Statistical analysis of follicular cell adenomas in males given 100 and 300 ppm and in females showed no statistical significance and no dose–effect relationship. In males at 1000 ppm, a statistically significant increase in these tumours was found, accompanied by increased organ weights and increased incidence and/or severity of follicular hypertrophy/hyperplasia.

Table 24. Incidences of non-neoplastic and neoplastic changes in the liver and thyroids of rats treated orally with pyraziflumid for two years

Sex	Male					Female				
Dose (ppm)	**0**	**50**	**100**	**300**	**1000**	**0**	**50**	**100**	**300**	**1000**
					Non-neoplastic changes					
Liver										
Centrilobular hypertrophy	1	3	6	32**	42**	4	3	7	38**	48**
Centrilobular vacuolation	3	2	10*	12*	35**	7	8	6	5	5
Hepatocellular Pigment,	2	3	1	23**	30**	22	25	28	36**	45**
Foci; cellular alteration minimal	37	37	41	38	45*	38	41	43	39	44
Angiectasis	4	2	3	5	5	1	2	4	4	14**
Increase in multinucleated hepatocytes	0	0	0	0	0	0	0	0	1	5*
Number of tissues examined	50	50	50	50	50	50	50	50	50	50
Thyroid										
Diffuse follicular cell hypertrophy	32	30	37	39	49**	17	19	26	35**	48**
Follicular cell hyperplasia	2	3	3	11**	11**	4	4	2	4	5
Number of tissues examined	50	50	50	50	49	50	50	50	49	50
					Neoplastic changes					
Liver										
Hepatocellular adenoma	0	0	0	0	1	0	1	1	0	6
as percentage	0	0	0	0	2	0	2	2	0	12
Number of animals examined	50	50	50	50	50	50	50	50	50	50
Thyroid										
Follicular cell carcinoma	0	0	0	0	2	0	0	0	0	0
as percentage	0	0	0	0	4	0	0	0	0	0
Follicular cell adenoma	2	5	7	6	17	0	2	3	7	4
as percentage	4	10	14	12	34	0	4	6	14	8
Number of animals examined	50	50	50	50	49	50	50	50	49	50

* $p < 0.05$; ** $p < 0.01$ Fisher's Exact test (one tailed); Source: Stamp, 2015b

The NOAEL was 50 ppm, equal to 2.2 mg/kg bw per day, based on macroscopical and histopathological signs of liver and thyroid toxicity observed in both sexes, and indications of minor kidney toxicity in females at 100 ppm, equal to 4.3 mg/kg bw per day. The NOAEL for neoplastic changes was 300 ppm (equal to 13 mg/kg bw per day) based on increased incidences of thyroid tumours in males and hepatocellular adenomas in females at 1000 ppm (equal to 46 mg/kg bw per day) (Stamp, 2015a, b).

2.4 Genotoxicity

Pyraziflumid was tested for genotoxicity in three in vitro assays and two in vivo assays. All tests gave negative results, apart from the finding of polyploidy in an in vitro test in Chinese hamster lung (CHL) cells Genotoxicity results are shown in Table 25.

Table 25. Overview of genotoxicity tests with pyraziflumid

End-point	Test system	Concentrations/doses tested	Purity	Results	Reference
		In vitro			
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2uvrA	0.195–12.5 mg/ml 19.5–313 µg/plate (−S9); 78.1–1250 µg/plate (+S9)	96.8%	Negative[a]	Oguma (2012)
Chromosomal aberration	Chinese hamster lung cell (CHL/IU)	5–1400 µg/mL in DMSO (−S9) 0.5–10 µg/mL in DMSO (+S9)	99.2%	Chromosomal aberration: negative. Polyploidy: Positive[b]	Tsukushi (2014a)
Mammalian cell gene mutation	L5178Y $tk^{+/-}$ (3.7.2C) mouse lymphoma cells	Initial test (3 h): 3.9–250 µg/mL (± S9, Mutation experiment 3 h: 5–100 µg/mL in DMSO (± S9) Mutation experiment 24 h: 5–40 µg/mL in DMSO (−S9)	94.8%	Negative[c]	Munechika (2017)
		In vivo			
Mouse micronucleus	Male Slc/ICR mouse, bone marrow	0, 500, 1000, 2000 mg/kg bw on two consecutive days	94.8%	Negative[d]	Tsukushi (2014b)
Comet assay	Female Rcc Han Wistar rat	0, 500, 1000, 2000 mg/kg bw on two consecutive days	94.8%	Negative[e]	Kasamoto (2015)

bw: body weight; CHL: Chinese hamster lung; DMSO: dimethyl sulfoxide;

S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] No increase in the numbers of revertant colonies was recorded for any of the strains of bacteria used, with or without metabolic activation. Precipitation of the test substance was observed at concentrations of 313 µg/plate and above without metabolic activation and 1250 µg/plate and above with metabolic activation. Batch no. 9JB0003P.

[b] The cells were exposed to pyraziflumid for 6 h with or without metabolic activation and then cultivated for another 17 h; this represented about 1.5 normal cell cycle lengths. Continuous exposure of cells to pyraziflumid for 23 and 45 h; equivalent to about 1.5 and 3.0 normal cell cycle lengths, respectively, were also examined without S9 mix. Pyraziflumid did not induce structural chromosome aberrations after short-term (6 h) exposure either with or without S9 mix. Furthermore, the number of cells with structural chromosome aberration was not increased when exposed to pyraziflumid for long-term exposure of pyraziflumid equivalent to 1.5 or 3.0 normal cell cycle lengths. No treatment-related increase in mutant frequencies was observed at any dose with or without the S9 mix. On the other hand, the number of polyploid cells increased in a concentration-dependent manner in short-term and long-term exposure experiments in the absence or presence of S9 mix. Batch no. 2JB00013P.

[c] Precipitate was observed at concentrations of 125 µg/mL and higher. In the absence of S-9 a concentration of 60 µg/mL gave 12% relative total growth (RTG) and with S-9, a concentration of 40 µg/mL gave 13% RTG. At higher concentrations in each condition, the RTG was below 10% due to high toxicity. In the mutation experiment for 24-hour treatment, seven concentrations ranging from 5 to 40 µg/mL, were tested in the absence of S-9. Two days after treatment, viability and mutant frequency were analysed. A concentration of 25 µg/mL gave 13% RTG. Above those concentrations, RTG was below 10% due to high toxicity. Under the conditions of this study it was concluded that pyraziflumid did not induce mutation at the *tk* locus of L5178Y mouse lymphoma cells. Batch no. 2JB00013P.

[d] Male Slc/ICR mice (five mice/group) received pyraziflumid at dose levels of 0, 500, 1000 and 2000 mg/kg bw per day, once daily for two consecutive days. A positive control group was administered a single intraperitoneal injection of Mitomycin C. Bone marrow smears were obtained from each animal 24 h after the last dosing. Pyraziflumid did not show

a clastogenic effect on immature erythrocytes in the bone marrow of mice under the study conditions. It was noted that no change in the ratio of immature erythrocyte to total erythrocyte was found. Batch no. 2JB00013P.

[e] An in vivo alkaline comet assay was conducted using RccHanTM:WIST female rats. Dosage levels tested were 2000, 1000 and 500 mg/kg. Pyraziflumid was administered orally to the rats (six rats per group) once daily on two consecutive days at 21-hour intervals. Three hours after the second dosing liver samples were collected and the isolated single cells embedded in agarose on a slide and subjected to electrophoresis under alkaline condition. No statistically significant increase in the percentage tail DNA was observed following pyraziflumid treatment. Pyraziflumid showed no potential to induce DNA damage in the liver under the conditions of this study. Batch no. 2JB00013P.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a two-generation reproductive toxicity study, groups of 24 male and 24 female Sprague Dawley (Crl:CD(SD)) rats were exposed to pyraziflumid (batch no. 2JB0013P; purity 94.8%) in the diet at 0, 50, 100, 300 or 1000 ppm, equal to:

- 0.0, 2.8, 5.6, 17 and 57 mg/kg bw per day for F0 males,
- 0.0, 3.6, 7.1, 21 and 72 mg/kg bw per day for F1 males,
- 0.0, 2.6, 5.3, 16 and 57 mg/kg bw per day for F0, gestating females,
- 0.0, 3.2, 6.3, 19 and 66 mg/kg bw per day for F1, gestating females,

F0 adults were treated over a 10-week premating period and throughout the three-week mating period and gestation. Female F0 adults continued to be treated throughout the 21-day lactation period and were killed and necropsied after the F1 pups weaned. On postnatal day 4 (PND 4), litters were culled to eight pups. Once weaned, one male pup and one female pup per litter were selected to become F1 parents for the F2 offspring. The remaining F1 weanlings were euthanized and subjected to gross external examination and necropsy. Treatment of the selected F1 rats continued for at least 10 weeks prior to mating and continued during the three-week mating and gestation. These F1 males were killed and necropsied one week after the birth of the F2 pups. The F1 females were killed and necropsied after the F2 pups were weaned. The rats were checked daily for mortality and clinical signs. Detailed observations were made weekly on all parental rats and on maternal F0 and F1 animals on gestation days (GDs) 0, 7, 14 and 20, and on PNDs 0, 7, 14 and 21. Body weights and feed consumption of parental rats were recorded weekly. In addition, body weights and feed consumption of females were recorded on GDs 0, 7, 14 and 20, and on PNDs 1, 7, 14 and 21. Individual pup weights were recorded on PNDs 0, 4, 7, 14 and 21. Estrous cycle length, fertility parameters (including spermatological examinations), and lengths of gestation were determined. All the litters were examined for number of pups, sex of pups, number of stillbirths, number of live births and any gross anomalies. All pups were checked daily for mortality and clinical signs. Each pup received a detailed physical examination on PNDs 1, 4, 7, 14 and 21. The age of preputial separation or vaginal opening was determined for all F1 weanlings selected for further treatment. The culled pups and pups euthanized at weaning were macroscopically examined. After weaning of the F1 and F2 pups, the respective F0 and F1 parents were euthanized and necropsied. The weights were recorded of ovaries, uterus (including cervix and oviduct), testes, epididymides, seminal vesicles with coagulating glands and their fluids, prostate (ventral lobe), brain (with medulla oblongata but without olfactory bulb), liver, kidneys, spleen, pituitary gland, thyroid glands with parathyroid glands, and adrenal glands from all parental F0 and F1 animals. With F1 and F2 weanlings, the weights were recorded of the brain (with medulla oblongata but without olfactory bulb), thymus, liver, spleen and uterus (including cervix and oviduct) for one pup of each sex per litter. A range of organs and tissues of the parental animals was examined histopathologically. Amongst F1 and F2 weanlings selected for organ weight measurement the following were examined histopathologically: thyroid glands; liver (F1 and F2 male and female weanlings); uterus, horns and cervix (F1 female weanlings); thymus (F1 and F2 male weanlings and F1 female weanlings) and spleen (F2 female weanlings). In addition, any organs or tissues showing grossly visible alterations were also examined histopathologically.

There were no treatment-related effects on mortality or clinical observations. In the F0 parental males, body weights and body weight gains in the treatment groups were comparable throughout the study period to those in the control group. For the F1 parental males, body weights were significantly

reduced at 1000 ppm compared to the control group during weeks 0 to 4 of the premating period (see Table 26). Body weight gains were also significantly decreased at 1000 ppm during weeks 0 to 3 of the premating period compared to the control group. The reduced body weight of the F1 parental males at 100 ppm was not considered to be test substance-related because it was not apparently dose-dependent. A decrease in body weight gain in week 1 of lactation only in parental females at 1000 ppm was not considered to be test substance-related as it was not observed in other weeks and it was not observed in F1 parental females at 1000 ppm.

In the F0 parental females at 1000 ppm, statistically significant reductions in feed consumption were seen in week 4 of the premating period (9%) and week 3 of the lactation period (14%), which may have been treatment-related. Among the F1 parental females, feed consumptions of the treatment groups were not significantly reduced.

Table 26. Premating body weight in F_1 males.

Dose level (ppm)	0	50	100	300	1000
			Mean body weight (g)		
Week 1	118 ± 13	111 ± 11	111 ± 9	111 ± 10	106 ± 10**
Week 2	180 ± 16	171 ± 17	170 ± 13	171 ± 14	162 ± 15**
Week 3	244 ± 20	234 ± 20	234 ± 15	232 ± 18	225 ± 19**
Week 4	310 ± 23	303 ± 24	302 ± 19	297 ± 22	292 ± 25**
			Mean body weight gain (g)		
Week 1	47 ± 4.7	45 ± 5.6	45 ± 3.6	44 ± 4.9	41 ± 4.2**
Week 2	109 ± 9.5	104 ± 12	105 ± 7.1	103 ± 11	97 ± 9.8**
Week 3	173 ± 14	168 ± 16	169 ± 9.7	165 ± 16	160 ± 15**

** $p < 0.01$ Source: Inagaki, 2014

There were no effects due to treatment in either generation on the number of days until copulation, mating, fertility, gestation indices, gestation duration, number of implantation sites, estrous cycling, sperm parameters or post-implantation losses. The numbers of primordial ovarian follicles in the F1 parental females at 1000 ppm were comparable to controls.

Necropsy of the F0 and F1 parental females at 1000 ppm showed dark discolouration in the liver. Absolute and relative liver and thyroid weights were significantly increased in the F0 and F1 parental males at 1000 ppm (Table 27). In addition, relative thyroid weight was increased in F0 males at 300 ppm. In the 1000 ppm group the incidence of follicular cell hypertrophy in the thyroid glands was significantly increased in the F0 parental males, and the incidence of single cell necrosis in the liver was significantly increased in the F0 parental females. In F0 and F1 males of the 300 ppm group and in both sexes of the 1000 ppm group, centrilobular fatty degeneration of the liver was observed. Incidences of centrilobular hepatocyte hypertrophy in the liver were significantly increased in both sexes of both generations.

Histopathology showed follicular cell hypertrophy in the thyroid glands of F0 parental males at 1000 ppm, centrilobular hepatocyte hypertrophy of the liver in both sexes in both generations at 300 and 1000 ppm, centrilobular fatty degeneration of the liver in the F0 and F1 parental males at 300 ppm and in both sexes at 1000 ppm, and single cell necrosis of the liver in the F0 parental females at 1000 ppm. No other treatment-related histopathological changes were observed. Necropsy and histopathology revealed no treatment-related abnormalities in the reproductive organs.

In the offspring of both generations, there were no treatment-related effects on the number of pups delivered, live births and stillbirths, sex ratio, incidence of gross external abnormalities and clinical findings, viability indices, or gross pathological abnormalities. In the offspring, at 1000 ppm, the trend in body weights was to decrease or they were significantly decreased (up to 10%) on lactation days 14 and 21 in both sexes of both generations.

For weanlings of the F1 generation relative liver weights were significantly increased at 1000 ppm in males (20%) and in females (20%). For weanlings of the F2 generation relative liver weights were significantly increased in males at 300 ppm (13%) and at 1000 ppm (20%), and in females

at 1000 ppm (19%). Statistically significant increases in relative liver weight were also seen in male F1 weanlings at 300 ppm, in male F2 weanlings at 100 ppm and in female F2 weanlings at 300 ppm, however these increase were small (< 10%).

Relative thymus weight was increased in male and/or female weanlings from both generations at 1000 ppm. Because there were no absolute weight changes or associated changes observed in histopathological examination of the thymus, the relationship to treatment was considered equivocal. An increase in relative uterus weight in the female F1 weanlings at 1000 ppm was considered incidental because no similar findings were noted in F2 weanlings and there were no histopathological changes.

Histopathological examination revealed increased incidences of centrilobular hepatocyte hypertrophy in the livers of weanlings of both sexes from the F1 generation at 300 and 1000 ppm, and in both sexes of the F2 weanlings at 1000 ppm; these were the only histopathological changes observed.

The incidence of hypertrophy at 300 ppm correlated poorly with the relative change in liver weight in male and female F1 and F2 offspring. Although clinical chemistry was not performed in this study, repeat-dose studies for up to 52 weeks did not show any changes in plasma liver enzymes at this dose. In the absence of any other histopathological changes, the hypertrophy observed in the livers of offspring at 300 ppm was not considered adverse.

Table 27. Effects of pyraziflumid on organ weights in parental males

Dose (ppm)	0	50	100	300	1000
			F0 Generation		
Absolute liver weight (g)	16.9 ± 1.9	17.1 ± 2.7	17.7 ± 3.1	18.0 ± 2.4	20.9 ± 3.9***
Relative liver weight (%)	2.88 ± 0.22	2.86 ± 0.27	2.87 ± 0.31	3.04 ± 0.39	3.34 ± 0.39***
Absolute thyroid weight (g)	0.0307 ± 0.0046	0.0315 ± 0.0062	0.0339 ± 0.0075	0.0349 ± 0.0064	0.0369 ± 0.0078**
Relative thyroid weight (%)	0.0052 ± 0.0008	0.0053 ± 0.0009	0.0056 ± 0.0011	0.0060 ± 0.0012*	0.0060 ± 0.0011*
			F1 Generation		
Absolute liver weight (g)	19.0 ± 2.4	19.8 ± 3.3	20.0 ± 3.3	20.1 ± 3.3	23.1 ± 3.8**
Relative liver weight (%)	3.04 ± 0.27	3.13 ± 0.31	3.19 ± 0.31	3.25 ± 0.35	3.72 ± 0.41**
Absolute thyroid weight (g)	0.0334 ± 0.0074	0.0361 ± 0.0071	0.0348 ± 0.0058	0.0353 ± 0.0050	0.0432 ± 0.0132***
Relative thyroid weight (%)	0.0053 ± 0.0010	0.0058 ± 0.0012	0.0056 ± 0.0010	0.0058 ± 0.0009	0.0069 ± 0.0017***

** $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ Source: Inagaki, 2014

The NOAEL for parental toxicity was 100 ppm (equal to 5.3 mg/kg bw per day) based on adverse effects on liver and thyroid as indicated by changes in organ weights and histopathology at 300 ppm (equal to 16 mg/kg bw per day).

The NOAEL for offspring toxicity was 300 ppm (equal to 16 mg/kg bw per day) based on reduced body weight gain at 1000 ppm (equal to 57 mg/kg bw per day).

The NOAEL for reproductive toxicity was 1000 ppm (equal to 57 mg/kg bw per day) the highest dose tested (Inagaki, 2014).

(b) Developmental toxicity

Rat

In a developmental toxicity study, pyraziflumid (batch no. 2JB0013P; purity 94.8%; formulated in a 0.5% aqueous sodium CMC suspension) was administrated by gavage to 24 pregnant Sprague Dawley (Crl:CD(SD)) rats from GD 6 to GD 19, at doses of 0, 20, 100 or 500 mg/kg bw per day. The dams were checked daily for mortality and clinical signs. Body weights and feed consumption were recorded on

GDs 0, 6, 9, 12, 15, 18 and 20. On the days of body weight measurements a detailed physical examination was conducted. The fetuses were delivered by caesarean section on GD 20. The uteri and ovaries were examined, placentae weighed and the number of fetuses, early and late resorptions, total implantations and corpora lutea were recorded. Gravid uterine weights were recorded, and net body weights from which net body weight changes were calculated. The fetuses were weighed, sexed and examined for external, visceral and skeletal malformations and developmental variations.

No mortality was observed. An increased incidence of fur loss was observed in the 500 mg/kg bw per day group. Statistically, incidences of fur loss at 20 and 100 mg/kg bw per day were not significantly different from controls. In the high-dose animals, a reduction in body weight gain was observed at 100 and 500 mg/kg bw per day throughout the treatment period (Table 28). Statistically significant reductions in body weight gain observed at 20 mg/kg bw per day were small and considered not toxicologically relevant. Final body weights were 426, 416, 407 and 401 g at 0, 120, 100 and 500 mg/kg bw per day respectively, reaching statistical significance at the mid and high dose. Feed consumption was reduced throughout the treatment period at 100 and 500 mg/kg bw per day (up to 12% and 22%respectively).

Table 28. Rat body weight gains (g) during treatment with pyraziflumid

	Body weight gain during periods of gestation				
Dose (mg/kg bw per day)	**GD 6–9**	**GD 6–12**	**GD 6–15**	**GD 6–18**	**GD 6–20**
0	14 ± 4	27 ± 7	46 ± 6	86 ± 9	121 ± 13
20	12 ± 3	25 ± 6	40 ± 6*	78 ± 8*	111 ± 10*
100	7 ± 6**	20 ± 6**	35 ± 8**	72 ± 16**	103 ± 21**
500	5 ± 6**	17 ± 7**	30 ± 8**	68 ± 8**	97 ± 14**

Values represent mean ± standard deviation; Source: Takahashi, 2014a

* $p \leq 0.05$; ** $p \leq 0.01$, Dunnett's test

Statistically. gravid uterine weight was significantly reduced at the high dose (80 g compared to 87 g in controls). Macroscopic examination revealed no effects due to treatment. Examination of the ovary and uterus (weight and gross necropsy) revealed no treatment-related specific effects in any of the treatment groups.

No treatment-related effects on numbers of live fetuses, percentage incidence of resorptions and fetal deaths, sex ratios, fetal body weights or placental weights were found. External, visceral and skeletal examinations also revealed no treatment-related effects.

The NOAEL for maternal toxicity was 20 mg/kg bw per day, based on reduced body weight gain and feed intake at 100 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity was 500 mg/kg bw per day, the highest dose tested (Takahashi, 2014a).

Rabbit

In a developmental toxicity study, pyraziflumid (batch no. 2JB0013P; purity 94.8%; formulated in a 0.5% aqueous sodium CMC suspension) was administered daily by gavage to groups of 25 female Japanese White (Kbl:JW) rabbits from GD 6 to GD 27 at a dose of 0, 10, 30 or 100 mg/kg bw per day. The doses were based on a preliminary study in eight females per group using dose levels of 0, 30, 100, 300, and 1000 mg/kg bw per day. The preliminary study was not submitted to JMPR, but it was reported that the majority of rabbits at 300 mg/kg bw per day, and all rabbits at 1000 mg/kg bw per day, showed abrupt and severe decreases in body weight gain and feed consumption immediately after initiation of test substance administration. Animals were euthanized when their group mean feed consumption amounted to less than 20% of the control value. Several females in the 300 mg/kg group showed abortion or death before the euthanasia.

In the main study the does were checked daily for mortality and clinical signs. Body weights and feed consumption were recorded on GDs 0, 6, 9, 12, 15, 18, 21, 24, 27 and 28. On days when body weight measurements were taken a detailed physical examination was conducted. The fetuses were delivered by caesarean section on GD 28. The uteri and ovaries were examined, placentas were weighed and the

numbers of fetuses, early and late resorptions, total implantations and corpora lutea were recorded. Gravid uterine weights were recorded, and net body weights and net body weight changes calculated. Fetuses were weighed, sexed and examined for external, visceral and skeletal malformations and any developmental variations.

No mortality was observed. Abortion was observed in one female in the control group (on GD 25) and in two females at 100 mg/kg bw per day (on GDs 21 and 24). The incidence of abortion at the high dose was above the range of the historical control data (20 studies; number of animals aborting in the control group ranged from 0–1), but statistically was not significantly different from the incidence in the concurrent controls. At 100 mg/kg bw per day net body weight gain was lower than in control rabbits from GD 12 resulting in a net weight gain of 187 g at the high dose compared with a weight gain of 225 g for the controls. Although statistically the differences in net body weight gains were not significantly different at the high dose, the values from GD 16 onward in this group were lower than the lowest values in the historical control data. Feed consumption in the high-dose group was also consistently lower than that of controls, and again, although no statistically significant difference was found, the values were lower than the lowest values in the historical control data. Macroscopic examination revealed no treatment-related effects. There were no treatment-related effects on gravid uterine weights, numbers of corpora lutea or implantations.

No treatment-related effects on numbers of live fetuses, percentage incidences of resorptions and fetal deaths, sex ratios, fetal body weights or placental weights were found. Nor did external, visceral and skeletal examinations reveal any treatment-related effects.

The NOAEL for maternal toxicity was 30 mg/kg bw per day, based on reduced body weight gain, feed consumption and a possible slight increase in the incidence of abortion at 100 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity was 100 mg/kg bw per day, the highest dose tested (Takahashi, 2014b).

2.6 Special studies

(a) Immunotoxicity

No data were submitted relating to immunotoxicity.

(b) Neurotoxicity

Acute neurotoxicity

In a limited acute neurotoxicity study, groups of Sprague Dawley (Crl:CD(SD)) rats (three of each sex per group) were administered a single oral gavage dose of pyraziflumid (batch no. 2JB0013P; purity 94.8%; dissolved in an aqueous solution containing 0.5% CMC and 0.1% Tween 80) at 0, 200, 600 or 2000 mg/kg bw. General activity and behaviour were observed before administration and 1, 2, 4, 6, and 24 hours after dosing in accordance with Irwin's multidimensional observation method.

No treatment-related effects were observed on general activity or behaviour (Yokotani, 2014a).

In a range-finding acute neurotoxicity study, groups of Sprague Dawley (Crl:CD(SD)) rats (three of each sex per group) were administered a single oral gavage dose of pyraziflumid (batch no. 2JB0013P; purity 94.8%; dissolved in an aqueous solution containing 0.5% CMC and 0.1% Tween 80) at 0, 500, 1000 or 2000 mg/kg bw. The animals were checked for clinical signs and body weights were recorded prior to dosing on day 0 and prior to euthanasia on day 1. Detailed clinical observations were performed at approximately 1, 2, 4, and 8 hours after dosing.

No mortality or clinical signs were observed. Whereas control animals gained weight over the day after dosing, all treatment groups showed slight body weight loss. Detailed clinical examination showed an alteration in gait (hunched posture) and low arousal in one high-dose female eight hours after dosing. No other treatment-related effects were observed (McElroy, 2017a).

In a second acute neurotoxicity study, groups of Sprague Dawley (Crl:CD(SD)) rats (10 of each sex per group) were administered a single oral gavage dose of pyraziflumid (batch no. 2JB0013P; purity 94.8%; dissolved in an aqueous solution containing 0.5% CMC and 0.1% Tween 80) at

concentrations of 0, 500, 1000 or 2000 mg/kg bw. The animals were observed daily for mortality and clinical signs. Body weights were recorded weekly. Functional observational battery (FOB) and locomotor activity data were recorded for all animals prior to treatment, at the time of peak effect on study day 0 (approximately eight hours after dose administration, estimated on the basis of the ADME data), and on days 7 and 14. At termination on day 15, brain weights and brain dimensions (excluding olfactory bulbs) were recorded. In addition, a neuropathological evaluation of selected tissues from the central and peripheral nervous systems was performed on five animals of each sex in the control and 2000 mg/kg bw groups.

No deaths occurred. No treatment-related clinical signs were noted. The weekly measurements revealed no treatment-related effects on body weight gain. No treatment-related effects were seen during FOB evaluations at the time of peak effect (approximately eight hours post dosing) or on days 7 or 14. Multivariate analysis of variance on the locomotor activity data from eight hours post dosing (with pretest values included as covariate) showed that pyraziflumid caused statistically significant decreases ($p < 0.01$) in total activity counts in both sexes in the 500, 1000, and 2000 mg/kg groups at eight hours post dosing (Table 29). The activity counts recognised a combination of fine motor skills, (for example, grooming or interruption of one photobeam) and ambulatory motor activity counts (interruption of two or more consecutive photobeams) in an overall testing session lasting 0–60 minutes. The effects were more pronounced in females than males. The motor activity test showed no effect of treatment at days 7 or 14. Habituation during the 60 minutes test was not affected by treatment.

There were no treatment-related macroscopic or microscopic findings in the brain or effects on brain weights or brain dimensions.

Table 29. Effect of pyraziflumid treatment on total activity and ambulatory motor activity of rats

Dose (mg/kg bw)		0	500	1000	2000
			Males		
Total activity counts	Pretest	1533	1908	2245	2007
	Day 0 at 8 hours	1472	1472	1830	1331
	Day 7	2085	2250	2594	2160
	Day 14	1938	2570	2262	2253
Ambulatory motor activity counts	Pretest	409	495	572	507
	Day 0 at 8 hours	318	298	346	279
	Day 7	449	540	600	488
	Day 14	427	575	480	468
			Females		
Total activity counts	Pretest	1934	1797	1935	1925
	Day 0 at 8 hours	2208	1454	1400	992
	Day 7	2363	2580	2594	2579
	Day 14	2619	2569	2676	2266
Ambulatory motor activity counts	Pretest	594	514	560	576
	Day 0 at 8 hours	650	379	333	250
	Day 7	647	750	737	742
	Day 14	710	707	752	613

Source: McElroy, 2017b

The NOAEL for neurotoxicity was 2000 mg/kg bw. The LOAEL for general toxicity is 500 mg/kg bw, the lowest dose tested, based on a transient decrease in total and ambulatory motor activity observed eight hours after treatment in females only at this dose in the absence of any neuropathological changes (McElroy, 2017b).

(c) Mode of action

In vivo

Study 1

In a seven-day study, the effects of dietary administration of pyraziflumid on serum thyroid hormone levels and hepatic enzyme activity were evaluated in groups of 12 male Wistar Hannover (RccHan:WIST) rats. The rats received pyraziflumid (batch no. 2JB0013P; purity 94.8%) for seven days in the diet at 0, 50, 300, 1000 or 2000 ppm (equal to 0, 4.0, 24, 78 and 158 mg/kg bw per day). The rats were checked daily for mortality and clinical signs. Body weight and feed consumption were measured on day 3 and day 7. Prior to the start of treatment and on days 3 and 7 blood was collected for the analysis of serum thyroid stimulating hormone (TSH), total thyroxine (T4), total triiodothyronine (T3), free T4 and free T3. On day 7 animals were necropsied, their livers and the thyroids weighed and examined histopathologically. Liver microsomal samples were prepared and total hepatic microsomal P450 content and T4 uridine diphosphate glucuronosyltransferase (T4 UDP-GT) activity were determined.

No treatment-related mortality or clinical signs were noted. No effects on body weight or feed consumption were observed. Necropsy revealed no effect of treatment. Dose-dependent increases in absolute and relative weights of the liver were found at 1000 ppm (18% and 15%, respectively) and 2000 ppm (18% and 21%, respectively). Dose-dependent increases in absolute and relative weights of the thyroid were found at 1000 ppm (11% and 13%, respectively) and 2000 ppm (12% and 15%, respectively), although these increases did not reach statistical significance. Histopathological examination of the liver showed centrilobular and/or diffuse hepatocellular hypertrophy at 300 ppm and above. In the thyroids, follicular cell hypertrophy was observed at 300 ppm and above.

Serum TSH levels were increased on days 3 and 7 at 1000 ppm (44% and 44%, respectively) and 2000 ppm (74% and 153%, respectively). No significant changes in total and free T4 and T3 levels were found. Total hepatic microsomal P450 content was dose-dependently increased at 300 ppm (66%), 1000 ppm (115%) and 2000 ppm (115%). Activity of T4 UDP-GT was dose-dependently increased at 300 ppm (88%), 1000 ppm (141%) and 2000 ppm (233%) (Nagai, 2019b).

Study 2

A study was performed to investigate the effects of pyraziflumid on hepatocellular proliferation and liver enzyme activity. Pyraziflumid (batch no. 2JB0013P; purity 94.8%) was administered in the diet to groups of eight female Wistar Hannover (RccHan:WIST) rats for one, two or four weeks at 0, 1000 or 2000 ppm. For the one-week study this was equal to 0, 70 and 142 mg/kg bw per day, for the two-week study 0, 77 and 144 mg/kg bw per day and for the four-week study 0, 72 and 140 mg/kg bw per day. The rats were checked daily for mortality and clinical signs. Body weight and feed consumption were measured weekly. Prior to termination BrdU at a dose of 15 mg/kg bw was injected subcutaneously, once daily, for seven consecutive days. At termination, all animals were necropsied. Livers were weighed and histopathological examination carried out. Proliferative activity of hepatocytes was assessed using a BrdU labelling index to compare treated with non-treated animals. Liver microsomal samples were prepared from animals treated for one week and these were analysed for levels of total P450 content and ethoxyresorufin-*O*-deethylase (EROD: CYP1A family) activity and pentoxyresorufin-*O*-dependentylase (PROD: CYP2B family) activity.

No treatment-related mortality or clinical signs were noted. A lower body weight (5–7%) was found in the groups receiving 2000 ppm compared to the controls. Feed consumption was not affected. A statistically significant dose- and time-related increase in liver weight was observed at 1000 ppm (9–17%; weeks 2 and 4) and at 2000 ppm (14–28%; weeks 1, 2 and 4). Necropsy showed a dark liver in one female dosed at 2000 ppm for four weeks. Centrilobular hepatocellular hypertrophy was observed in animals dosed at 1000 and 2000 ppm. The incidence and severity of this finding increased with dose and duration of treatment. No change in BrdU labeling was observed, indicating that hepatocellular proliferation was comparable among dose groups at all sampling points. A 2.3-fold increase in total liver microsomal P450 content was observed with both 1000 and 2000 ppm treatments for one week. EROD activity was increased at dose levels of 1000 ppm (18-fold) and 2000 ppm (35-fold). PROD activity was increased at 1000 ppm (256-fold) and 2000 ppm (184-fold) (Nagai, 2015c).

Study 3

In a 15-week dietary toxicity study, effects on thyroid hormone levels and liver enzyme activity were investigated in groups of ten male Wistar Hannover (RccHan:WIST) rats after administration of pyraziflumid (batch no. 2JB0013P; purity 94.8%). Animals received feed with dietary pyraziflumid concentrations of 0, 300, 1000 or 2000 ppm (equal to 0, 17, 55 and 113 mg/kg bw per day). The rats were checked daily for mortality and clinical signs. Body weight and feed consumption were measured weekly. Prior to treatment and at 2, 4, 8 and 13 weeks of treatment, blood samples were collected from the animals for the analysis of serum TSH, T4 and (T3). At termination animals were necropsied and the liver, thyroids and pituitary were weighed. These organs were examined histopathologically (the pituitary was examined histopathologically only in the high-dose group). Liver microsomal samples were prepared and total liver P450 content, T4 UDP-GT and T3 UDP-GT activities were determined.

No treatment-related mortality or clinical signs were noted. No effects on body weight or feed consumption were observed. At necropsy dark-coloured changes were observed in the liver, the thyroid and the kidneys at 2000 ppm. Dose-dependent increases in absolute and relative weights of the liver were found at 1000 ppm (30% and 26%, respectively) and at 2000 ppm (34% and 35%, respectively). Dose-dependent increases in absolute and relative weights of the thyroid were found at 300 ppm (12% and 15%, respectively), at 1000 ppm (39% and 35%, respectively) and at 2000 ppm (58% and 59%, respectively). Histopathological examination of the liver showed hepatocellular hypertrophy and centrilobular hepatocellular vacuolation in animals of all treatment groups. At 2000 ppm follicular cell hypertrophy and colloid alteration in the thyroid gland were observed.

Serum TSH level was increased at 1000 ppm (up to 19%) and 2000 ppm (up to 82%), in particular during the first four weeks of treatment. There was no effect on serum T4 or T3 levels at any time point. Total hepatic microsomal P450 content was dose-dependently increased at 300 ppm (67%), 1000 ppm (86%) and 2000 ppm (93%). Activity of T4 UDP-GT was dose-dependently increased at 300 ppm (58%), 1000 ppm (110%) and 2000 ppm (159%). Activity of T3 UDP-GT tended to be slightly lower than in the controls than in any of the treatment groups (up to 33%) (Nagai, 2015d).

In vitro

The effect of pyraziflumid (batch no. 2JB0013P; purity 94.8%) on thyroid peroxidase (TPO) activity was studied in an in vitro test system. Blood and thyroid tissue was collected from 15 male Wistar Hannover (RccHan:WIST) rats and microsomes prepared from pooled thyroid glands of five rats. The fluorescent peroxidase substrate Amplex UltraRed (ThermoFisher Scientific) was used to measure TPO activity in thyroidal microsomes obtained from untreated rats. Pyraziflumid or one of four positive control substances (methimazole, 6-propyl-2-thiouracil, 2,2′,4,4′-tetrahydroxybenzophenone and sulfamethazine) were added to the test system to determine concentration–response curves and to determine the half-maximal inhibitory concentration (IC_{50}).

Pyraziflumid had no effect on TPO-catalyzed Amplex UltraRed oxidation up to concentrations of 50 µM, pyraziflumid's maximum solubility. Values of IC_{50} for positive control substances were 0.025, 0.16, 0.17 and 20 µM for methimazole, 6-propyl-2-thiouracil, 2,2′,4,4′-tetrahydroxybenzophenone and sulfamethazine, respectively.

This in vitro study indicates that pyraziflumid does not directly inhibit TPO activity in rats (Nagai, 2019a).

(d) Effects on the cardiovascular system

The effects of pyraziflumid (batch no. 2JB0013P; purity 94.8%, administered by gavage in a solution containing 0.5% w/v sodium CMC and 0.1% v/v Tween 80 on blood pressure and heart rate were investigated in groups of six female Sprague Dawley (Crl:CD(SD)) rats at doses of 0, 200, 600 or 2000 mg/kg bw. Blood pressure and heart rate were measured using the tail-cuff method before treatment and 2, 4, 6, and 24 hours after administration.

No effects were noted on the blood pressure or heart rate of female rats at any dose (Yokotani, 2014b).

(e) Effects on the respiratory system

The effects of pyraziflumid (batch no. 2JB0013P; purity 94.8%, administered by gavage in a 0.5% (w/v) carboxymethylcellulose sodium/0.1 % Tween 80 (v/v) solution) on the respiration rate were investigated in groups of six female Sprague Dawley (Crl:CD(SD)) rats at doses of 0, 200, 600 or 2000 mg/kg bw. The respiration rate was measured before, and 2, 4, 6, and 24 hours after administration using a respiration-monitoring system.

No effects were noted on the respiration rate of female rats at any dose level (Yokotani, 2014c)

(f) Effects on small intestinal transport

The effects of pyraziflumid (batch no. 2JB0013P; purity 94.8%, administered by gavage in a solution containing 0.5% w/v sodium CMC and 0.1% v/v Tween 80 on small intestinal transport were investigated by the transport length of charcoal in groups of six female Sprague Dawley (Crl:CD(SD)) rats at doses of 0, 200, 600 or 2000 mg/kg bw. At 320 minutes after dosing, a suspension of 5% activated charcoal was administered by gavage. Forty minutes after charcoal administration rats were euthanised and charcoal transport measured.

No effects were noted on small intestinal transport in female rats at any dose level (Yokotani, 2014d)

(g) Studies with metabolites and impurities

No studies with metabolites or impurities were submitted.

3. Observations in humans

As this is a new compound no data on the effects of the compound in humans were available.

Comments

Biochemical aspects

Toxicokinetic studies were performed on pyraziflumid with a ^{14}C label in the pyrazinyl, aniline or difluorophenyl rings. Following the administration of a single oral dose of 1 or 100 mg/kg bw of [^{14}C]pyraziflumid to rats absorption was relatively rapid, with a peak concentration of radioactivity in plasma (depending on the position of the radiolabel) at the low dose after 3–12 hours, and the high dose after 9–24 hours. Excretion was relatively rapid, with more than 84–92% of the radioactivity excreted within 72 hours following a 1 mg/kg bw dose, indicating that pyraziflumid has a low potential for accumulation. At a dose of 1 mg/kg bw urinary and faecal excretion in males was 9–13% and 84–88%, respectively after 168 hours. In studies with pyrazinyl-labelled pyraziflumid up to 5% of radiolabel was excreted in expired air. Studies in bile duct-cannulated male rats indicated that the majority of an administered dose of [^{14}C]pyraziflumid at 1 mg/kg bw was excreted in the bile, 83–85%, and that at least 90% of the administered dose was absorbed. There were no major differences in excretion or metabolism due to dose or sex. Tissue distribution was widespread, but levels in tissues were low. Highest levels were found in the gastrointestinal tract, liver, fat, kidneys, thyroid and adrenals (Yoshizane, 2014a, b, 2015; Yasunaga, 2014).

Up to 10 metabolites of pyraziflumid were identified in metabolism studies. Pyraziflumid is metabolized mainly via hydroxylation of the pyrazinyl and aniline rings followed by conjugation with glucuronic acid. There is some limited cleavage of the molecular backbone of pyraziflumid, yielding pyraziflumid-oxamic acid, pyraziflumid-acid and pyraziflumid-amide. The major metabolites were 4-hydroxy-pyraziflumid and 3-hydroxy-pyraziflumid, which are excreted unchanged or as their glucuronide conjugates. No parent compound was found in urine or bile. No marked sex- or dose-related differences in metabolism were seen (Yoshizane, 2014a, b, 2015; Yasunaga, 2014). Studies in vitro with hepatic liver microsomes indicated that metabolism by humans was qualitatively similar to that in other mammalian species.

Toxicological data

The acute oral median lethal dose (LD_{50}) in rats was greater than 2000 mg/kg bw (Toga, 2014), the acute dermal LD_{50} was greater than 2000 mg/kg bw (Munechika, 2013a) and the acute inhalation median lethal concentration (LC_{50}) was greater than 2.1 mg/L (Sieber, 2013). Pyraziflumid was not irritating to the skin of rabbits (Munechika, 2013b) or to the eyes of rabbits (Munechika, 2013c). Pyraziflumid was not skin sensitizing in a local lymph node assay (LLNA) in mice (Munechika, 2013d).

In repeat-dose oral toxicity studies with pyraziflumid in mice, rats and dogs the main targets for toxicity were the liver and thyroid.

In a 28-day range-finding study in mice using dietary pyraziflumid concentrations of 0, 300, 1000, 3000 or 10 000 ppm (equal to 0, 49, 164, 520 and 1744 mg/kg bw per day for males, 0, 66, 195, 759 and 2475 mg/kg bw per day for females), the NOAEL was 3000 ppm (equal to 520 mg/kg bw per day), based on increased relative liver weight in males and changes in blood biochemistry in both sexes at 10 000 ppm (equal to 1744 mg/kg bw per day) (Nagai, 2015a).

In a 90-day study in mice using dietary pyraziflumid concentrations of 0, 800, 3000 or 8000 ppm (equal to 0, 119, 433 and 1183 mg/kg bw per day for males, 0, 146, 514 and 1456 mg/kg bw per day for females), the NOAEL was 800 ppm (equal to 119 mg/kg bw per day), based on reductions in cholesterol and increases in liver weight in males, and hepatocyte hypertrophy and increased periportal hepatocyte fat in both sexes at 3000 ppm (equal to 433 mg/kg bw per day) (Coleman 2013).

A 13-week study in rats was performed using dietary pyraziflumid concentrations in males of 0, 100, 500 or 5000 ppm (equal to 0, 7.1, 36 and 435 mg/kg bw/day), this highest dose being reduced to 2000 ppm (151 mg/kg bw per day) from week 9 due to excessive toxicity. Females received 0, 100, 500 or 2000 ppm (equal to 0, 8.6, 42 and 172 mg/kg bw per day) throughout the study period. The NOAEL was 100 ppm (equal to 7.1 mg/kg bw per day), based on changes in clinical signs, clinical chemistry and effects on liver and thyroid in both sexes, and effects on kidney in females at 500 ppm (equal to 36 mg/kg bw per day) (Stamp, 2010).

A 90-day study in dogs was performed using dietary pyraziflumid concentrations of 0, 200, 1000 and 10 000 ppm. In males the high dose was reduced to 5000 ppm from week 3 due to severe toxicity in these animals. The dietary doses were equal to 0, 6.0, 29 and 167 mg/kg bw per day for males and 0, 6.2, 31 and 320 mg/kg bw per day for females. The NOAEL was 1000 ppm (equal to 29 mg/kg bw per day), based on effects on body weight and feed consumption in males, and several signs of liver toxicity in both sexes at 10 000↓5000 ppm (equal to 167 mg/kg bw per day) (Kuwahara 2013).

In a one-year oral toxicity study in dogs, pyraziflumid was administered in the diet at dose levels of 0, 200, 1000 or 5000 ppm. Due to severe toxicity the treatment of the high-dose animals was discontinued from day 9 for males and day 4 for females. After a withdrawal and recovery period, treatment of the high dose group recommenced at 2000 ppm. The dietary doses were equal to 0, 5.4, 28 and 51 mg/kg bw per day for males, 0, 5.5, 28, and 48 mg/kg bw per day for females. The NOAEL was 1000 ppm (equal to 28 mg/kg bw per day) based on mortality, clinical signs, body weight loss and reduced feed consumption in males, and on clinical biochemistry and histopathological signs of liver and kidney toxicity in both sexes at 5000↓2000 ppm (equal to 48 mg/kg bw per day) (Kuwahara 2015).

The overall NOAEL for the 90-day and one-year dog studies was 1000 ppm (equal to 29 mg/kg bw per day), and the overall LOAEL was 5000↓2000ppm (equal to 48 mg/kg bw per day).

In an 18-month carcinogenicity study in mice using dietary pyraziflumid concentrations of 0, 200, 2000 or 8000 ppm (equal to 0, 21, 227 and 905 mg/kg bw per day for males, 0, 25, 251 and 1030 mg/kg bw per day for females) the NOAEL was 200 ppm (equal to 21 mg/kg bw per day) based on histopathological signs of liver toxicity observed in both sexes, accompanied by slight reductions in body weight gain in males, particularly at the first week of treatment at 2000 ppm (equal to 227 mg/kg bw per day). There were no treatment-related neoplastic lesions (Stamp, 2015a).

In a two-year combined toxicity and carcinogenicity study in rats using dietary pyraziflumid concentrations of 0, 50, 100, 300 and 1000 ppm (equal to 0.0, 2.2, 4.3, 13 and 46 mg/kg bw per day for males, 0.0, 2.9, 5.7, 18 and 66 mg/kg bw per day for females) the NOAEL was 50 ppm (equal to 2.2 mg/kg bw per day) based on macroscopic and histopathological signs of liver and thyroid toxicity

observed in both sexes, and indications of minor kidney toxicity in females at 100 ppm (equal to 4.3 mg/kg bw per day). Thyroid adenomas and carcinomas in both sexes and hepatocellular adenomas in females were observed. The NOAEL for neoplastic changes was 300 ppm (equal to 13 mg/kg bw per day) based on increased incidences of thyroid tumours in males and hepatocellular adenomas in females at 1000 ppm (equal to 46 mg/kg bw per day) (Stamp, 2015b).

A 1–4 week study was conducted to elucidate a possible mechanism for the increase in hepatocellular adenomas that had been observed (particularly in female rats) after dietary treatment with pyraziflumid at 1000 ppm in a two-year study. Pyraziflumid at 1000 ppm (equal to 70 mg/kg bw per day) caused hepatic enlargement associated with induction of the liver enzymes of the CYP sub-family CYP2B and to a lesser extent CYP1A. Activation of the nuclear receptors AhR, CAR, PXR, PPARα and PPARγ, was not investigated. The data are consistent with the induction of liver adenomas by pyraziflumid through activation of CAR, although it was noted that no hepatocyte proliferation was found after pyraziflumid treatment at doses of 1000 and 2000 ppm for 1–4 weeks (Nagai, 2015c).

Several mechanistic studies were performed to investigate the MOA responsible for the increased incidence of thyroid tumours observed at doses of 300 ppm and higher in rats. In repeat-dose studies, pyraziflumid, at dietary doses of 300 ppm (equal to 17 mg/kg bw per day) or higher, caused hepatocellular and thyroid follicular cell hypertrophy and an increase in total microsomal P450 levels in liver (Nagai, 2015c, d). Induction of CYP2B activity, and to a lesser extent CYP1A activity, was observed at doses of 1000 ppm (lowest dose tested) and higher (Nagai, 2015c). In a seven-day dietary study and a 15-week dietary study, the activity of T4-UDP-GT was also increased at doses of 300 ppm and higher, whereas T3-UDP-GT activity was unchanged (Nagai, 2015b, d). The Meeting noted that thyroid-stimulating hormone (TSH) plasma levels were increased at doses of 1000 ppm and higher but not at 300 ppm, and that plasma levels of thyroxine (T4) and triiodothyronine (T3) were not affected by treatment at doses up to 2000 ppm (Nagai, 2019b). An in vitro study with thyroid microsomes found no direct inhibition of thyroid peroxidase (TPO) by pyraziflumid (Nagai, 2019a). The data suggest that pyraziflumid may cause an enhanced hepatic clearance of thyroid hormones by induction of T4-UDP-GT and a compensatory stimulation of the thyroid by TSH. However, the lowest dose at which an increased incidence of thyroid tumours was observed did not affect plasma TSH levels, nor the levels of T4 and T3. Thus, the mechanistic studies were not sufficient to elucidate unequivocally the MOA of thyroid activation in rats.

The Meeting concluded that pyraziflumid is carcinogenic in rats, but not in mice.

Pyraziflumid was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of induction of gene mutation or structural chromosomal aberrations was found (Oguma, 2012; Tsukushi, 2014a, b; Munechika, 2017; Kasamoto, 2015). Pyraziflumid induced polyploidy in vitro in Chinese hamster lungs cells (Tsukushi, 2014a), a cell line that is known to be susceptible to induction of polyploidy by various factors acting via non-specific mechanisms.

Although it cannot be unequivocally concluded that the parent compound does not have the ability to induce polyploidy in vivo as no specific test was conducted, the negative finding obtained in the in vivo micronucleus test in mice (Tsukushi, 2014b) can be considered reliable indirect evidence of the absence of polyploidy induction.

In addition, the induction of polyploidy is a threshold phenomenon since it is not related to direct interaction with the DNA. Taking all these considerations into account, the observation of polyploidy in the in vitro test was considered to be of low concern.

The Meeting concluded that pyraziflumid is unlikely to be genotoxic.

In view of the lack of genotoxicity, the absence of carcinogenicity in mice, and the fact that increases in liver and thyroid tumours in rats were observed only at a high dose by non-genotoxic mode of action, the Meeting concluded that pyraziflumid is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation reproductive toxicity study in rats administered pyraziflumid in the diet at 0, 50, 100, 300 or 1000 ppm (equal to 0, 2.8, 5.6, 17 and 57 mg/kg bw per day for F0 males, 0, 3.6, 7.1, 21 and 72 mg/kg bw per day for F1 males, 0, 3.5, 7.0, 21 and 70 mg/kg bw per day for F0 premating females) the NOAEL for parental toxicity was 100 ppm (equal to 5.3 mg/kg bw per day) based on adverse effects on liver and thyroid, as indicated by changes in organ weights and histopathology at 300 ppm (equal to 16 mg/kg bw per day).

The NOAEL for offspring toxicity was 300 ppm (equal to 16 mg/kg bw per day) based on reduced body weight gain at 1000 ppm (equal to 57 mg/kg bw per day). The NOAEL for reproductive toxicity was 1000 ppm (equal to 57 mg/kg bw per day) the highest dose tested (Inagaki, 2014).

In a developmental toxicity study in rats using gavage doses of pyraziflumid at 0, 20, 100 or 500 mg/kg bw per day, the NOAEL for maternal toxicity was 20 mg/kg bw per day based on reduced body weight gain and feed intake at 100 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 500 mg/kg bw per day, the highest dose tested (Takahashi, 2014a).

In a developmental toxicity study in rabbits administered pyraziflumid by gavage at a dose of 0, 10, 30 or 100 mg/kg bw per day from gestation day (GD) 6 to GD 27, the NOAEL for maternal toxicity was 30 mg/kg bw per day, based on reduced body weight gain, feed consumption and a slightly increased incidence of abortion at 100 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 100 mg/kg bw per day, the highest dose tested (Takahashi, 2014b).

The Meeting concluded that pyraziflumid is not teratogenic.

In an acute neurotoxicity study in which rats were administered pyraziflumid by gavage at a dose of 0, 500, 1000 or 2000 mg/kg bw and then observed for 14 days, the NOAEL for neurotoxicity was 2000 mg/kg bw. The LOAEL for general toxicity was 500 mg/kg bw, the lowest dose tested, based on a transient decrease in total and ambulatory motor activity observed eight hours after treatment at this dose, in the absence of any neuropathological changes (McElroy, 2017b).

Although there were no indications of neuropathological effects due to pyraziflumid, the Meeting concluded that pyraziflumid may cause transient, acute neurobehavioural effects at high doses.

No studies on immunotoxicity were available. In the available toxicity studies on pyraziflumid no indications of an immunotoxic potential were seen.

The Meeting concluded that pyraziflumid is unlikely to be immunotoxic.

Special studies in rats revealed no effect of pyraziflumid at doses up to 2000 mg/kg bw on the cardiovascular and respiratory systems or on small intestinal transport.

Toxicological data on metabolites and/or degradates

No studies were available on the toxicity of pyraziflumid metabolites. The major metabolites identified in livestock were pyraziflumid-4′-OH (BC-01) and its glucuronide conjugate, and pyraziflumid-amide (BC-10). In the rat at least 90% of pyraziflumid was absorbed and subsequently its metabolites were largely excreted in bile (83–85%). Pyraziflumid-4′-OH (BC-01) and its glucuronide are the major metabolites in bile (approximately 6% and 48% of AD, respectively). Of the administered radiolabel 6–13% was excreted in urine, of which a major part was pyraziflumid-4′-OH and its glucuronide (2–7% of the administered dose, or 29–58% of total excretion in urine). Thus it is concluded that the toxicity of pyraziflumid-4′-OH and its glucuronide is covered by that of pyraziflumid.

The metabolite pyraziflumid-amide (BC-10) only occurs at low levels in urine (up to 2% of excreted radioactivity). QSAR analysis (OECD QSAR Toolbox) indicated that pyraziflumid-amide may bind to DNA and have the ability to induce chromosomal aberrations. As no genotoxicity studies were available for this metabolite the threshold of toxological concern (TTC) for genotoxicity can be applied for the assessment of chronic toxicity, that is 0.0025 µg/kg bw per day.

Microbiological data

No data for the impact of pyraziflumid on the human gut microbiome was available.

Human data

As this is a new compound no data for the effects of the pyraziflumid in humans were available.

The Meeting concluded that the existing database on pyraziflumid 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 0–0.02 mg/kg bw for pyraziflumid on the basis of a NOAEL of 2.2 mg/kg bw per day in a two-year rat study, based on macroscopic and histopathological signs of liver and thyroid toxicity seen in both sexes, and indications of minor kidney toxicity in females, observed at 4.3 mg/kg bw per day. A safety factor of 100 was used. The upper bound of the ADI gives a margin of ×2300 relative to the LOAEL for the observed thyroid tumours and hepatocellular adenomas in rats.

The Meeting established an acute reference dose (ARfD) of 2 mg/kg bw for pyraziflumid on the basis of a LOAEL of 500 mg/kg bw, based on a reduction in locomotor activity in an acute neurotoxicity study in rats. A safety factor of 300 was used, which includes an additional factor of ×3 for the use of a LOAEL instead of a NOAEL.

Levels relevant to risk assessment of pyraziflumid

Species	Study	Effect	NOAEL	LOAEL
Mouse	18-month study of carcinogenicity[a]	Toxicity	200 ppm, equal to 21 mg/kg bw per day	2000 ppm, equal to 227 mg/kg bw per day
		Carcinogenicity	8000 ppm, equal to 905 mg/kg bw per day[c]	-
Rat	Acute neurotoxicity study[b]	Neurotoxicity	-	-
		Toxicity	-	500 mg/kg bw[e]
	Two-year study of toxicity and carcinogenicity[a]	Toxicity	50 ppm, equal to 2.2 mg/kg bw per day	100 ppm, equal to 4.3 mg/kg bw per day
		Carcinogenicity	300 ppm, equal to 13 mg/kg bw per day	1000 ppm, equal to 46 mg/kg bw per day
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	1000 ppm, equal to 57 mg/kg bw per day[c]	-
		Parental toxicity	100 ppm, equal to 5.3 mg/kg bw per day	300 ppm, equal to 16 mg/kg bw per day
		Offspring toxicity	300 ppm, equal to 16 mg/kg bw per day	1000 ppm, equal to 57 mg/kg bw per day
	Developmental toxicity study[b]	Maternal toxicity	20 mg/kg bw per day	100 mg/kg bw per day
		Embryo/fetal toxicity	500 mg/kg bw per day[c]	-
Rabbit	Developmental toxicity study[b]	Maternal toxicity	30 mg/kg bw per day	100 mg/kg bw per day
		Embryo/fetal toxicity	100 mg/kg bw per day[c]	-
Dog	13-week and one-year studies of toxicity[d]	Toxicity	1000 ppm, equal to 29 mg/kg bw per day	5000↓2000 ppm, equal to 48 mg/kg bw per day

[a] Dietary administration [b] Gavage administration [c] Highest dose tested
[d] Two or more studies combined [e] Lowest dose tested

Acceptable daily intake (ADI) applies to pyraziflumid, pyraziflumid-4'-OH (BC-01) and its glucuronide conjugate, expressed as pyraziflumid

0–0.02 mg/kg bw

Acute reference dose (ARfD) applies to pyraziflumid, pyraziflumid-4'-OH (BC-01) and its glucuronide conjugate, expressed as pyraziflumid

2.0 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 [compound]

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Relatively rapid (T_{max} 3–12 hours at 1 mg/kg bw, 9–24 hours at 100 mg/kg bw) and almost complete (> 90%) in rats
Dermal absorption	No data
Distribution	Widely distributed, highest concentrations found in gastrointestinal tract, liver, fat, kidneys, thyroid and adrenals
Potential for accumulation	Low
Rate and extent of excretion	Relatively rapid; 84–92% in 72 hours
Metabolism in animals	Extensively metabolized; major metabolites are 4-hydroxy-pyraziflumid, 3-hydroxy-pyraziflumid and their glucuronides.
Toxicologically significant compounds in animals and plants	Pyraziflumid
Acute toxicity	
Rat, LD_{50}, oral	> 2000 mg/kg bw
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	> 2.1 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Not irritating
Mouse, dermal sensitization	Not sensitizing (LLNA)
Guinea pig, dermal sensitization	No data
Short-term studies of toxicity	
Target/critical effect	Liver, thyroid
Lowest relevant oral NOAEL	7.1 mg/kg bw per day (rat)
Lowest relevant dermal NOAEL	1000 mg/kg bw per day, highest dose tested (rat)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Liver (rat and mouse), thyroid and kidney (rat)
Lowest relevant NOAEL	2.2 mg/kg bw per day (rat)
Carcinogenicity	Carcinogenic in rats (thyroid and liver tumours), but not in mice[a]
Genotoxicity	Unlikely to be genotoxic
Reproductive toxicity	
Target/critical effect	No reproductive effects, liver and thyroid effect (parental), pup body weigh gain
Lowest relevant parental NOAEL	5.3 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	16 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	57 mg/kg bw per day, highest dose tested (rat)
Developmental toxicity	
Target/critical effect	No developmental toxicity, body weight gain and feed consumption (rat, rabbit), slight effect on abortions (rabbit)
Lowest relevant maternal NOAEL	20 mg/kg bw per day (rat)
Lowest relevant embryo/fetal NOAEL	100 mg/kg bw per day, highest dose tested (rabbit)

Neurotoxicity	
Acute neurotoxicity NOAEL	2000 mg/kg bw (LOAEL general toxicity: 500 mg/kg bw, lowest dose tested) (rat)
Subchronic neurotoxicity NOAEL	No data
Developmental neurotoxicity NOAEL	No data
Immunotoxicity	No data
Studies on toxicologically relevant metabolites	No data
Microbiological data	No data is available for the impact on the human gut microbiome.
Human data	No data

[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)	100
ARfD	2.0 mg/kg bw[a]	Acute neurotoxicity study (rat)	300

[a] Applies to pyraziflumid, pyraziflumid-4′-OH (BC-01) and its glucuronide conjugate, expressed as pyraziflumid

References

All studies referenced below comply with GLP unless otherwise stated.

Coleman DG (2013). NNF-0721: Preliminary carcinogenicity study by dietary administration to CD-1 mice for 13 weeks. Study No. LMS0065, T-33019. Huntingdon Life Sciences, Cambridgeshire, UK, on behalf of Nihon Nohyaku Co. Ltd. (Unpublished).

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Spiropidion

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Explanation

Spiropidion is the common name approved by the International Organization for Standardization (ISO) for 3-(4-chloro-2,6-dimethylphenyl)-8-methoxy-1-methyl-2-oxo-1,8-diazaspiro[4.5]dec-3-en-4-yl ethyl carbonate (IUPAC), with the Chemical Abstracts Service (CAS) number 1229023-00-0. It is a proinsecticide incorporating a novel tetramic acid derivative. The pesticidal mode of action (MOA)is by inhibiting the enzyme acetyl-CoA carboxylase.

Spiropidion 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. The Meeting noted that in short term toxicity studies in rodents and in the 90-day dog study a number of control blood samples subject to bioanalysis were contaminated, either with the parent compound spiropidion (SYN546330) and/or the enol-metabolite SYN547305, and that the reason for the contamination was not clearly identified. However, the Meeting concluded that the contamination did not invalidate the results and interpretation of the study′s toxicological findings. No additional information from a literature search was identified that complemented the toxicological information submitted for the current assessment.

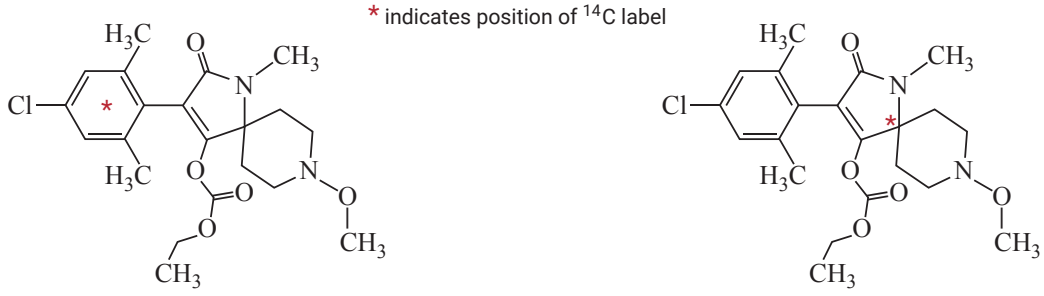
Evaluation for acceptable intake

1. Biochemical aspects

The absorption, distribution, metabolism and excretion (ADME) of spiropidion (code SYN546330) were investigated in rats following the administration of spiropidion labelled with ^{14}C at either the phenyl or the spirodecanone ring in a single oral low dose, a single oral high dose or by intravenous (i.v.) administration of radiolabelled spiropidion in a single low dose. The rates and routes of elimination were also assessed in bile duct-cannulated animals after administration of a single oral low dose. In addition, a comparative in vitro metabolism study was conducted using rat and human liver microsomes.

The structures of the two radiolabelled forms of spiripidion are shown in Fig. 1.

Figure 1. Position of radiolabels in spiropidion used in ADME studies



[*Phenyl-U*-^{14}C]spiropidion

[*Spirodecanone-5*-^{14}C]spiropidion

Redrawn from Tomlinson, Hutton & Strathdee, 2016

1.1 Absorption, distribution and excretion

In a preliminary pharmacokinetics, absorption, and excretion study, male and female Han Wistar rats were administered [*phenyl-U*-^{14}C]spiropidion (purity 96.8%) and [*spirodecanone-5*-^{14}C]spiropidion (SYN546330; purity 97.7%) in single oral doses of 5 mg/kg bw, 250 mg/kg bw (spirodecanone label only) or 1000 mg/kg bw (phenyl label only), or a single i.v. dose of 1 mg/kg bw body weight (bw). An initial phenyl-labelled dose of 1000 mg/kg bw was administered to male and female rats, however following clinical signs this was reduced to 250 mg/kg bw for the spirodecanone label. Excretion samples were obtained over a seven-day period, with expired air collected following oral doses over the first two days. In each group, blood samples were taken over predetermined time intervals up to three days post dose (oral administration) or two days post dose (i.v. administration). The vehicle for the oral doses was 0.5% (w/v) aqueous carboxymethyl cellulose (CMC). The intravenous vehicle was ethanol : PEG 400 : saline at 5 : 5 : 90 (v/v/v). Radioactivity in all samples was quantified by liquid scintillation counting (LSC), either by direct analysis or following sample oxidation.

With both male and female rats, 95% or more of the administered radioactivity was recovered at 168 hours post dose following single oral doses of either labelled form at 5 mg/kg bw, a single oral dose with the spirodecanone label at 250 mg/kg bw, or after the single i.v. dose at 1 mg/kg bw. The majority of the administered radioactivity (91–95%) was eliminated by 48 hours after dosing. There were no marked differences observed in urinary or faecal excretion between the sexes after either dose, which achieved completion. The major route of elimination was via the urine, with less abundant faecal elimination. Elimination via expired air was negligible (< 0.1%).

Table 1. Cumulative recovery of radioactivity in the excreta of rats following a single oral low dose, a single oral high dose and a single low intravenous dose of [phenyl-U-^{14}C]spiropidion and [spirodecanone-5-^{14}C]spiropidion at 168 hours

	Percentage of administered dose											
	Oral administration								Intravenous administration			
	5 mg/kg bw				1000 mg/kg bw		250 mg/kg bw		1 mg/kg bw			
Time after dosing (hours)	Phenyl radiolabel		Spirodecanone radiolabel		Phenyl radiolabel		Spirodecanone radiolabel		Phenyl radiolabel		Spirodecanone radiolabel	
	M (*n* = 1)	F (*n* = 1)	M (*n* = 1)	F (*n* = 1)	M (*n* = 1)	F (*n* = 1)	M (*n* = 1)	F (*n* = 1)	M (*n* = 1)	F (*n* = 1)	M (*n* = 1)	F (*n* = 1)
Urine												
0–8	52	48	48	34	6.2	7.3	17	27	53	42	53	52
8–24	18	18	11	22	14	8.9	38	27	15	22	13	16
24–48	1.0	2.5	1.0	3.1	12 [a]	4.2 [b]	5.7	5.3	1.2	2.8	0.5	1.3
48–72	0.2	0.6	0.1	0.5	NS	NS	<0.1	0.9	0.2	0.3	0.1	0.2
0–168	71	70	61	61	32 [a]	20 [b]	60	61	69	67	67	70
Faeces												
0–24	18	17	23	26	23	9.2	8.3	12	18	14	21	17
24–48	4.6	6.6	8.3	2.2	17 [a]	NS [b]	21	15	4.9	6.6	2.8	3.2
48–72	0.5	0.5	1.9	0.8	NS	NS	3.7	4.1	0.6	1.0	0.2	0.5
0–168	24	24	34	30	40 [a]	9.2 [b]	34	32	24	22	25	21
Cage wash												
Predose	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
0–24	0.9	1.3	2.3	7.3	4.3	2.4	3.3	3.9	2.3	6.1	3.5	4.2
24–48	0.3	0.5	0.2	0.4	7.9 [a]	12 [b]	0.5	1.1	0.4	0.4	0.1	0.3
48–72	0.1	<0.1	0.2	0.3	NS	NS	0.1	0.1	0.3	0.1	<0.1	0.2
0–168	1.3	2.0	2.7	8.3	12 [a]	15 [b]	3.9	5.8	3.1	6.8	3.7	4.8
Expired Air												
0–48	<0.1	<0.1	<0.1	<0.1	<0.1 [a]	<0.1 [b]	<0.1	<0.1	NE	NE	NE	NE
Total excreted	**96**	**96**	**97**	**99**	**84**	**44**	**98**	**98**	**96**	**96**	**95**	**96**
GI tract	< 0.1	< 0.1	< 0.1	< 0.1	5.6	50	< 0.1	< 0.1	<0.1	<0.1	<0.1	<0.1
Carcass	0.3	0.2	0.3	0.4	1.3	6.0	0.3	0.2	0.2	0.2	0.3	0.1
Total Recovery	**96**	**96**	**97**	**99**	**91**	**101**	**98**	**98**	**96**	**97**	**95**	**96**

M: male; F: female; Source: Adapted from Tomlinson, Hutton & Strathdee, 2016

[a] animal sacrificed at 48 h post dose due to clinical signs; [b] animal sacrificed at 29 h post dose due to clinical signs;

NE: Not evaluated; NS: No sample;

Analysis of pharmacokinetic parameters showed that following a single oral low dose of 5 mg/kg bw [*phenyl-U*-^{14}C] or [*spirodecanone-5*-^{14}C]spiropidion to male and female rats, the peak concentration (C_{max}) of radioactivity in blood and plasma was observed at time (T_{max}) two hours post dose. Thereafter, blood concentrations generally steadily decreased.

Following a single oral high dose of 1000 mg/kg bw of [*phenyl-U*-^{14}C]spiropidion or 250 mg/kg bw of [*spirodecanone-5*-^{14}C]spiropidion to male and female rats, the C_{max} of radioactivity in blood and plasma was observed at six hours post dose in males and two hours post dose in females. Thereafter, in animals given 1000 mg/kg bw of [*phenyl-U*-^{14}C]spiropidion, male whole-blood concentrations initially decreased (6–8 hours) then appeared to plateau at 48 hours post dose. Female whole-blood concentrations also appeared to plateau between four and eight hours post dose. In animals given 250 mg/kg bw [*spirodecanone-5*-^{14}C]spiropidion, concentrations generally decreased steadily until 72 hours and 48 hours in blood and plasma respectively. Estimated half-life ($t_{½}$) for [*spirodecanone-5*-^{14}C]spiropidion in male and female plasma was 10.2 hours and 8.60 hours respectively; in blood the $t_{½}$ was 37.9 hours in males and 17.4 hours in females. In animals given 1000 mg/kg bw [*phenyl-U*-^{14}C]spiropidion estimated $t_{½}$ values were 33.8 hours and 8.14 hours in male and female plasma, in blood 49.4 hours and 12.6 hours in males and females respectively.

Following a single intravenous dose of 1 mg/kg bw [^{14}C]SYN546330 to male and female rats, blood concentrations decreased steadily up to 48 hours, irrespective of the radiolabel position. The blood terminal $t_{½}$ was 38.1 hours in males and 14.0 hours in females for the phenyl-labelled spiropidion, and 18.2 hours in males and 8.77 hours in females, in the case of the spirodecanone-labelled spiropidion.

The absolute bioavailability (F_{abs}) of a 5 mg/kg bw oral dose was estimated to be greater than 76%. In addition, the percent of dose recovered in the urine after oral and i.v. dosing was very similar, suggesting absorption could be essentially complete (Tomlinson, Hutton & Strathdee, 2016).

Since no significant differences were in evidence for a difference in the absorption, excretion or pharmacokinetic behaviour between phenyl-labelled and spirodecanone-labelled spiropidion, all other ADME studies were conducted with spiropidion labelled at the phenyl position.

Table 2. Mean pharmacokinetic parameters in blood and plasma of rats following oral and intravenous administration of [phenyl-U-^{14}C]spiropidion and [spirodecanone-5-^{14}C]spiropidion at 168 hours

	Percentage of administered dose											
	Oral administration								**Intravenous administration**			
	5 mg/kg bw				**1000 mg/kg bw**		**250 mg/kg bw**		**1 mg/kg bw**			
	Phenyl radiolabel		**Spirodecanone radiolabel**		**Phenyl radiolabel**		**Spirodecanone radiolabel**		**Phenyl radiolabel**		**Spirodecanone radiolabel**	
	M (*n*=6)	**F (*n*=6)**	**M (*n*=6)**	**F (*n*=6)**	**M (*n*=6)**	**F (*n*=6)**	**M (*n*=6)**	**F (*n*=6)**	**M (*n*=6)**	**F (*n*=6)**	**M (*n*=6)**	**F (*n*=6)**
Blood												
C_{max} (µg equiv./g)	2.71	3.14	3.22	2.99	90.7	76.7	60.9	48.9	NA	NA	NA	NA
T_{max} (h)	2.0	2.0	1.0	2.22	6.0	2.0	6.0	2.2	NA	NA	NA	NA
$t_{½}$ (h)	31.5[#]	22.1[#]	11.2[#]	25.3[#]	49.4[#]	12.6[#]	37.9	17.4	38.1[#]	14.0[#]	18.2	8.77
$AUC_{(0-t)}$	15.1	13.9	15.5	14.2	3 510	1 080	703	698	2.19	3.01	3.82	3.52
F_{abs} (%)	121	86.3	76.4	80.5	110	40.4	71.1	87.1	NA	NA	NA	NA
Plasma												
C_{max} (µg equiv./g)	3.53	4.51	1.23	3.47	99.4	101	147	83.4	NS	NS	NS	NS
T_{max} (h)	2.0	2.0	2.0	2.2	24	2.0	0.5	1.0	NS	NS	NS	NS
$t_{½}$ (h)	17.4	16.8[#]	13.8[#]	22.7	33.8[#]	8.14	10.2	8.6	NS	NS	NS	NS
$AUC_{(0-t)}$	16.0	19.3	10.1	18.1	3 270	1 270	838	682	NS	NS	NS	NS

M: male; F: female; Source: Adapted from Tomlinson, Hutton & Strathdee, 2016

NA: Not applicable; NS: No sample; C_{max}: Maximum concentration; T_{max}: Time taken to reach C_{max};

$t_{½}$: Terminal elimination half-life;

$AUC_{(0-t)}$ (µg equiv. h/g): The area under the concentration–time curve from the start of dose administration to the time after dosing at which the last quantifiable concentration was observed

F_{abs}: Absolute bioavailability calculated as the ratio of the dose-normalised AUC (oral) to dose-normalised AUC (intravenous)

[#] Coefficient of determination was < 0.8

In an absorption, excretion, biliary elimination study, four Han Wistar rats/sex per group were administered [*phenyl-U*-^{14}C]spiropidion (purity 98.4%) in a single oral dose of 5 mg/kg bw or 250 mg/kg bw to intact and bile duct-cannulated rats. An additional group of four males were given a single 1 mg/kg bw dose of [*phenyl-U*-^{14}C]spiropidion intravenously. Excretion samples were obtained over a seven-day (oral) or four-day (intravenous) period for intact animals, or three-day periods for bile duct-cannulated animals. After this period the rats were humanely killed, and residual radioactivity was measured in selected tissues (oral, non-cannulated) and the remaining carcass. The vehicle for the oral doses was 0.5% (w/v) aqueous CMC. The intravenous vehicle was ethanol : PEG 400 : saline in the ratio 5 : 5 : 90 (v/v/v). Radioactivity in all samples was quantified by LSC, either by direct analysis or following sample oxidation.

Following a single oral administration of [^{14}C]spiropidion at 5 mg/kg bw to non-cannulated rats, a mean of 96–97% of the administered dose (AD) was eliminated in urine and faeces (including cage wash) over seven days. The majority of administered radioactivity (94%) was excreted in the first 48 hours. The routes and rates were similar for both males and females, with the majority of the dose excreted in the urine (57–61%). Faecal excretion accounted for 29–36% of AD.

Following a single oral administration of [^{14}C]spiropidion at 250 mg/kg bw to non-cannulated rats, a mean of 103–105% of the AD was eliminated in urine and faeces (including cage wash) over seven days. The majority of administered radioactivity (97–100%) was excreted in the first 48 hours. The routes and rates were similar for both males and females, with 49–53% of the dose excreted in the urine and 45–47% in the faeces.

Following a single oral administration of [^{14}C]spiropidion at 5 mg/kg bw to bile duct-cannulated rats, a mean of 96–97% of the AD was eliminated in urine, bile and faeces (including cage wash) over three days. The routes and rates were similar for both males and females. The majority of the dose was excreted in the urine (65–66%) in the first 48 hours. Elimination via the faeces accounted for 14–15% of the dose and biliary excretion accounted for 5.7–11% of AD.

Following a single oral administration of [^{14}C]spiropidion at 250 mg/kg bw to bile duct-cannulated rats, a mean of 97–100% of the AD was eliminated in urine, bile and faeces (including cage wash) over three days. The routes and rates were broadly similar for males and females. The majority of the dose was excreted in the urine (45–64%) in the first 48 hours. Elimination via the faeces accounted for 19–30% of the dose and biliary excretion accounted for 13–14% of AD.

Following a single intravenous administration of [^{14}C]spiropidion at 1 mg/kg bw to non-cannulated male rats, a mean of 103% of the AD was eliminated in urine and faeces (including cage wash) over four days. The majority of administered radioactivity (97%) was excreted in the first 24 hours. The majority of the dose was excreted in the urine (64%). Faecal excretion accounted for 30% of AD.

Irrespective of dose or sex, the routes and rates of elimination were broadly similar. Absorption was similar following both doses, indicating absorption was not saturated. At each dose level, the absorption was similar in males and females, with oral absorption ranging from 69–86%.

Table 3. Cumulative recovery of radioactivity in excreta of rats following a single oral low dose, a single oral high dose and a single low i.v. dose of [phenyl-U-^{14}C]spiropidion at 168 hours.

	Percentage of administered dose								
	Oral (non-cannulated)				**Oral (bile duct-cannulated)**				**Intravenous**
	5 mg/kg bw		**250 mg/kg bw**		**5 mg/kg bw**		**250 mg/kg bw**		**1 mg/kg bw**
	M (*n* = 4)	**F (*n* = 4)**	**M (*n* = 4)**	**F (*n* = 4)**	**M (*n* = 4)**	**F (*n* = 4)**	**M (*n* = 4)**	**F (*n* = 4)**	**M (*n* = 4)**
Urine									
0–8 h	43	41	20	23	50	47	20	34	43
8–24 h	12	17	26	23	13	17	22	22	19
24–48 h	1.0	2.1	1.9	6.5	1.3	1.9	3.0	7.8	1.2
48–72 h	0.2	0.5	0.3	0.5	0.3	0.4	0.2	0.8	0.1
0–168 h	57	61	49	53	65[a]	66[a]	45[a]	64[a]	64[b]

	Percentage of administered dose								
	Oral (non-cannulated)				Oral (bile duct-cannulated)				Intravenous
	5 mg/kg bw		250 mg/kg bw		5 mg/kg bw		250 mg/kg bw		1 mg/kg bw
	M (*n* = 4)	F (*n* = 4)	M (*n* = 4)	F (*n* = 4)	M (*n* = 4)	F (*n* = 4)	M (*n* = 4)	F (*n* = 4)	M (*n* = 4)
Faeces									
0–24 h	29	21	26	22	13	13	26	14	27
24–48 h	5.2	6.6	18	18	1.1	1.6	3.0	4.2	2.7
48–72 h	0.9	0.8	2.3	4.2	0.2	0.3	0.5	1.1	0.4
72–96 h	0.4	0.4	0.5	0.6	–	–	–	–	0.2
0–168 h	36	29	47	45	14[a]	15[a]	30[a]	19[a]	30[b]
Bile									
0–1 h	–	–	–	–	1.5	0.4	0.7	0.4	–
1–2 h	–	–	–	–	3.3	1.0	1.7	1.3	–
2–4 h	–	–	–	–	2.6	1.6	3.1	3.3	–
4–8 h	–	–	–	–	1.6	1.7	3.5	3.2	–
8–12 h	–	–	–	–	0.7	0.5	2.9	1.9	–
12–24 h	–	–	–	–	0.7	0.4	2.3	1.6	–
24–48 h	–	–	–	–	0.3	0.1	0.3	1.3	–
0–72 h	–	–	–	–	11	5.7	14	13	–
Cage wash	2.9	7.4	8.7	5.1	6.3	10	7.7	3.4	9.0
Total excreted	–	–	–	–	96	97	97	100	103
Absorption	–	–	–	–	86	84	69	81	–
Tissues	0.2	0.2	0.2	<0.1	–	–	–	–	–
GI tract	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	–
GI tract contents	<0.1	<0.1	<0.1	<0.1	<0.1	0.1	0.2	0.1	–
Carcass	0.1[c]	0.1[c]	0.2[c]	0.2[c]	0.7	0.6	0.2	0.3[c]	0.5
Total Recovery	**96**	**97**	**105**	**103**	**97**	**98**	**97**	**100**	**103**

[a] 0–72 hours; [b] 0–96 hours; M: male; F: female; Source: adapted from Punler, Tomlinson & Hutton, 2016a

[c] Mean includes results calculated from data less than 30 dpm above background; GI: Gastrointestinal

Seven days after administration of 5 or 250 mg/kg bw of [^{14}C]spiropidion, radioactive residues in the majority of tissues were not detectable. The highest mean tissue concentration was observed in the liver, with the tissue distribution of radioactivity being similar in both sexes following both doses. Higher concentrations in the liver and kidney are consistent with the urinary and biliary elimination of absorbed [^{14}C]spiropidion (Punler, Tomlinson & Hutton, 2016a).

In a study to investigate tissue distribution and depletion of radioactivity, 20 Han Wistar rats/sex per group were administered [^{14}C]spiropidion (purity 98.7%) in a single oral dose of 5 or 250 mg/kg bw. The vehicle was 0.5% (w/v) aqueous CMC. Following dosing, groups of four rats/sex per time point were humanely killed. The first time point chosen was based around the C_{max} observed in blood. For the 5 mg/kg bw dose groups the times chosen were 2 h, 8 h (females only), 12 h (males only), 24, 48 and 96 h. For the 250 mg/kg bw dose group, times chosen were 2 h (females only), 4 h (males only), 12, 24 h (males only), 36 h (females only), 48 and 96 h. Residual radioactivity was measured in selected tissues/organs and the remaining carcasses. Where appropriate, terminal phase half-lives of depletion were calculated for individual tissues. Radioactivity in all samples was quantified by LSC by direct analysis or following sample oxidation.

In male and female plasma/blood the t_{max} was observed at two hours for animals given 5 mg/kg bw and at four hours for animals given 250mg/kg bw. Radioactivity then declined steadily during the 96 hours after dosing. In animals given 5 mg/kg bw, circulating concentrations of total radioactivity were primarily associated with the plasma fraction at 12 hours for males and 24 hours for females, then at later time points up to 96 hours post dose they were associated with the cellular fraction. In animals given 250 mg/kg bw, circulating concentrations of total radioactivity were mainly associated with the plasma fraction at 12 hours post dose (males and females), after which they were associated with the cellular fraction, at later time points up to 96 hours post dose.

A high concentration of radioactivity was observed in the gastrointestinal (GI) tract and contents, consistent with biliary and/or faecal elimination of the oral dose. Radioactivity in the GI tract content accounted for 25% of the AD at two hours and declined to 0.7% at 96 hours in animals given 5 mg/kg bw. In animals given 250 mg/kg bw, radioactivity in the GI tract accounted for 65% of the AD at two hours and then declined to 0.4% at 96 hours.

Other tissues/organs with notable levels of radioactivity were liver and kidneys. Among these tissues/organs the highest radioactivity was reported in liver, where in animals given 5 mg/kg bw radioactivity it accounted for 25% of AD at two hours, declining to 0.4% at 96 hours. Radioactivity in all other tissues was equal to or lower than 0.1% of AD.

Table 4. Distribution of radioactivity in selected tissues/organs 2, 12, 24, 48 and 96 hours after oral administration of [^{14}C]SYN546330 to male and female rats at dose levels of 5 and 250 mg/kg bw

Tissue/time point	Percentage of administered dose			
	5 mg/kg bw		250 mg/kg bw	
	M (*n* = 4)	F (*n* = 4)	M (*n* = 4)	F (*n* = 4)
GI tract				
2 h	13	10	ND	9.9
4 h	-	-	6.6	-
8 h	-	6.8	-	-
12 h	4.1	ND	5.6	5.7
24 h	0.7	0.8	2.9	ND
36 h	-	-	-	1.3
48 h	0.1	0.3	0.2	0.4
96 h	0.2	0.2	0.1	<0.1
GI tract contents				
2 h	25	17	-	64
4 h	-	-	65	-
8 h	-	18	-	-
12 h	19	-	48	38
24 h	4.9	3.8	13	-
36 h	-	-	-	5.2
48 h	0.6	1.0	1.0	1.3
96 h	0.7	0.4	0.4	0.1
Kidneys				
2 h	1.4	0.9	-	0.3
4 h	-	-	0.2	-
8 h	-	0.4	-	-
12 h	0.1	-	0.2	0.1

Tissue/time point	Percentage of administered dose			
	5 mg/kg bw		250 mg/kg bw	
	M (n = 4)	F (n = 4)	M (n = 4)	F (n = 4)
24 h	<0.1	<0.1	0.1	-
36 h	-	-	-	<0.1
48 h	<0.1	<0.1	<0.1	<0.1
96 h	<0.1	<0.1[a]	<0.1	<0.1
Liver[a]				
2 h	25	30	–	2.5
4 h	–	–	2.9	–
8 h	–	17	–	–
12 h	6.0	–	2.1	1.7
24 h	2.3	3.3	1.2	–
36 h	–	–	–	0.9
48 h	0.8	1.9	0.4	0.4
96 h	0.4	0.7	0.2	0.1
Residual carcass				
2 h	15	9.4	–	7.5
4 h	–	–	6.0	–
8 h	–	6.1	–	–
12 h	6.1	–	6.2	5.5
24 h	3.2	3.0	2.8	–
36 h	–	–	–	2.9
48 h	2.3	2.1	1.3	1.6
96 h	1.2	1.3	1.0	1.0
Tissue and carcass				
2 h	81	68	–	84
4 h	–	–	81	–
8 h	–	48	–	–
12 h	36	–	62	52
24 h	11	11	21	–
36 h	–	–	–	10
48 h	3.8	5.3	2.8	3.8
96 h	2.5	2.5	1.6	1.3

[a] Mean includes results calculated from data less than 30dpm above background;

GI: Gastrointestinal; M: Males; F: Females; ND: Not determined;

Source: Adapted from Punler et al., 2016a

The terminal phase $t_{½}$ estimates for tissue depletion of total radioactivity in plasma ranged from 26.0 hours to 29.1 hours following the 5 mg/kg bw dose and 11.2–12.5 hours following the 250 mg/kg bw dose. In other tissues the majority of $t_{½}$ estimates were in a similar range, or estimates were shorter than in plasma. However, longer $t_{½}$ estimates were obtained for whole blood (43.7–68.7 hours for low dose, 26.7–32.4 hours for high dose).

Estimates for tissue depletion half-life appeared similar in male and female animals at both doses.

Table 5. Tissue depletion half-lives of total radioactivity in tissues following a single oral dose of 5 or 250 mg/kg bw of [^{14}C]spiropidion to male and female rats

Tissue	Values are expressed as $t_{½\ elim}$ (h)			
	Male (n = 4)		Female (n = 4)	
	5 mg/kg bw	250 mg/kg bw	5 mg/kg bw	250 mg/kg bw
Adrenals	3.47	8.75	4.80	6.59
Bone: mineral	24.4	10.2	26.8	10.1
Brain	4.63	9.30	6.04	6.83
Fat: renal	3.42	6.05	6.64	7.26
Heart	22.7*	18.9	24.6	11.3
Kidneys	31.8	16.0	30.5	17.9
Liver	30.9	24.8	31.2	19.7
Lungs	41.3	20.9*	28.8	21.7
Muscle	22.9	14.1	23.6	8.74
Ovaries	NA	NA	4.80	6.45
Pancreas	19.3	6.06	8.65	7.25
Plasma	29.1	11.2	26.0	12.5
Spleen	49.0	21.6	29.1	21.7
Testes	34.8	17.9	NA	NA
Thymus	17.1	5.39	8.12	6.67
Thyroid	27.0*	11.5	5.78	6.96
Uterus	NA	NA	8.42	7.52
Whole blood	68.7*	26.7*	43.7	32.4

NA: Not applicable; *Source:* Adapted from Punler et al., 2016a

* The coefficient of determination of terminal phase was < 0.8

In a pharmacokinetic study 24 Han Wistar rats/sex per group were administered [*phenyl-U*-^{14}C]-spiropidion (purity 98.4%) in a single oral dose of 5 mg/kg bw or 250 mg/kg bw. An additional group of four males and four females were given a single i.v. dose of 1 mg/kg bw. Blood samples were taken over a four-day period (oral) or three-dau period (i.v.) to determine the pharmacokinetics of total radioactivity in blood (following oral and i.v. administration) and plasma (following oral administration). Oral bioavailability was determined by comparing the dose-normalised exposures following oral and i.v. administration of [^{14}C]spiropidion. Radioactivity in all samples was quantified by LSC, by direct analysis or following sample oxidation.

Absorption was rapid with a blood and plasma T_{max} at 1–2 hours and 1–4 hours post dose in animals given 5 and 250 mg/kg bw, respectively. Total systemic exposure was comparable for whole blood and plasma at the same dose levels. Systemic exposure to total radioactivity (based on AUC_{0-t} estimates) increased in a broadly proportional manner between the 5 and 250 mg/kg bw doses with no obvious trend for non-proportionality. The absolute bioavailability, F_{abs}, was estimated to be in excess of 105%. In general, there were no consistent sex-related differences noted in the pharmacokinetics of total radioactivity.

Blood to plasma ratios suggested that at earlier time points, total radioactivity remained predominantly in plasma rather than in the cellular component of whole blood. The blood : plasma ratio appeared to increase at later time points, becoming either evenly distributed or greater in the cellular fraction.

Following a single i.v. dose of 1 mg/kg bw of [^{14}C]spiropidion to male and female rats, the concentration of radioactivity in blood steadily declined during the 72 hours following dosing. The systemic exposure (AUC_{0-t}) was comparable irrespective of sex, and the estimated $t_{½}$ of total radioactivity was longer in males (42 hours) than females (22 hours) (Punler, Tomlinson & Hutton, 2016b).

Table 6. Mean whole blood and plasma concentrations and pharmacokinetic parameters following a single oral dose (5 or 250 mg/kg bw) or a single i.v. dose (1 mg/kg bw) of [^{14}C]spiropidion to male and female rats (µg equiv. of spiropidion/mL)

	Oral				Intravenous	
	5 mg/kg bw		250 mg/kg bw		1 mg/kg bw	
	Males (n = 24)	Females (n = 24)	Males (n = 24)	Females (n = 24)	Males (n = 4)	Females (n = 4)
			Whole blood			
Sampling time (h)						
0.25	0.725	1.150	8.9	9.3	0.552	0.441
0.5	1.012	1.935	25.3	31.0	0.368	0.300
1	1.511	2.704	34.6	46.0	0.284	0.277
2	1.776	1.920	38.0	55.7	0.227	0.226
4	0.827	0.823	40.9	28.4	0.123	0.141
8	0.463	0.513	34.5	30.2	0.066	0.101
12	0.243	0.315	32.0	13.9	0.036	0.070
24	0.069	0.071	9.9	6.3	0.010	0.016
30	0.043	0.035	2.4	1.7	ND	ND
48	0.045	0.026	2.3	1.4	0.005	0.004
72	0.029	0.019	1.4	0.9	0.005	0.003
96	0.031	0.020	1.5	1.0	ND	ND
C_{max} (µg equiv./g)	1.78	2.70	40.9	55.7	NA	NA
C_0 (for i.v.)	NA	NA	NA	NA	0.831	0.831
C_{max}/D	0.363	0.546	0.171	0.232	NA	NA
T_{max} (h)	2	1	4	2	NA	NA
$t_{½}$ (h)	64.7[#]	44.1[#]	74.7[#]	65.5[#]	41.8	21.5
$AUC_{(0-t)}$	13.8	15.4	851	605	2.23	2.66
$AUC_{(0-t)}/D$	2.82	3.11	3.56	2.52	2.04	2.31
F_{elim} (%)	131	130	167	105	NA	NA
			Plasma			
Sampling time (h)						
0.25	1.158	1.890	16.3	14.8	ND	ND
0.5	1.459	2.926	37.6	46.9	ND	ND
1	2.316	4.047	49.4	68.3	ND	ND
2	2.321	2.800	43.9	74.1	ND	ND
4	0.683	1.041	42.0	33.4	ND	ND
8	0.562	0.713	33.5	37.9	ND	ND
12	0.246	0.417	30.8	14.0	ND	ND
24	0.057	0.090	7.5	5.6	ND	ND
30	0.025[a]	0.034	1.1	1.1	ND	ND
48	0.014[a]	0.016[a]	0.5[a]	0.6[a]	ND	ND
72	0.011	0.012	0.2[a]	0.2[a]	ND	ND
96	0.006[a]	0.017	0.2[a]	0.3[a]	ND	ND
C_{max} (µg equiv./g)	2.32	4.05	49.4	74.1	ND	ND
C_{max}/D	0.474	0.818	0.206	0.309	ND	ND

	Oral				Intravenous	
	5 mg/kg bw		250 mg/kg bw		1 mg/kg bw	
	Males (n = 24)	Females (n = 24)	Males (n = 24)	Females (n = 24)	Males (n = 4)	Females (n = 4)
T_{max} (h)	2	1	1	2	ND	ND
$t_{½}$ (h)	24.3	38.4[#]	9.98	26.5[#]	ND	ND
$AUC_{(0-t)}$	13.8	20.2	735	635	ND	ND
$AUC_{(0-t)}/D$	2.81	4.08	3.07	2.65	ND	ND

ND: Not determined; NA: Not applicable; Source: adapted from Punler, Tomlinson & Hutton, 2016b

[a] Mean includes results calculated from data less than 30dpm above background;

C_0: theoretical concentration at time zero after intravenous bolus dosing; D: Dose;

$t_{½}$: Terminal elimination half-life; C_{max} : Maximum concentration achieved T_{max} : Time taken to reach C_{max};

$AUC_{(0-t)}$ (µg equiv. h/g): The area under the concentration–time curve from the start of dose administration to the time after dosing at which the last quantifiable concentration was observed

F_{abs}: Absolute bioavailability calculated as the ratio of the dose-normalised AUC (oral) to dose-normalised AUC (intravenous)

[#] Coefficient of determination of terminal phase was < 0.8.

In an investigative, non-GLP pharmacokinetic study, oral doses of 3, 30 or 300 mg/kg bw of spiropidion (SYN546330) and 3 mg/kg bw of spiropidion's first downstream metabolite SYN547305 (formed by ester hydrolysis of the ethoxycarbonyl moiety of spiropidion) were administered to groups of three male and three female rats over seven days. Single i.v. doses of 1 mg/kg bw of SYN546330 and SYN547305 were administered to one male and one female animal at each dose, for each compound. Blood samples were taken over 12 hours on day 1 (oral and i.v.), before dosing on days 2–7 (oral), and then 48 hours after the last administered dose on day 7 (oral). this was done to determine the pharmacokinetics of the two compounds in blood. Samples were analysed by liquid chromatography with tandem mass spectrometry (LC-MS-MS).

Intravenous administration of spiropidion at a dose level of 1 mg/kg bw or an oral administration at dose levels of 3 or 30 mg/kg bw per day resulted in generally very low (< 10 ng/mL or below the level of quantitation, LOQ) concentrations of unchanged spiropidion on the first day of administration. By contrast, on the first day animals administered 300 mg/kg bw per day exhibited a quantifiable (around 20 ng/mL) concentration of unchanged spiropidion. After repeated administration for seven days, concentrations of unchanged spiropidion in blood were generally low and variable after all doses. Therefore, the pharmacokinetics of spiropidion (SYN546330) could not be reliably interpreted after i.v., single or repeat oral administration. The low and variable blood concentrations of unchanged spiropidion (SYN546330) is likely due to the rapid hydrolysis of spiropidion (SYN546330) to metabolite SYN547305. This is supported by substantially higher concentrations of the metabolite SYN547305 than corresponding concentrations of unchanged spiropidion (SYN546330) after both i.v. and oral dosing.

After i.v. dosing of spiropidion (SYN546330) the observed concentrations of metabolite SYN547305 in males were higher than those in females, with a C_{max} of 107 ng/mL in males and 21 ng/mL in females observed at one hour post dose. The total area under the concentration–time curve (AUC) was 279 and 152 ng h/mL for males and females respectively. This suggests that metabolite SYN547305 is cleared from blood faster in females.

After single oral administration of spiropidion (SYN546330) maximal concentrations of metabolite SYN547305 were observed approximately two hours post administration, supporting the presumption of rapid hydrolysis of spiropidion (SYN546330). Mean peak concentrations of metabolite SYN547305 increased in both male and female from ca 85 ng/mL at 3 mg/kg bw to 4000 ng/mL at 300 mg/kg bw. The AUC also increased with increasing dose after both single and repeat administration. Due to the variability in the data, no obvious correlation could be concluded regarding proportionality of AUC or C_{max} to dose. No sex-specific differences were evident from single-dosed animals.

Following i.v. administration of metabolite SYN547305, peak concentrations were of 239 ng/mL in males and 91.6 ng/mL in females. This is higher than after administration of spiropidion SYN546330, but it also reflects the same pattern, with higher concentrations observed in the males and in the AUC. The exposure to metabolite SYN547305 after its oral administration was around 3–4-fold higher than after oral administration of spiropidion SYN546330 (Lewsley & Hewitt, 2013).

Table 7. Pharmacokinetic parameters following repeated dosing of spiropidion (SYN546330) (3, 30 or 300 mg/kg bw per day) and metabolite SYN547305 (3 mg/kg bw per day) for 7 days or a single i.v. dose of 1 mg/kg bw of spiropidion or metabolite SYN547305 to male and female rats.

Pharmaco-kinetic parameter	M (n = 1)	F (n = 1)	M (n = 3)	F (n = 3)	M (n = 3)	F (n = 3)	M (n = 3)	F (n = 3)
	1 mg/kg bw i.v		3 mg/kg bw per day oral		30 mg/kg bw per day oral		300 mg/kg bw per day oral	
	Spiropidion (SYN546330) data after administration of spiropidion (SYN546330)							
Dose day 1								
C_{max} (ng/mL) [SD]	7.31	11.2	NC	NC	7.60 (9.4)	4.35 (2.3)	19.2 (16.2)	19.7 (7.9)
T_{max} (h) [SD]	3	3	NC	NC	2.7 (1.2)	1.0 (0.9)	1.7 (0.6)	1.7 (0.6)
$AUC_{(last)}$ [SD]	17.2*	26.3*	NC	NC	6.12 (4.8)	2.84 (2.2)	81.0 (53.6)	108 (53.6)
Dose day 7								
C_{max} (ng/mL) [SD]	50.4	21.4	50.4 (NC)	21.4 (10.5)	180 (62.4)	65.3 (50.8)	51.1 (27.0)	42.8 (NC)
T_{max} (h) [SD]	2	3.5	2 (NC)	3.5 (4.0)	1.00 (0.0)	3.83 (5.4)	1.0 (0.0)	0.5 (NC)
$AUC_{(last)}$ [SD]	NC	68.2	NC	68.2 (16.6)	266 (117)	143 (73)	169 (45.2)	393 (NC)
	Metabolite SYN547305 data after administration of spiropidion (SYN546330)							
Dose day 1								
C_{max} (ng/mL) [SD]	107	21.0	89.0 (77.1)	85.1 (35.7)	1200 (713)	1850 (306)	3950 (681)	4580 (1330)
T_{max} (h) [SD]	1.0	1.0	2.0 (0.0)	2.0 (0.0)	1.3 (0.6)	0.8 (0.3)	3.3 (2.3)	2.0 (0.0)
$AUC_{(last)}$ [SD]	279	152	497 (481)	564 (204)	4010 (2630)	6460 (768)	34100 (11100)	35800 (10200)
Dose day 7								
C_{max} (ng/mL) [SD]	NC	NC	88.6 (92.0)	109 (25.9)	1410 (773)	2660 (425)	6440 (1380)	4310 (NC)
T_{max} (h) [SD]	NC	NC	1.3 (0.6)	2.0 (0.0)	1.0 (0.0)	1.3 (0.6)	4.0 (3.5)	6.0 (NC)
$AUC_{(last)}$ [SD]	NC	NC	528 (463)	797 (228)	4550 (2980)	10 800 (2310)	72900 (21400)	90700 (NC)
	Metabolite SYN547305 data after administration of metabolite SYN547305							
Dose day 1								
C_{max} (ng/mL) [SD]	239	91.6	386 (171)	443 (91.9)	NC	NC	NC	NC
T_{max} (h) [SD]	1	1	1.33 (0.58)	0.75 (0.43)	NC	NC	NC	NC

Pharmaco-kinetic parameter	M (n = 1)	F (n = 1)	M (n = 3)	F (n = 3)	M (n = 3)	F (n = 3)	M (n = 3)	F (n = 3)
	1 mg/kg bw i.v		3 mg/kg bw per day oral		30 mg/kg bw per day oral		300 mg/kg bw per day oral	
$AUC_{(last)}$ [SD]	783	245	1430 (388)	2400 (537)	NC	NC	NC	NC

* Unreliable data estimation; M: male; F: female; Source: adapted from Lewsley & Hewitt, 2013.

C_{max} : Maximum concentration achieved T_{max} : Time taken to reach C_{max};

AUC_{last} : Area under the concentration–time curve until the last measurement (ng.h/mL);

NC: Not calculated: for males no explanation given, for females because two females were removed from the study due to severe clinical signs;

1.2 Biotransformation

The metabolic fate of spiropidion was investigated in Han Wistar rats. The test item (purity 98.4%) was administered by gavage to eight male rats per group as a single dose at 5 or 250 mg/kg bw. Only male rats were investigated as there were no substantial metabolic differences between sexes in a preliminary study. An additional group of three bile duct-cannulated males was given [^{14}C]spiropidion as a single dose at 5 or 250 mg/kg bw. The vehicle was 0.5% (w/v) aqueous CMC. In non-cannulated rats urine was collected pre-dose and at eight hours post dose, faeces were collected at before dosing, and urine and faeces were then collected at daily intervals until termination (96 hours post dose). In non-cannulated pharmacokinetic animals, blood samples were taken at 0.5, 1, 4, 12 and 24 hours (termination) post dose. In bile duct-cannulated rats, urine and faeces were collected before dosing and daily up to 72 hours post dose. Bile was collected before dosing and for the periods 0–1, 1–2, 2–4, 4–8, 8–12, 12–24, 24–48 and 48–72 hours post dose. The nature and identity of metabolites present in samples of urine, faeces, bile and plasma obtained from male rats were investigated by radio-chromatography and mass spectrometry in samples pooled by time point as follows: urine (0–48 h), faeces (0–72 h) and plasma (0–24 h, AUC pool) samples were prepared from non-cannulated rats; urine (0–48 h), faeces (0–48 h) and bile (0–24 h) samples were prepared from bile duct-cannulated rats. Urine, faeces extracts, bile and plasma extracts were analysed by high-performance liquid chromatography (HPLC) with concurrent radio detection to allow the quantification of radio-labelled metabolites. All metabolites accounting for more than 5% of the AD were identified using liquid chromatography–mass spectrometry (LC-MS) analysis and comparison of retention times with certified reference standards, where available.

The results of the excretion balance were similar to the those determined by Punler, Tomlinson & Hutton (2016a) in which, irrespective of dose or sex, the routes and rates of elimination were broadly similar. In that study, absorption was similar following both doses, indicating that absorption was not saturated. At each dose level the absorption was similar in males and females, with oral absorption ranging from 69–86%.

A total of 71.0% and 76.0% of the AD was identified in the urine and faeces samples obtained from the 5 and 250 mg/kg bw intact rats, respectively, while a total of 82.1% and 84.1% of the AD was identified in urine, faeces and bile obtained from the 5 and 250 mg/kg bw bile duct-cannulated rats, respectively.

Following oral administration of [^{14}C]SYN546330, it was rapidly metabolised, with parent only detected in faeces at 2.7% of AD in intact rats and 7% of AD in bile duct-cannulated rats. The parent SYN546330 was primarily metabolised to SYN547305 via the loss of the ethoxy carbonyl moiety. Direct *N*-demethoxylation was a relatively minor metabolic route for SYN546330, accounting for 1.1–2.5% of the total radioactivity (TR) AUC in plasma. Metabolite *N*-demethoxy-SYN546330 was only detected in the faeces from the 250 mg/kg bw dose group at 1.2% of AD. Metabolite SYN547305 accounted for the largest percentage of the total radioactivity AUC (47.9%) in plasma. However, following subsequent metabolism it only accounted for 1.4% of the 5 mg/kg bw dose in excreta and 13.8% of the 250 mg/kg bw dose in faeces only, with no SYN547305 detected in bile.

SYN547305 was further metabolised via demethoxylation to SYN548430, direct glucuronidation, *N*-dealkylation to SYN547435, demethoxylation, oxidation and hydroxylation, and reduction.

SYN548430 was the only other metabolite that accounted for more than 10% of the circulating radioactivity, at 17.4–19.0% of the total radioactivity AUC. Metabolite SYN548430 accounted for up to 61% of the dose in excreta, and in urine represented 43% and 35% of the 5 and 250 mg/kg bw doses, respectively. A further ca 8% was recovered in bile.

Like SYN547305, its glucuronide was only a minor metabolite in excreta with less than 1% of the dose in urine and less than 1.3% of the dose in bile.

Hydroxy oxidised desmethoxy-SYN547305 in plasma accounted for approximately 5% of the total radioactivity AUC. It was excreted in urine, accounting for 5.0–7.2% (bile duct-cannulated) of the AD, and in bile accounting for 3.0–4.6% of AD.

Both SYN547435 and reduced SYN547305 accounted for less than1% of AD in urine.

Table 8. Metabolites quantification in excreta of intact rats given a single oral dose of 5 or 250 mg/kg bw [^{14}C]spiropidion (SYN546330).

	Percentage of administered dose					
	5 mg/kg bw			250 mg/kg bw		
Compound	Urine (0–48 h)	Faeces (0–72 h)	Total excreta	Urine (0–48 h)	Faeces (0–72 h)	Total excreta
SYN546330 (spiropidion)	ND	ND	ND	ND	2.7	2.7
Unidentified (10.6–10.7 min)	0.2	ND	0.2	ND	ND	ND
Unidentified (14.6–15.3 min)	0.5	0.8	1.3	0.3	ND	0.3
SYN548430	42.8	18.6	61.4	35.1	16.1	51.2
Unidentified (21.9 min)	0.2	ND	0.2	ND	ND	ND
Unidentified (22.3–22.5 min)	0.3	ND	0.3	ND	ND	ND
SYN547305 glucuronide	0.9	ND	0.9	0.7	ND	0.7
Unidentified (24.7–25.1 min)	0.6	1.3	1.9	0.3	1.1	1.4
Unidentified (26.3 min)	ND	1.2	1.2	ND	ND	ND
Hydroxy oxidised desmethoxy-SYN547305	6.3	ND	6.3	6.2	ND	6.2
Unidentified (29.2–29.5 min)	ND	1.1	1.1	ND	1.1	1.1
Unidentified (32.1–32.3 min)	ND	0.9	0.9	ND	ND	ND
Reduced SYN547305	0.6	ND	0.6	0.8	ND	0.8
Unidentified (37.0–37.9 min)	ND	0.5	0.5	ND	ND	ND
SYN547435	0.4	ND	0.4	0.6	ND	0.6
Unidentified (38.1 min)	ND	1.0	1.0	ND	ND	ND
SYN547305	0.6	0.8[a]	1.4	ND	13.8	13.8
Desmethoxy SYN546330[b]	ND	ND	ND	ND	1.2	1.2
Post extraction solids	0.4	6.8	7.2	0.4	3.9	4.3
Total identified	51.6	19.4	71.0	43.4	32.6	76.0
Total unidentified	1.8	6.8	8.6	0.6	3.4	4.0
Total accounted for	53.4	26.2	79.6	44.0	36.0	80.0
Losses/gains	0.2	9.0	9.2	0.3	11.0	11.3
Total	53.6	35.2	88.8	44.3	47.0	91.3

[a] Identified by comparison of retention times with 250 mg/kg bw sample; Source: Strathdee & Pulner, 2017

[b] Tentative identification as no fragment ions were observed; ND: Not detected

LOQ: Limit of quantitation: 0.1% dose and for faeces and 250 mg/kg bw urine samples, <0.1% dose for 5 mg/kg bw urine sample

Table 9. Quantification of metabolites in excreta of bile cannulated rat given a single oral dose of 5 or 250 mg/kg bw [^{14}C]spiropidion SYN546330

	Percentage of administered dose							
	5 mg/kg bw				250 mg/kg bw			
Compound	Urine (0–48 h)	Faeces (0–48 h)	Bile (0–24 h)	Total excreta	Urine (0–48 h)	Faeces (0–48 h)	Bile (0–24 h)	Total excreta
SYN546330	ND	ND	ND	ND	ND	7.0[A]	ND	7.0
Unidentified (10.6–10.7 min)	0.2	ND	ND	0.2	0.1	ND	ND	0.1
Unidentified (14.6–15.3 min)	0.3	0.2	0.2	0.7	0.3	ND	ND	0.3
SYN548430	52.5	5.7	7.6	65.8	36.8	5.0	8.4	50.2
Unidentified (21.9 min)	0.5	ND	ND	0.5	ND	ND	ND	ND
Unidentified (22.3–23.3 min)	0.4	ND	0.4	0.8	0.6	ND	ND	0.6
SYN547305 glucuronide	0.9	ND	1.3	2.2	0.9	1.0	0.7	2.6
Unidentified (24.7–25.1 min)	0.5	ND	0.4	0.9	0.2	ND	ND	0.2
Unidentified (26.3 min)	ND	0.2	ND	0.2	ND	ND	ND	ND
Hydroxy oxidised desmethoxy-SYN547305	7.2	ND	4.6	11.8	5.0	ND	3.0	8.0
Unidentified (29.2–29.5 min)	ND	0.2	ND	0.2	ND	ND	ND	ND
Unidentified (32.1–32.3 min)	ND	0.2	ND	0.2	ND	ND	ND	ND
Reduced SYN547305	0.8	ND	ND	0.8	0.4	ND	ND	0.4
Unidentified (37.0–37.9 min)	ND	0.2	ND	0.2	ND	ND	ND	ND
SYN547435	0.4	ND	ND	0.4	0.2	ND	ND	0.2
SYN547305	0.7	0.4[b]	ND	1.1	0.2	15.5[a]	ND	15.7
Post-extraction solids	0.8	1.2	0.1	2.1	1.1	0.6	0.2	1.9
Total identified	62.5	6.1	13.5	82.1	43.5	28.5	12.1	84.1
Total unidentified	1.9	1.0	1.0	3.9	1.2	<0.1	<0.1	<1.4
Total accounted for	64.4	7.1	14.5	86.0	44.7	28.5	12.1	85.5
Losses/Gains	1.0	2.3	0.1	3.4	1.2	2.2	0.2	3.4
Total	65.4	9.4	14.6	89.4	45.9	30.7	12.3	88.9

[a] Shift in retention time (ca 3 minutes) observed compared to other phases; Source: Strathdee & Pulner, 2017

[b] Identified by comparison of retention time with component identified in the 250 mg/kg bw intact faeces sample;

LOQ: Limit of quantitation: 0.1% dose and for urine, bile and 5 mg/kg bw faeces samples and 0.1% dose for 250 mg/kg bw faeces sample; ND: Not detected

Table 10. Metabolites quantification in plasma of intact rat given a single oral dose of 5 or 250 mg/kg bw [^{14}C]spiropidion SYN546330.

	AUC_{24} (% total radioactivity AUC)	
Compound	**5 mg/kg bw**	**250 mg/kg bw**
SYN548430	19.0	17.4
Unidentified (24.1–24.4 min)	1.6	1.8
Hydroxy oxidised desmethoxy-SYN547305	5.2	5.3
Unidentified (32.4 min)	0.8	3.5
Unidentified (34.1–34.4 min)	0.5	1.1
Hydroxy SYN547305	6.6	3.0
SYN547305	45.0	47.9
Unidentified (42.1 min)	ND	1.4
Desmethoxy-SYN546330	1.1	2.5
Unidentified (63.6 min)	ND	1.0
Post-extraction solids	4.5	4.3
Total identified	76.9	76.1
Total unidentified	2.9	8.8
Total accounted for	79.8	84.9
Losses/gains	20.2	15.1
Total	100.0	100.0

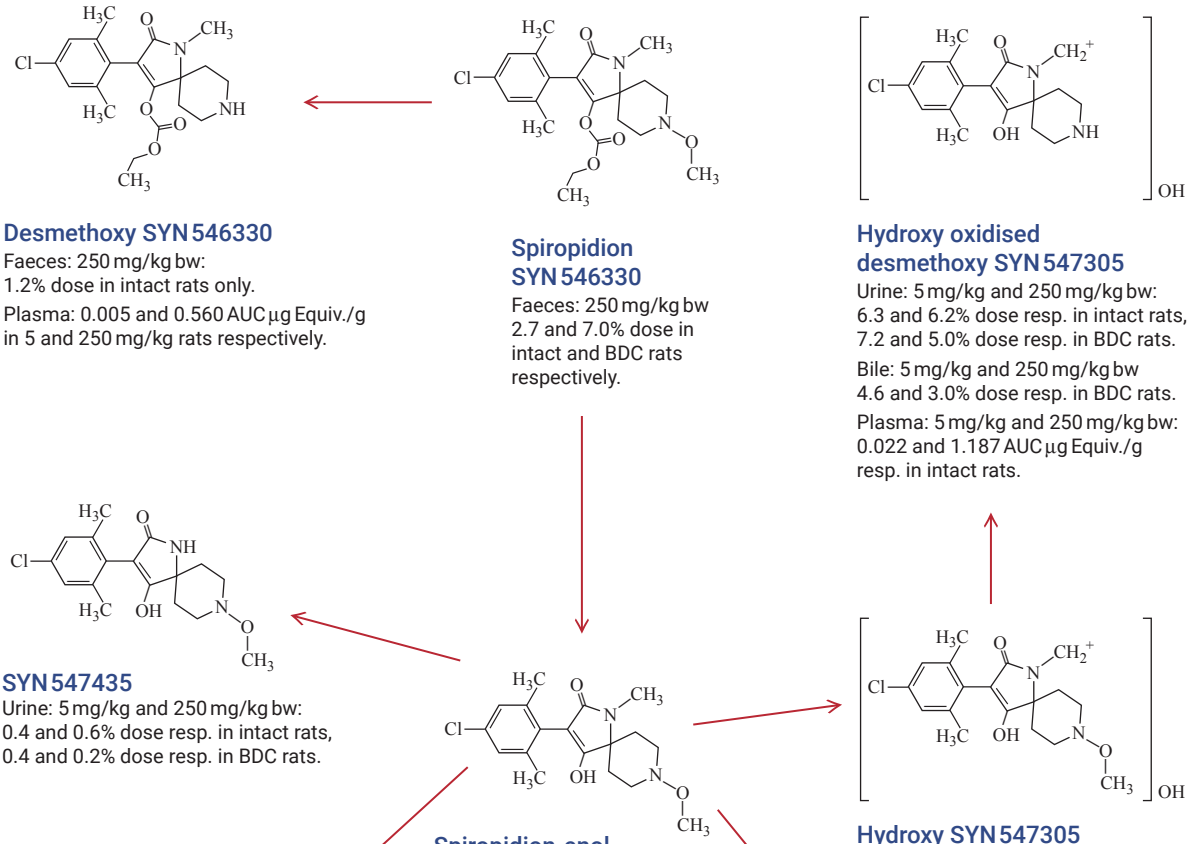
ND: Not determined Source: Strathdee & Pulner, 2017

AUC: Area under the concentration–time curve (µg equiv./g)

LOQ: Limit of quantitation: 0.002% and 0.089% of AUC radioactivity for 5 and 250 mg/kg bw samples, respectively

It is concluded that metabolites observed in urine, faeces, bile and plasma samples were broadly similar, both qualitatively and quantitatively, irrespective of dose. The primary metabolic route appears to be ester hydrolysis of the eth oxycarbonyl moiety to form SYN547305, followed by the loss of the methoxy moiety from the piperidine ring to form SYN548430. No cleavage of the ring structures was observed (Strathdee & Pulner, 2017).

Figure 2. Biotransformation pathway for spiropidion following oral administration to rats



Desmethoxy SYN 546330
Faeces: 250 mg/kg bw:
1.2% dose in intact rats only.
Plasma: 0.005 and 0.560 AUC µg Equiv./g in 5 and 250 mg/kg rats respectively.

Spiropidion SYN 546330
Faeces: 250 mg/kg bw
2.7 and 7.0% dose in intact and BDC rats respectively.

Hydroxy oxidised desmethoxy SYN 547305
Urine: 5 mg/kg and 250 mg/kg bw:
6.3 and 6.2% dose resp. in intact rats,
7.2 and 5.0% dose resp. in BDC rats.
Bile: 5 mg/kg and 250 mg/kg bw
4.6 and 3.0% dose resp. in BDC rats.
Plasma: 5 mg/kg and 250 mg/kg bw:
0.022 and 1.187 AUC µg Equiv./g resp. in intact rats.

SYN 547435
Urine: 5 mg/kg and 250 mg/kg bw:
0.4 and 0.6% dose resp. in intact rats,
0.4 and 0.2% dose resp. in BDC rats.

Spiropidion-enol SYN 547305
Urine:
0.6% dose in 5 mg/kg bw intact rats,
0.7 and 0.2% dose resp. in
5 mg/kg and 250 mg/kg BDC rats.
Faeces: 5 mg/kg and 250 mg/kg bw
0.8 and 13.8% dose resp. in intact rats,
0.4 and 15.5% dose resp. in BDC rats.
Plasma: 5 mg/kg and 250 mg/kg bw
0.195 and 10.635 AUC µg Equiv./g resp. in intact rats.

Hydroxy SYN 547305
Plasma: 5 mg/kg and 250 mg/kg bw:
0.028 and 0.670 AUC µg Equiv./g resp. in intact rats.

Reduced SYN 547305
Urine: 5 mg/kg and 250 mg/kg bw:
0.6 and 0.8% dose resp. in intact rats,
0.8 and 0.4% dose resp. in BDC rats.

SYN 548430
Urine: 5 mg/kg and 250 mg/kg bw
42.8 and 35.1% dose resp. in intact rats,
52.5 and 36.8% dose resp. in BDC rats.
Faeces: 5 mg/kg and 250 mg/kg bw
18.6 and 16.1% dose resp. in intact rats,
5.7 and 5.0% dose resp. in BDC rats.
Plasma: 5 mg/kg and 250 mg/kg bw
0.082 and 3.858 AUC µg Equiv./g resp. in intact rats.
Bile: 5 mg/kg and 250 mg/kg bw
7.6 and 8.4% dose resp. in BDC rats.

SYN 547305 glucuronide
Urine: 5 mg/kg and 250 mg/kg bw:
0.9 and 0.7% dose resp. in intact rats,
0.9 and 0.9% dose resp. in BDC rats.
Faeces: 1.0% dose in BDC rats at 250 mg/kg bw.
Bile: 5 mg/kg and 250 mg/kg bw
1.3 and 0.7% dose resp. in BDC rats.

Redrawn from Strathdee & Punler, 2017

1.3 In vitro rat and human metabolism

Using a concentration of 10μM in each case and in duplicate, [*phenyl-U*-^{14}C]spiropidion and [*spirodecanone-5*-^{14}C]spiropidion were incubated separately (at ca 37°C ± 0.5°C) with 0.5 mg/mL of either pooled Han Wistar rat liver microsomes (24 and 65 samples for females and males, respectively) or pooled human liver microsomes (150 samples, mixed gender) for 0, 5, 10, 30 and 60 minutes. To check the stability of [^{14}C]-spiropidion under experimental conditions, incubations in the absence of microsomes were also conducted for 0 and 60 minutes for each radiolabel test item. Incubation samples were analysed by HPLC with radioactive monitoring and the proportions of the metabolites and spiropidion (SYN546330) quantified.

The metabolic viability of the rat and human liver microsomes was assessed using [^{14}C]testosterone as a model substrate. The viability of the liver microsomes was confirmed by the production of 6β-hydroxytestosterone and depletion of testosterone with both microsome preparations, thus the data generated following incubation with radiolabelled spiropidion were considered valid.

The proportions of [*phenyl-U*-^{14}C]spiropidion or [*spirodecanone-5*-^{14}C]spiropidion present in samples at 0 minutes with rat liver microsomes were 87% and 90%, respectively; with human microsomes the values were 86% and 90%, respectively. After 60 minutes incubation, it was observed that extensive metabolic activity had taken place with both rat and human liver microsomes, only 1% and 6%, respectively, of [*phenyl-U*-^{14}C] or [*spirodecanone-5*-^{14}C]spiropidion remaining unmetabolised.

Irrespective of the radiolabel position seven metabolite fractions (M1, M2, M4, M10, M11, M16 and M17) were consistently detected above the limit of quantification (≥1%) with rat or human liver microsomes. All the metabolite fractions observed with human microsomes were observed with rat microsomes.

After 0 and 60 minutes incubation in the absence of microsomal protein, ca 80–90% and 72–79%, respectively of SYN546330 remained in the incubate.

It was concluded that the metabolism of [^{14}C]spiropidion by human liver microsomes was extensive, but qualitatively comparable to that seen with rat liver microsomes, irrespective of radiolabel position. In validated test systems, all the human metabolites formed from [^{14}C]spiropidion were detected in comparable rat preparations (Paul, 2018).

Table 11. Metabolites detected following 60 minute incubations of phenyl- and spirodecanone-^{14}C labelled spiropidion (SYN546330) with pooled rat and human liver microsomes

	Percentage of radioactivity detected			
	[*phenyl-U*-^{14}C]spiropidion		[*spirodecanone-5*-^{14}C]spiropidion	
Metabolite	**RLM**	**HLM**	**RLM**	**HLM**
Spiropidion (SYN546330)	1.1	6.3	BLQ	5.6
M1	19.3	14.3	19.7	14.2
M2	20.7	13.6	19.4	14.7
M4	22.1	11.3	25.2	13.2
M10	15.3	33.4	14.2	33.8
M11	10.4	11.8	10.6	11.3
M16	2.5	3.1	2.5	2.3
M17	1.6	<LOQ	1.5	<LOQ

RLM: Rat liver microsomes; HLM: Human liver microsomes; Source: Paul, 2018

<LOQ: Below the limit of quantification

2. Toxicological studies

2.1 Acute toxicity

The results of acute oral, dermal and inhalation toxicity studies with spiropidion, along with the results of dermal and eye irritation, skin sensitization and phototoxicity studies, are summarized in Table 12 below, and the studies are described in more detail thereafter.

Table 12. Acute toxicity of spiropidion

Species	Strain	Sex	Route	Purity	Result	Reference
Rat	Crl:WI	Female	Oral	98.4%	LD_{50}: >2000 mg/kg bw	Tarcai, 2016a
Rat	Crl:WI	Male & Female	Dermal	98.4%	LD_{50}: >5000 mg/kg bw	Tarcai, 2016b
Rat	Crl:WI	Male & Female	Inhalation	98.8%	LC_{50} >1.12 mg/L	Tóth, 2017
Rabbit	New Zealand White	Male	Skin irritation	98.4%	Not irritating	Török-Bathó, 2015
Chicken	COBB 500	Female	Eye irritation	98.4%	In vitro study	Váliczkó, 2015
Rabbit	New Zealand White	Male	Eye irritation	98.4%	Minimally irritating	Váliczkó, 2016
Mouse	CBA/CaOla Hsd	Female	Skin sensitization	98.4%	Skin sensitizer EC3 value: 0.13%	Pooles, 2015
NA	BALB/c 3T3	NA	Phototoxicity	98.4%	Not phototoxic	Gehrke, 2018

LC_{50}: Median lethal concentration; LD_{50}: Median lethal dose; NA: Not applicable

(a) Lethal doses

An acute oral toxicity study (up-and-down procedure) was conducted with seven female Crl:WI rats. The animals were treated with a single oral (gavage) dose of spiropidion (purity 98.4%) in the following order: 1750, 5000, 1750, 550 and 2000 mg/kg bw in 0.5 % (w/v) CMC, acidified to pH 5; the dose volume was 10 mL/kg bw. The animals were fasted overnight prior to treatment and food was returned three hours after dosing. After dosing all animals were observed individually at 30 minutes, 1, 2, 3, 4 and 6 hours post treatment and once each day for 14 days thereafter. The body weights of the animals were recorded on day –1 and days 0 (prior to administration), 7 and 14, or at death.

The first animal (1750 mg/kg bw) showed decreased activity (score 1), vocalisation, irritability, hunched back, inco-ordination (score 1) and piloerection and was symptom-free beginning on day 7 post dose.

The second animal (5000 mg/kg bw) showed decreased activity (score 1 and 2), hunched back, piloerection and liquid faeces and was found dead on day 2. Diffuse, dark red discoloration in all lobes of the collapsed lungs was noted.

In the third animal (1750 mg/kg bw) decreased activity (score 1 and 2), irritability, hunched back, inco-ordination (score 1 and 2) and piloerection were observed. Respiratory rate decreased (score 1) before the day of death. The animal was found in moribund condition and euthanised on day 6. At necropsy, dark red diffuse discoloration in the glandular mucosa of the stomach and opaque liquid material in the dilated duodenum, jejunum and ileum were observed. In addition, collapsed lungs were observed.

The fourth animal (550 mg/kg bw) was clear of symptoms during the observation period. Following this, in agreement with the Sponsor, the highest dose level was changed from 5000 to 2000 mg/kg bw as it was considered an appropriate highest dose level based on observations on the first four animals.

At 2000 mg/kg bw, the following clinical signs were noted: decreased activity (score 1) in two of three animals, inco-ordination (score 1) in all three animals, intermittent tremors in one of three animals,

irritability and hunched back in all three animals. Piloerection in all three animals and vocalisation in two of teh three rats were also observed. Each animal at this dose level was symptom-free beginning on day 6 at the latest.

There were no treatment-related changes in the body weights of the surviving animals.

The acute oral median lethal dose (LD_{50}) of spiropidion in female rats was greater than 2000 mg/kg bw (Tarcai, 2016a).

Five male and five female Crl:WI rats were treated with a single, semi-occlusive dermal application of spiropidion (purity 98.4%) at a dose of 5000 mg/kg bw. The test item was applied undiluted to a shaved area on approximately 10% of the total body surface. The application period was 24 hours, followed by a 14-day observation period. Clinical observations along with a check of viability and mortality were performed on all animals at one and five hours after dosing and daily for 14 days thereafter. Body weight was measured prior to dosing on day 0 and on days 7 and 14. Rats were euthanized and subjected to a gross macroscopic examination at the end of the two-week observation period (day 14).

No mortality occurred during the 14-day observation period. No clinical signs were observed after treatment with the test item or during the 14-day observation period. All rats were symptom-free during the entire study.

There were no treatment-related changes in body weight. The body weight of the animals was within the range commonly recorded for this strain and age. There were no macroscopic observations at necropsy. The acute dermal LD_{50} of spiropidion in rats was greater than 5000 mg/kg bw for both sexes (Tarcai, 2016b).

Acute inhalation toxicity of milled spiropidion (purity 98.4%) was assessed in rats (Crl:WI) by a single four-hour, nose-only exposure followed by a 14-day observation period. The day of exposure was designated day 0. The study was composed of three sighting groups and one main study group, for which two animals per sex were used for each of the sighting groups and five animals per sex for the main study. The mean achieved concentrations were 5.07 mg/L (sighting group 1), 2.53 mg/L (sighting group 2), 1.22 mg/L (sighting group 3) and 1.12 mg/L (main study). The particle size distribution of the test aerosol was determined regularly during the exposure period. The mass median aerodynamic diameter (MMAD) ranged from 3.01 µm to 3.51 µm and the geometric standard deviation from 2.00 to 2.70, these figures for all the groups. Clinical observations and body weights were recorded throughout the study and at the end of the scheduled period the animals were euthanised and subjected to a gross examination post mortem.

Two males and two females were found dead on day 1 in group 1 (5.07 mg/L) and one female was found dead on day 2 in group 2 (2.53 mg/L). There were no mortalities in group 3 (1.22 mg/L) or the main group (1.12 mg/L). Results for each group were as follows:

Group 1 (5.07 mg/L) – clinical observations reported before death were laboured respiration (slight to moderate), decreased activity (moderate to severe), inco-ordination (moderate to severe), clonic convulsion (whole body), prone position and cold to touch.

Group 2 (2.53 mg/L) – reported clinical signs were laboured respiration (slight to severe), gasping respiration, noisy respiration (slight to moderate), sneezing, increased aggressiveness, decreased activity (slight to severe), hunched back and clonic convulsion (whole body). Two of the surviving animals were symptom-free from day 9 and in one male noisy respiration (moderate) and laboured respiration (moderate) was noted till the end of the observation period. Body weight loss was up to 15.7%.

Group 3 (1.22 mg/L) – reported clinical signs were laboured respiration (slight to severe), gasping respiration, activity decreased (slight), noisy respiration (slight to moderate), sneezing, and piloerection. The animals were symptom-free from day 3. Body weight loss was up to 13%.

Main group (1.12 mg/L) – reported clinical signs were laboured respiration (slight to moderate), noisy respiration (slight), respiratory rate increased (slight), sneezing, inco-ordination (slight), activity decreased (moderate), and hunched back. In one female (#9175) continuous tremor (first and second third of the animal) was recorded immediately after the exposure. The animals were symptom free-from day 4. Body weight loss was up to 13.4%.

At necropsy, enlargement and dark/red diffuse discoloration of the lungs, dry red material at the perinasal/perioral fur was observed in one female (of two) exposed at 2.53 mg/L (group 2) and four animals (of four) exposed to the concentration of 5.07 mg/L (group 1) which were all found dead. These findings were considered to be treatment-related. In surviving animals subjected to the necropsy on day 14, no macroscopic changes at concentration levels of 2.53 (group 2), 1.22 (group 3) and 1.12 mg/L (main group) were recorded.

The acute inhalation median lethal concentration (LC_{50}) of spiropidion in rats was greater than 1.12 mg/L for both sexes (Tóth, 2017).

(b) Dermal irritation

In a primary skin irritation test, 0.5 g of spiropidion (purity 98.4%) was applied to approximately 6 cm^2 of shaved skin on three male young adult New Zealand White rabbits, under a semi-occlusive dressing for four hours. A minimal amount of water was used to ensure good contact with the skin. Skin reactions were scored at 1, 24, 48 and 72 hours after removal of the dressing. Irritation was scored at 24, 48 and 72 hours by a numerical scoring system and the primary irritation index (PII) was calculated.

No clinical signs, skin irritation reactions or body weight changes were observed in any animal during the observation period of 72 hours after the removal of the patches (Török-Bathó, 2015).

(c) Ocular irritation

Spiropidion (purity 98.4%) was tested in vitro on isolated chicken eyes (COBB 500). A 30 mg aliquot of spiropidion was applied to the centre of the cornea of three isolated chicken eyes. The positive control eyes were treated in a similar way with 30 mg of imidazole. The negative control eye was treated with 30 µL of physiological saline (Salsol solution at 0.9%). After 10 seconds, the surfaces were rinsed with physiological saline. The eyes were evaluated before treatment and at approximately 30, 75, 120, 180 and 240 minutes after the post-treatment rinse. Corneal thickness and corneal opacity were measured at all time points. Fluorescein retention was measured on two occasions, at base line ($t = 0$) and approximately 30 minutes after the post-treatment rinse.

No significant corneal swelling was observed during the four hour observation period. Corneal opacity (severity 0.5 or 1) and fluorescein retention change (severity 0.5 or 1) were observed on all three eyes. No other corneal effects were observed, although the test item was stuck on the cornea surfaces on all three eyes after the post-treatment rinse and the cornea surfaces were not cleared 240 minutes after the post-treatment rinse.

Based on the results of the test, spiropidion did not appear to be a severe irritant nor a nonirritant. It was concluded that an in vivo study is required (Váliczkó, 2015).

In an in vivo eye irritation study spiropidion (purity 98.4%) was tested by installation of a single dose of 0.1 g into the conjunctival sac of the left eye of three young adult male New Zealand White rabbits. The untreated right eye served as the control. Scoring of irritation effects was performed for all animals approximately 1, 24, 48 and 72 hours after test material installation. Eye irritation scores were evaluated according to the scoring system by Draize and OECD 405 (2012). Observations with fluorescein staining were made in all animals approximately 24 hours before treatment and then 24, 48 and 72 hours after the treatment. Rabbits were treated with analgesic and anaesthetic as per the regulatory guideline.

No initial pain reaction/pain reaction (IPR/PR) was observed. Conjunctival redness (score 1) and discharge (score 1 or 2) were seen in all rabbits at one hour after treatment. The test item remained in the eye sac at the one-hour observation in all animals. At 24, 48 and 72 hours after application no clinical signs and no conjunctival or corneal effects were observed in any animal. Fluorescein staining was negative in all animals during the experiment. All animal control eyes were symptom-free during the study.

Spiropidion (SYN546330) was judged to be minimally irritant to the eye of the rabbit (Váliczkó, 2016).

(d) Dermal sensitization

In a dermal sensitization study conducted following the local lymph node assay, spiropidion (purity 98.4%) was tested in four groups, each of five CBA/Ca (CBA/CaOlaHsd) female mice, with 50 µL (25 µL per ear) of the test item in acetone : olive oil mixture (4 : 1) at concentrations of 10%, 2.5%, 1% and 0.1% w/w. A further group of five animals was treated with the same acetone/olive oil mix alone as a control. Concentrations tested were identified in a preliminary screening test, in which a concentration of 25% had previously been tested and found to be unsuitable due to toxicity (the animal was euthanised due to clinical signs including hunched posture, lethargy, increased respiratory rate, elevated tail and body weight loss).

No mortality or signs of systemic toxicity were observed for the test item-treated animals during the study. No treatment-related effects were observed on body weight. The stimulation indexes were 2.80, 8.47, 10.89 and 18.27 for concentrations of 0.1%, 1.0%, 2.5% and 10% respectively. Therefore, the concentration of test item expected to cause a three-fold increase (EC3 value) in the incorporation of tritium-labelled methyl thymidine (^{3}H-TdR) was calculated to be 0.13%.

It was concluded that spiropidion (SYN546330) is a skin sensitizer in mice (Pooles, 2015).

(e) Phototoxicity

The phototoxicity potential of spiropidion (purity 98.4%) was evaluated in an in vitro 3T3 NRU phototoxicity test, in two independent experiments. The compound was dissolved in dimethyl sulfoxide (DMSO) and diluted in a 1:100 ratio in Earle's balanced salt solution. Cells were treated for one hour at different concentrations (500.00, 158.11, 50.00, 15.81, 5.00, 1.58, 0.50 and 0.158 µg/mL) of the test solution at 37 ± 1°C, and for a further 50 minutes in the absence and in the presence of a noncytotoxic dose of UV-A light (range 315–400 nm). One day after treatment cytotoxicity was analysed by measuring the uptake of neutral red stain and comparing this to the controls. Chlorpromazine was tested concurrently in a full scale phototoxicity test (eight concentrations) on two plates along with the test item. As negative control DMSO was used at 1% in place of the test item.

Under the given conditions, spiropidion showed a cytotoxic effect with and without irradiation in experiments I and II. At the highest test item concentration, relative cell viability in the + UV-A experiment was 0.6% (experiment I) and 1.0% (experiment II) and in the – UV-A experiment 0.1% (experiment I) and 0.5 (experiment II) compared to the untreated controls. The calculated EC_{50} (concentration needed to produce 50% of maximum response) and photo irritation factor (PIF) values were as follows:

Experiment I: EC_{50} –UV-A, 29.64 µg/mL; EC_{50} + UV-A, 18.75 µg/mL; PIF = 1.59

Experiment II: EC_{50} –UV-A, 27.75 µg/mL; EC_{50} + UV-A, 17.55 µg/mL; PIF = 1.58

The controls confirmed the validity of the study. It is concluded that spiropidion showed a cytotoxic effect, but no phototoxic effects (Gehrke, 2018).

2.2 Short-term studies of toxicity

The short-term toxicity of spiropidion was evaluated in mice, rats and dogs.

(a) Oral administration

Mouse

In a dose selection study, spiropidion (SYN546330; purity 98.8%) was administered to four groups (five of each sex per group) of CD-1 mice, approximately seven weeks-old at a dietary concentration of 0, 250, 700 or 2500 ppm (equal to 0, 42.1, 117.4 and 448.6 mg/kg bw per day for males, 0, 45.2, 126.1 and 465.4 mg/kg bw per day for females) for 28 days. Stability of the test article and achieved concentration were evaluated. All animals were observed daily for viability/mortality and clinical signs. Food consumption and body weights were recorded daily during the treatment period. Blood samples were taken at termination of the study for haematology and clinical chemistry analysis. In addition, blood samples were collected on days 2, 9 and 28 to estimate toxicokinetic parameters (C_{max}, T_{max} and $AUC_{(0-t)}$) for spiropidion and metabolite SYN547305. All animals were subjected to detailed

post mortem gross examination (internal and external). The following organs were wighed from all animals: brain, epididymides, adrenals, pituitary gland, prostate, seminal vesicle and coagulating gland, heart, kidneys, liver, thymus, ovaries/testes, spleen and uterus. Histopathological evaluation of all tissues was undertaken for all control and high-dose animals and any gross abnormalities (where appropriate) from low and intermediate dose animals.

Achieved concentrations of the compound in the diet and its homogeneity were evaluated to be acceptable.

Spiropidion was not detected in samples from the control diet.

Two males (day 21 and 28) and two females (day 9 and 22) given 2500 ppm were found dead. The cause of death could not be determined. One female given 2500 ppm (animal 43) was prematurely killed on day 5 due to the following clinical signs: subdued behaviour with hunched posture, rolling gait, eyes partially closed, intermittent tremors, piloerection and slow respiration. There were no findings observed at necropsy of this animal. Microscopic evaluation revealed moderate decreased cellularity in the bone marrow of the sternum and femur. Moderate to marked lymphocytolysis was observed in the thymus, white pulp of the spleen, mandibular and mesenteric lymph nodes. The findings in the lymphoid system were not observed in any of the scheduled kill or animals found dead. Mild necrosis was observed in the cortex of the adrenal gland.

There were no treatment-related clinical signs throughout the treatment period, with the exception of animal 43.

Body weight at 2500 ppm lower (statistically significant) throughout the treatment period, ranging from 10% to 18% in both sexes. In the same animals, body weight gain (loss) was significantly lower throughout the treatment period with a body weight loss of 8% and 7% in males and females, respectively. Body weight and body weight gain were lower throughout the treatment period in males at 250 and 700 ppm however, with the exception of the first few days of treatment, changes were of small (within 10% of the control), therefore were not considered toxicologically relevant. Body weight and cumulative body weight change in females receiving 250 or 700 ppm were considered to be similar to those of the controls.

Food consumption was generally lower in animals receiving 2500 ppm up to day 14 (males) and day 17 (females). Lower food consumption was also noted in animals receiving 250 and 700 ppm, however due to the small magnitude of the changes, these findings were considered not to be toxicologically relevant.

Haematological investigation did not reveal any treatment-related effects.

Due to mortality and insufficient samples for analysis in both sexes, clinical biochemistry evaluation of the high-dose group was performed on a limited number of samples (two animals per group). For electrolytes only a few samples (from one or two animals) were obtained for all dose groups including controls. Higher levels of alanine transaminase (ALT) and total bilirubin (statistically significant) were reported in females at 2500 ppm. Phosphate concentration was higher in females at 700 ppm, however, there were insufficient samples for analysis at the high dose level. However since no treatment-related changes of inorganic phosphate were observed in the 90-day mouse study up to 1500 ppm, the reported increase in the present study was deemed likely to be incidental. Changes in other parameters were reported, but not with statistical significance: an apparent dose-related decrease in creatine phosphokinase in males at 700 and 2500 ppm, a decreased glucose level in males and females at 2500 ppm, an increase in glycerides in males and females at 2500 ppm (with an unclear dose response in females), and a decrease of cholesterol in females at 2500 ppm. It was noted that for creatine phosphokinase, glucose, glycerides and cholesterol, the results were obtained from two animals per dose group, and each individual value is within, or slightly outside, their concurrent control range. However, given the low number of samples analysed, a quantitative evaluation of the above parameters was not possible. Overall, considering the lack of histopathological correlates and that no alteration of these parameters was observed in the 90-day mouse study up to a dose level of 1500 ppm (Shearer, 2018b), the toxicological significance of these findings was considered doubtful.

A less than statistically significant change in the following organ weights was reported; decrease of absolute and relative kidney weights of 18% and 10% of the control, respectively, were reported

in females at 2500 ppm. Given the small size of the effect and absence of histopathological correlates kidney weight changes were not considered to be toxicologically relevant. Decreased absolute and relative uterine weights at 700 ppm and 2500 ppm were reported. The decrease at 700 ppm was 12% and 19% compared with controls for the absolute and relative weights respectively, and at 2500 ppm the decrease was 27% and 21% compared with controls for the absolute and relative weight respectively. Results from the 2500 ppm group were obtained from two animals for both of which individual values were within their concurrent control ranges for both absolute and relative weight. Given the lack of histopathological correlates and that no alteration of uterine weights were observed in the 90-day mouse study (Shearer, 2018a), the toxicological significance of these findings was considered doubtful. A decreased absolute brain weight of 10% compared with the control was reported in females at 2500 ppm. Given the absence of histopathological correlates and since no similar effect had been observed in longer duration studies this finding was considered to be incidental to treatment.

Histopathological investigation did not reveal any treatment-related effects.

The bioanalysis evaluation of blood samples showed that spiropidion was not quantifiable in any group at any time point. On day 9 metabolite SYN547305 was quantified in a few male samples and all females control animals, indicating that contamination occurred. Metabolite SYN547305 in control animals was not quantifiable at day 2 or 28. Despite a check of the animal and formulation data, the cause of the contamination could not be determined.

Toxicokinetic evaluation showed that on day 2 the level of metabolite SYN547305 at 2500 ppm was higher in females than in males. This difference was less apparent at 250 and 700 ppm. On day 9 females were shown to have higher levels of the metabolite at all doses, with greater difference at 700 ppm. A similar trend was observed on day 28, with higher levels in females than in males at the low and intermediate doses. However, it is difficult to draw a conclusion on trend since fewer animals were sampled.

Systemic exposure to metabolite SYN547305 was similar in males and females at the low and intermediate doses on day 2. For all other doses across days 2, 9 and 28 mean $AUC_{(0-t)}$ estimates were approximately 1.7- to 3.3-fold greater in females than males, with the exception of a 1.6-fold increase in exposure to metabolite SYN547305 for males compared to females in the high-dose group on day 28.

Table 13. Key findings of the 28 day dietary toxicity study in mice

Parameter	Males				Females			
	0 ppm	**250 ppm**	**700 ppm**	**2500 ppm**	**0 ppm**	**250 ppm**	**700 ppm**	**2500 ppm**
Mortality and weight (± SD)								
Found dead	0	0	0	2	0	0	0	2
Moribund	0	0	0	0	0	0	0	1
Body weight (g)	36.7 ± 2.1	33.8 ± 1.7	35.4 ± 1.7	33.1 ± 1.0*	29.3 ± 1.1	28.6 ± 2.2	29.7 ± 3.0	26.6 ± 3.5
Body weight gain 0–28 days (g)	2.9 ± 1.3	1.5 ± 0.8	1.6 ± 1.5	−0.6 ± 1.4**	2.0 ± 0.8	1.9 ± 1.4	2.0 ± 1.9	−2.4 ± 0.4**
Clinical chemistry (mean ± SD)								
Alanine transaminase (U/L)	59 ± 41 *n* = 5	40 ± 13 *n* = 5	50 ± 14 *n* = 5	30 ± 8 *n* = 2	33 ± 10 *n* = 5	37 ± 7 *n* = 5	38 ± 9 *n* = 5	65 ± 15** *n* = 2
Total bilirubin (µmol/L)	2.0 ± 0.6 *n* = 5	2.1 ± 0.4 *n* = 5	1.9 ± 0.4 *n* = 5	1.7 ± 0.0 *n* = 3	1.7 ± 0.0 *n* = 5	1.7 ± 0.0 *n* = 5	1.7 ± 0.1 *n* = 5	2.2 ± 0.1** *n* = 2
Creatine phosphokinase (U/L)	98 ± 65 *n* = 5	102 ± 119 *n* = 4	55 ± 9 *n* = 2	33 ± 1 *n* = 2	27 ± 2 *n* = 2	58 ± 35 *n* = 2	36 ± 10 *n* = 3	29 ± 4 *n* = 2

Parameter	Males				Females			
	0 ppm	250 ppm	700 ppm	2 500 ppm	0 ppm	250 ppm	700 ppm	2 500 ppm
Glucose (mmol/L)	13.56 ± 3.25 *n* = 5	12.69 ± 0.87 *n* = 5	13,24 ± 1.9 *n* = 4	11.86 ± 2.9 *n* = 2	13.4 ± 1.63 *n* = 3	11.9 ± 0.84 *n* = 4	12.9 ± 0.62 *n* = 3	10.73 ± 1.0 *n* = 2
Triglycerides (mmol/L)	1.51 ± 0.48 *n* = 5	1.53 ± 0.23 *n* = 5	1.56 ± 0.57 *n* = 4	2.14 ± 0.8 *n* = 2	1.39 ± 0.58 *n* = 5	1.06 ± 0.3 *n* = 5	1.52 ± 0.61 *n* = 3	2.06 ± 0.31 *n* = 2
Cholesterol (mmol/L)	3.0 ± 0.6 *n* = 5	3.5 ± 0.6 *n* = 5	2.9 ± 0.3 *n* = 5	3.2 ± 0.5 *n* = 3	2.6 ± 0.3 *n* = 5	2.5 ± 0.5 *n* = 5	2.5 ± 0.5 *n* = 5	2.0 ± 0.1 *n* = 2
Organ weight (mean ± SD)								
Kidney; absolute (g)	0.573 ± 0.082 *n* = 5	0.539 ± 0.071 *n* = 5	0.57 ± 0.088 *n* = 5	0.543 ± 0.070 *n* = 3	0.368 ± 0.032 *n* = 5	0.344 ± 0.022 *n* = 5	0.355 ± 0.039 *n* = 5	0.313 ± 0.028 *n* = 2
Kidney; relative to bw (%)	1.66 ± 0.23 *n* = 5	1.69 ± 0.19 *n* = 5	1.68 ± 0.24 *n* = 5	1.74 ± 0.27 *n* = 3	1.352 ± 0.085 *n* = 5	1.29 ± 0.093 *n* = 5	1.27 ± 0.129 *n* = 5	1.23 ± 0.062 *n* = 2
Uterus; absolute (g)	NA	NA	NA	NA	0.19 ± 0.04 *n* = 5	0.2 ± 0.04 *n* = 5	0.17 ± 0.04 *n* = 5	0.15 ± 0.0 *n* = 2
Uterus; relative to bw (%)	NA	NA	NA	NA	0.71 ± 0.13 *n* = 5	0.76 ± 0.22 *n* = 5	0.6 ± 0.17 *n* = 5	0.59 ± 0.08 *n* = 2
Brain; absolute (g)	0.53 ± 0.02 *n* = 5	0.51 ± 0.02 *n* = 5	0.52 ± 0.02 *n* = 5	0.52 ± 0.01 *n* = 3	0.53 ± 0.0 *n* = 5	0.52 ± 0.03 *n* = 5	0.52 ± 0.03 *n* = 5	0.48 ± 0.01 *n* = 2
Histopathology								
Uterus								
moderate atrophy	-	-	-	-	0	NA	NA	1
Toxicokinetic parameters of metabolite SYN547305 (*n* = 5 mice/sex per group)								
Day 2								
C_{max} (ng/mL)		304	807	1150		453	1020	3870
AUC $_{(0-t)}$		3560	8820	12 700		4660	10 200	41 100
Day 9								
C_{max} (ng/mL)		200	598	1820		460	2010	2640
AUC $_{(0-t)}$		2150	6160	11 300		4530	20 600	24 600
Day 28								
C_{max} (ng/mL)		288	648	1940		545	1050	1360
AUC $_{(0-t)}$		3230	7080	21 900		5650	11 700	13 600

SD: Standard deviation; *n*: Number of animals evaluated; Source: adapted from Shearer, 2018a

-: Insufficient sample for analysis; NA: Not applicable; C_{max}: Maximum concentration

$AUC_{(0-t)}$: The area under the concentration–time curve from the start of dose administration to the time after dosing at which the last quantifiable concentration was observed (ng.h/mL)

* $p < 0.05$, ** $p < 0.01$, statistically significant difference from control group mean (Dunnett's)

The Meeting noted that a number of control blood samples subject to bioanalysis were contaminated with spiropidion-enol metabolite (SYN547305). The Meeting concluded that the contamination did not invalidate the results or interpretation of study′s toxicological findings.

The NOAEL was 700 ppm (equal to 117.4 mg/kg bw per day) based on mortality, reduced body weight, body weight loss and food consumption in both sexes at 2500 ppm (equal to 448.6 mg/kg bw per day) (Shearer, 2018a).

Spiropidion (purity 98.4%) was administered to four groups (ten of each sex per group) of approximately seven-week-old CD-1 mice at a dietary concentration of 0, 250, 700 or 1500 ppm (equal to 0, 35.2, 105 or 236 mg/kg bw per day for males, 0, 44.1, 115 or 252 mg/kg bw per day for females) for 90 days. All animals were checked for viability and observed cageside twice a day. Once a week, animals were removed from their cages and received a detailed clinical examination (appearance, movement and behaviour patterns, skin and hair condition, eyes and mucous membranes, respiration and excreta). Body weights and food consumption were measured and recorded once weekly during the pretrial and dosing periods. Visual inspection of water consumption was evaluated throughout the study. An adequate range of haematological and clinical chemistry parameters were evaluated on samples taken at terminal sacrifice. In addition, blood samples were collected on days 2, 28 and 85 to estimate toxicokinetic parameters (C_{max}, T_{max} and $AUC_{(0-t)}$) and blood estimation of spiropidion and metabolite SYN547305. All surviving animals were euthanised after at least 13 weeks of treatment and subjected to a detailed necropsy examination, with selected organs being weighed, except the thyroid which was microscopically examined. An adequate number of tissues from all animals in the control and high-dose groups were subjected to a comprehensive histopathological evaluation. In addition, gross lesions from the low and intermediate groups were also subjected to histopathological evaluation.

Achieved concentrations of the compound in the diet and its homogeneity were examined and assessed as acceptable.

Spiropidion was not detected in samples of the control diet.

No treatment-related mortality was observed. There were no treatment-related clinical observations.

Body weight and body weight gain were lower in all animals at 1500 ppm, achieving statistical significance at all time points for females (except at week 13) and at two time points for males. Most of the animals lost weight in the first two weeks of treatment and some continued to lose weight thereafter. Body weight was lower in animals receiving 700 ppm and females receiving 250 ppm at the end of treatment, however, statistical significance was not achieved in either group and in females there was no dose–response relationship.

Food utilisation was lower (statistically significant) over the treatment period (weeks 1–13) in males receiving 1500 ppm compared to controls, and over weeks 1–4 in all treated groups except males receiving 250 ppm when compared with controls.

No visual treatment-related effects on water consumption were observed.

Haematological investigation showed a statistically significant decrease (–18%) in platelets in females at 1500 ppm. Considering the magnitude of change, the lack of either a true dose response or histopathological correlates, this effect was considered of no toxicological relevance.

Evaluation of clinical chemistry parameters showed with statistical significance higher urea and blood nitrogen urea (BUN) levels in males at 1500 ppm compared with the controls. Urea and BUN were also higher compared with controls in males receiving 250 and 700 ppm, but the difference did not achieve statistical significance. Alkaline phosphatase (ALP) was to a statistically significant extent higher in females at 1500 ppm than in the controls. Albumin levels and albumin : globulin (A : G) ratio were lower in males at 700 ppm and in both sexes at 1500 ppm when compared with controls, in both cases statistically significant. Considering that all individual albumin values in males at 700 and 1500 ppm were in the range of individual concurrent control values, the changes to albumin levels in males were considered of low toxicological relevance. The small change in the albumin level of females at 1500 ppm was considered of low toxicological importance. A marginal decrease in total proteins was

observed in males and females at 1500 ppm (–4%), achieving statistical significance only in females. Total bilirubin was found to decrease in males and increase in females at 700 and 1500 ppm, achieving statistical significance only in females at the mid dose. It was noted that the direction of total bilirubin changes was inconsistent between sexes; there was no true dose response, statistical significance was only at the mid dose in females, and no treatment-related bilirubin changes were reported in other species.

A statistically significant increase in covariate liver weight was observed in males at 1500 ppm, as were statistically significant increases in covariate prostate weight in males at 250 and 1500 ppm. Due to the absence of a dose–effect relationship and a lack of histopathological correlates, this finding was considered incidental to treatment. Statistically significant decreases in absolute and covariate kidney and heart weights were observed in females at 1500 ppm. Given the small nature if this change and its lack of histopathological correlates the finding was considered not toxicologically relevant.

No treatment-related gross or histopathological findings were noted.

Bioanalytical analysis showed that spiropidion was present in quantifiable levels in five male (5.17 ng/mL) and seven female (371 ng/mL) control animals.

Quantifiable levels of metabolite SYN547305 were also present in three male (6.51 ng/mL) and one female (50.1 ng/mL) control animals. With the exception of one female, all samples were below 10% of the mean concentration found in the low-dose group. Despite a check of the animal and bioanalytical data the cause of the contamination could not be determined. As only 33/480 samples showed spiropidion or its metabolite (SYN547305) in concentrations marginally above the LOQ, the finding was deemed not to have an impact on the toxicokinetic results or their interpretation, and ultimately on the integrity of the study.

Toxicokinetic analysis showed that spiropidion was not quantifiable in the majority of the samples, particularly at the low doses. Systemic exposure to metabolite SYN547305 increased subproportionally across the dose ranges in both sexes. Following repeat administration, exposure estimates were generally comparable between sample days. There were no appreciable differences in systemic exposure to SYN547305 (CSCD710959) between sexes. Where some degree of difference was noted however, exposure was greater in females than in males.

Table 14. Key findings of the 90-day dietary toxicity study in mice

Parameters	Males				Females			
	0 control	250 ppm	700 ppm	1500 ppm	0 control	250 ppm	700 ppm	1500 ppm
Body weight (g) ± SD								
Week								
0	32.8 ± 1.2	33.3 ± 1.7	33.3 ± 2.2	33.3 ± 1.8	26.3 ± 1.5	26.6 ± 1.7	27.9 ± 2.4	26.3 ± 2.2
1	33.2 ± 1.4	33.4 ± 2.1	33.0 ± 2.4	32.1 ± 2.1	27.5 ± 1.5	26.8 ± 1.5	28.1 ± 2.0	25.6 ± 1.9*
2	34.2 ± 1.4	34.5 ± 2.3	33.5 ± 2.6	32.5 ± 2.1	29.0 ± 2.5	27.8 ± 2.2	29.0 ± 2.1	26.2 ± 1.9*
4	35.0 ± 1.4	35.0 ± 2.9	34.2 ± 2.9	32.8 ± 2.2	29.5 ± 2.6	27.9 ± 1.9	29.3 ± 1.8	26.5 ± 1.7**
5	36.1 ± 1.6	35.2 ± 2.9	33.9 ± 2.6	33.2 ± 1.6*	30.4 ± 2.7	28.5 ± 2.5	29.9 ± 2.0	27.1 ± 1.9*
6	36.4 ± 1.5	36.2 ± 3.2	34.4 ± 3.0	33.4 ± 1.9*	31.4 ± 3.6	28.9 ± 2.5	30.2 ± 2.0	27.3 ± 1.9**
9	38.7 ± 2.2	39.3 ± 4.6	37.7 ± 3.8	35.1 ± 1.7	33.7 ± 4.3	30.8 ± 2.7	32.3 ± 3.0	28.6 ± 1.9**
13	39.3 ± 2.1	39.8 ± 4.9	38.5 ± 3.5	36.4 ± 1.9	33.6 ± 4.8	31.8 ± 3.1	33.9 ± 3.0	29.8 ± 2.3

Parameters	Males				Females			
	0 control	250 ppm	700 ppm	1500 ppm	0 control	250 ppm	700 ppm	1500 ppm
Body weight gain (g) ± SD								
0–13 weeks	6.6 ± 2.1	6.5 ± 3.5	5.3 ± 2.5	3.1 ± 2.1*	7.3 ± 3.9	5.0 ± 3.2	6.0 ± 1.9	3.6 ± 1.4*
% difference		*−2*	*−20*	*−53*		*−32*	*−18*	*−51*
Food utilization (g body weight change / g food consumed) ± SD								
1–13 week	1.4 ± 0.3	1.4 ± 0.6	1.1 ± 0.5	0.6 ± 0.4**	1.6 ± 0.7	1.1 ± 0.5	1.3 ± 0.4	0.9 ± 0.2
% difference 1–13		*0*	*−21*	*−57*		*−31*	*−19*	*−44*
Haematology (mean ± SD)								
Platelet ($\times 10^{-9}$/L)	1371 ± 158	1225 ± 145	1227 ± 135	1214 ± 107	1315 ± 148	1192 ± 169	1252 ± 152	1078 ± 140**
Clinical biochemistry								
Urea (mmol/L)	6.4 ± 0.7	7.4 ± 1.1	7.6 ± 1.2	8.4 ± 1.6**	7.1 ± 1.8	7.6 ± 1.9	6.3 ± 1.1	6.8 ± 1.3
% difference		*+16*	*+19*	*+31*		*+7*	*−11*	*−4*
Blood urea nitrogen (mg/dL)	17.9 ± 1.9	20.6 ± 3.0	21.4 ± 3.5	23.5 ± 4.4**	19.9 ± 4.9	21.3 ± 5.4	17.7 ± 3.3	18.8 ± 3.6
% difference		*+15*	*+20*	*+31*		*+7*	*−11*	*−6*
Alkaline phosphatase (U/L)	57 ± 13	47 ± 9	53 ± 16	64 ± 15	56 ± 11	55 ± 12	64 ± 18	82 ± 23**
% difference		*−18*	*−25*	*+12*		*−2*	*+14*	*+46*
Albumin (g/L)	34 ± 3	32 ± 1	32 ± 1*	31 ± 2**	37 ± 1	36 ± 2	36 ± 1	34 ± 2**
% difference		*−6*	*−6*	*−9*		*−3*	*−3*	*−8*
A/G ratio	2.0 ± 0.6	1.7 ± 0.1	1.6 ± 0.1*	1.6 ± 0.1*	2.4 ± 0.2	2.2 ± 0.3	2.3 ± 0.3	2.1 ± 0.2*
% difference		*−15*	*−20*	*−20*		*−8*	*−4*	*−13*
Total protein (g/L)	52 ± 2	51 ± 2	51 ± 1	50 ± 3	52 ± 2	52 ± 2	52 ± 1	50 ± 2*
% difference		*−2*	*−2*	*−4*		*0*	*0*	*−4*
Total bilirubin (µmol/L)	2.3 ± 0.4	2.2 ± 1.0	1.8 ± 0.2	1.8 ± 0.2	1.9 ± 0.2	2.2 ± 0.3	2.3 ± 0.6*	2.3 ± 0.4
% difference		*−4*	*−22*	*−22*		*+16*	*+21*	*+21*
Organ weight (mean ± SD)								
Liver (g)								
Absolute	1.83 ± 0.2	1.97 ± 0.2	1.94 ± 0.1	2.02 ± 0.2	1.68 ± 0.29	1.59 ± 0.12	1.63 ± 0.18	1.47 ± 0.22
% difference		*+8*	*+6*	*+10*		*−5*	*−3*	*−13*
Covariance with body weight	1.80 ± 0.05	1.93 ± 0.05	1.94 ± 0.05	2.08 ± 0.05**	1.61 ± 0.05	1.61 ± 0.05	1.57 ± 0.05	1.61 ± 0.05
% difference		*+7*	*+8*	*+16*		*0*	*−3*	*0*
Relative to body weight (%)	4.90 ± 0.6	5.22 ± 0.4	5.38 ± 0.5	5.88 ± 0.5	5.26 ± 0.5	5.33 ± 0.6	5.17 ± 0.5	5.31 ± 0.4
% difference		*+6*	*+10*	*+20*		*+1*	*−2*	*+1*

Parameters	Males				Females			
	0 control	250 ppm	700 ppm	1500 ppm	0 control	250 ppm	700 ppm	1500 ppm
Kidney (g)								
Absolute	0.573 ± 0.06	0.554 ± 0.09	0.579 ± 0.06	0.566 ± 0.07	0.403 ± 0.05	0.369 ± 0.03	0.366 ± 0.04	0.324 ± 0.04*
% diff		*+5*	*+1*	*−1*		*−8*	*−9*	*−20*
Covariance with body weight	0.561 ± 0.017	0.535 ± 0.018	0.582 ± 0.017	0.593 ± 0.018	0.391 ± 0.01	0.371 ± 0.01	0.356 ± 0.01	0.344 ± 0.01*
% diff		*−5*	*+4*	*+6*		*−5*	*−9*	*−12*
Relative to body weight (%)	1.54 ± 0.14	1.46 ± 0.11	1.60 ± 0.14	1.65 ± 0.20	1.27 ± 0.11	1.24 ± 0.17	1.16 ± 0.09	1.17 ± 0.11
% diff		*−5*	*+4*	*+8*		*−2*	*−9*	*−7*
Heart (g)								
Absolute	0.22 ± 0.03	0.20 ± 0.04	0.20 ± 0.03	0.19 ± 0.02	0.18 ± 0.03	0.16 ± 0.01	0.17 ± 0.02	0.15 ± 0.02*
% diff		*−9*	*−9*	*−14*		*−11*	*−6*	*−17*
Covariance with body weight	0.22 ± 0.01	0.20 ± 0.01	0.20 ± 0.01	0.20 ± 0.01	0.18 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.15 ± 0.01
% diff		*−9*	*−9*	*−9*		*−11*	*−11*	*−17*
Relative to body weight (%)	0.589 ± 0.09	0.536 ± 0.07	0.564 ± 0.08	0.559 ± 0.07	0.568 ± 0.14	0.541 ± 0.03	0.529 ± 0.05	0.544 ± 0.04
% diff		*−9*	*−4*	*−5*		*−5*	*−7*	*−4*
Toxicokinetic parameters of metabolite SYN547305 (*n* = 10 mice/sex per group)								
Day 2								
C_{max}		494	922	1510		765	2310	3260
AUC $_{(0-t)}$		5520	9670	13 600		7810	20 100	29 400
Day 28								
C_{max}		355	792	1740		702	2330	3220
AUC $_{(0-t)}$		3670	8100	16 600		7780	21 900	30 600
Day 85								
C_{max}		303	1070	1970		461	1560	2460
AUC $_{(0-t)}$		3500	10 900	20 700		4600	15 700	22 900

SD: Standard deviation; C_{max}: Maximum concentration Source: adapted from Shearer, 2018b

$AUC_{(0-t)}$: The area under the concentration–time curve from the start of dose administration to the time after dosing at which the last quantifiable concentration was observed (ng.h/mL)

* $p < 0.05$, ** $p < 0.01$, statistically significant difference from control group mean (Dunnett's)

The Meeting noted that a number of control blood samples subject to bioanalysis were contaminated with either spiropidion or spiropidion-enol metabolite (SYN547305). Nevertheless the Meeting concluded that the contamination did not invalidate the results or interpretation of study′s toxicological findings.

The NOAEL was 700 ppm (equal to 105 mg/kg bw per day), based on reduced body weights, food utilization, changes in clinical biochemistry parameters in both sexes at 1500 ppm (equal to 236 mg/kg bw per day) (Shearer, 2018b*)*.

Rat

Study 1

Spiropidion (purity 98.8%) was administered for 28 days to four groups five animals/sex per group of approximately seven-week-old Han Wistar (Crl:WI(Han)) rats at a dietary concentration of 0, 500, 2000 or 3000 ppm (equal to 0, 44, 177 and 259 mg/kg bw per day in males, 0, 44, 178 and 264 mg/kg bw per day in females) . All animals were checked twice each day for viability. Once each week, starting pretrial, all animals received a detailed clinical examination which included: appearance, movement and behaviour patterns, skin and hair condition, eyes and mucous membranes, respiration and excreta. Body weight and food consumption were recorded twice during the pretrial period and daily during the treatment period. Visual inspection of water consumption was carried out throughout the study. Ophthalmoscopic examination was conducted once during the pretrial period and all control and high-dose animals were examined during week 4 of treatment. All animals received a detailed functional observation battery (FOB) assessment (including motor activity) during week 4 of treatment. An adequate number of haematological, coagulation and clinical chemistry parameters were evaluated at terminal sacrifice. Additional blood samples were collected on days 2, 9 and 28 from all animals to be used for whole blood bioanalysis and toxicokinetic evaluation (C_{max} and $AUC_{(0-t)}$) for spiropidion and metabolite SYN547305. All surviving animals were euthanised after 28 days of treatment and subjected to a detailed necropsy examination. The following organs were weighed: brain, epididymides, adrenals, pituitary, prostate, seminal vesicle and coagulating gland, heart, kidneys, liver, thymus, ovaries/testes, spleen and uterus). An adequate number of tissues were sampled for microscopic examination. Histopathology was conducted on all control and high-dose group animal tissues. Additionally, all gross lesions, the thyroid gland from both sexes and the Harderian gland (from females only) were processed from low and intermediate dose animals.

Achieved concentrations of the compound in the diet and its homogeneity were assessed to be acceptable.

Spiropidion was not detected in samples of the control diet.

No treatment-related mortality or clinical observations were noted.

Body weight and body weight gain were lower (statistically significant) in both sexes at 3000 ppm. An initial body weight loss up to day 3 in males and day 5 in females was recorded at 3000 ppm. In females at 2000 ppm a non-statistically significant reduction of body weight was recorded throughout the treatment period with values within 10% of the control. Body weight gain was significantly reduced in females at 2000 ppm. Food consumption at 3000 ppm was lower throughout the treatment period in males and lower in females up to day 11.

No treatment-related clinical or quantitative functional observations were noted. In males at 3000 ppm an increase of movements was noted throughout the recording period, occasionally achieving statistical significance, and this also resulted in a larger number of movements overall. However, it was noted that the number of movements fell to a similar level as the control at the end of the recording period and that the values were within the historical control range. It was suggested that these animals required a longer time for adaptation. In the absence of any other corroborating findings the large number of movements in males during the motor activity was considered incidental.

Haematological investigation revealed a slight but statistically significant decrease in mean cell volume (MCV) in males at 3000 ppm. Given the small size of change and a lack of changes in correlated parameters, the toxicological significance of this finding was considered equivocal. An increase in white blood cells (WBC) and lymphocytes was reported at all dose levels in females, achieving statistical significance at 2000 ppm and 3000 ppm, but with no true dose–response relationship. A slight but statistically significant increase in basophils was reported in females at 3000 ppm. Given the small size of change the increase in basophils was considered of no toxicological significance. Other statistically significant changes included an increase of prothrombin time (PT) in males at 3000 ppm, and a decrease in activated partial thromboplastin time (APTT) in males at 500 ppm and 3000 ppm, but this latter effect showed no dose–response relationship. An increase in platelets was reported in females at all dose levels, achieving statistical significance at 3000 ppm.

A dose-related increase in ALT was observed in all dosed groups, with statistical significance reached in both sexes at 3000 ppm and in females at 2000 ppm. The magnitude of change in males and

females at 500 ppm and in males at 2000 ppm was not considered toxicologically relevant. Alkaline phosphatase was higher than controls in males at all dose levels, achieving statistical significance at 3000 ppm. Changes at 500 and 2000 ppm were considered small and therefore of no toxicological relevance. Creatine phosphokinase (CPK) was higher in females at 2000 and 3000 ppm than in controls, with statistical significance reached at 3000 ppm. The size of change at 2000 ppm was not considered toxicologically significant. A statistically significant decrease in total proteins in both sexes at 3000 ppm and in females at 2000 ppm, and of albumin in both sexes at 2000 ppm and 3000 ppm were recorded. No clear dose–response relationship was observed for albumin in females. A statistically significant increase in glucose was reported for females at 3000 ppm. In females at 3000 ppm a significant increase of creatine was also reported, but with no dose–response relationship. Changes in total proteins, albumin, glucose and creatinine were not considered toxicologically significant. Cholesterol and triglyceride levels were lower in all groups than in controls, with statistical significance achieved at 2000 and 3000 ppm and at 500 ppm in males (cholesterol only) and females (triglycerides only).

Absolute and covariate prostate weights were lower in males (statistically significant) at 3000 ppm compared with controls. There were no other treatment-related organ weight changes.

No treatment-related gross findings were noted. Test substance-related histopathological findings consisted of increased incidence of diffuse, minimal to mild degeneration/atrophy of the Harderian gland in females at 2000 ppm and 3000 ppm, and a statistically significant increase in minimal diffuse thyroid follicular cell hypertrophy in both sexes at 2000 ppm and 3000 ppm.

Other microscopic findings were recorded in the lung in females (focal polymorphonuclear leukocytic perivascular infiltration), and in the kidneys in males (focal mononuclear cell infiltration, basophilic tubules and cysts), both at the highest dose tested. The nature of the latter findings represents a commonly observed feature in this strain and age of rat, and were of similar incidence and severity in control and treated animals; therefore they were considered unrelated to treatment.

Bioanalysis showed that spiropidion was below the LOQ in all samples analysed. Metabolite SYN547305 was quantifiable (5.44–16.5 ng/mL) at one time point in day 9 samples from two control females and at three time points on day 28 for another female. No explanation for this contamination was provided in the study report. Considering that only 5/540 control samples showed metabolite SYN547305 concentrations marginally above the LOQ (5 ng/mL) and that all samples were below 1.2% of the mean concentration of the low-dose group (500 ppm), this finding was judged not to have a significant impact on the toxicokinetics results or their interpretation, nor on the overall integrity of the study.

The absence of spiropidion in samples from all substance-treated groups indicated a complete metabolization of the parent compound. Systemic exposure to metabolite SYN547305 increased supraproportionally in males and subproportionally in females following continuous dietary administration on day 2. Systemic exposure to metabolite SYN547305 following repeated administration of spiropidion was generally comparable between day 2 and day 9, except in the high-dose males and intermediate and high-dose females where systemic exposure estimates were up to two-fold greater on day 9 than on day 2. With further dosing there was no appreciable difference between the estimated mean $AUC_{(0-t)}$ for day 28 when compared to that for day 9. There were no clear and consistent differences in exposure between sexes. Where slight differences were noted, mean $AUC_{(0-t)}$ estimates were approximately two-fold greater in males of the intermediate group on day 2, but between 1.5-fold and 1.7-fold greater in females than males in the low-dose groups on days 9 and 28. Overall, there were no meaningful differences in mean $AUC_{(0-t)}$ estimates between the sexes.

Table 15. Key findings of the 28-day dietary toxicity study in rat

	Males				Females			
	0 Control	**500 ppm**	**2000 ppm**	**3000 ppm**	**0 Control**	**500 ppm**	**2000 ppm**	**3000 ppm**
Body weight (g) ± SD	293 ± 13	291 ± 16	284 ± 26	251 ± 38*	183 ± 11	184 ± 15	168 ± 10	162 ± 12*
Bw gain (g) 0–28 days ± SD	115 ± 10	111 ± 13	103 ± 19	81 ± 30*	55 ± 9	59 ± 10	40 ± 4*	32 ± 9**

	Males				Females			
	0 Control	500 ppm	2000 ppm	3000 ppm	0 Control	500 ppm	2000 ppm	3000 ppm
Haematology								
MCV (fL)	57.4	55.4	56.5	54.3	57.0	57.0	54.7	54.9
± SD	± 1.7	± 0.4	± 1.6	± *2.1	± 1.5	± 2.5	± 1.3	± 1.2
% difference		*−3*	*−2*	*−5*		*0*	*−4*	*−4*
WBC (10^9/L)	8.7	8.3	9.4	9.8	4.5	6.3	9.1	8.3
± SD	± 1.2	± 2.3	± 1.0	± 1.9	± 2.2	± 2.1	± 2.3**	± 0.7*
% difference		*−5*	*+8*	*+13*		*+40*	*+102*	*+84*
Lymphocytes	7.4	7.2	8.5	8.2	3.6	5.5	8.0	7.1
(10^9/L) ± SD	± 0.8	± 2.2	± 1.0	± 1.7	± 2.0	± 1.9	± 2.2**	± 0.7*
% difference		*−3*	*+15*	*+11*		*+53*	*+122*	*+97*
Basophils (10^9/L)	0.05	0.03	0.04	0.05	0.02	0.02	0.03	0.04
± SD	± 0.01	± 0.01	± 0.02	± 0.02	± 0.02	± 0.01	± 0.02	± 0.01*
% difference		*−40*	*−20*	*0*		*0*	*+50*	*+100*
PT (s)	24.0	23.4	24.1	25.7	24.3	24.3	24.7	23.5
± SD	± 0.8	± 0.9	± 0.6	± 1.3*	± 1.0	± 2.0	± 2.2	± 0.7
% difference		*−3*	*0*	*+7*		*0*	*+2*	*−3*
APTT (s)	16.8	15.2	16.1	15.1	15.0	15.4	13.4	13.2
± SD	± 0.9	± 0.7*	± 0.7	± 0.8*	± 0.9	± 0.6	± 1.5	± 1.2
% difference		*−10*	*−4*	*−10*		*+3*	*−11*	*−12*
Platelets (10^9/L)	720	750	619	688	653	829	851	881
± SD	± 66	± 59	± 279	± 131	± 181	± 84	± 119	± 93*
% difference		*+4*	*−14*	*−4*		*+27*	*+30*	*+35*
Clinical biochemistry								
ALP (U/L)	130	145	154	203	83	73	67	80
± SD	± 14	± 27	± 39	± 26**	± 8	± 10	± 23	± 25
% difference		*+12*	*+18*	*+56*		*−12*	*−19*	*−4*
ALT (U/L)	40	45	55	69	34	42	78	83
± SD	± 6	± 7	± 12	± 17**	± 8	± 14	± 15**	± 24**
% difference		*+13*	*+38*	*+73*		*+24*	*+129*	*+144*
CPK (U/L)	412	314	833	473	234	261	321	411
± SD	± 307	± 126	± 894	± 410	± 61	± 117	± 61	± 89*
% difference		*−24*	*+102*	*+15*		*+12*	*+37*	*+76*
Total protein (g/L)	59	56	55	53	59	57	54	55
± SD	± 2	± 1	± 3	± 3**	± 2	± 1	± 3**	± 1**
% difference		*−5*	*−7*	*−10*		*−3*	*−8*	*−7*
Albumin (g/L)	42	39	38	38	45	42	39	41
± SD	± 1	± 2	± 2**	± 2**	± 1	± 2	± 2**	± 0**
% difference		*−7*	*−10*	*−10*		*−7*	*−13*	*−9*
Cholesterol								
(mmol/L)	1.6	1.2	1.0	0.9	1.4	0.9	0.8	0.7
± SD	± 0.2	± 0.4*	± 0.3**	± 0.2*	± 0.2	± 0.2	± 0.0*	± 0.2**
% difference		*−25*	*−38*	*−44*		*−36*	*−43*	*−50*
Triglycerides	1.85	1.45	1.13	0.92	1.37	0.62	0.72	0.73
(mmol/L) ± SD	± 0.7	± 0.3	± 0.3*	± 0.3**	± 0.5	± 0.2**	± 0.3*	± 0.1*
% difference		*−22*	*−39*	*−50*		*−55*	*−47*	*−47*

	Males				Females			
	0 Control	**500 ppm**	**2000 ppm**	**3000 ppm**	**0 Control**	**500 ppm**	**2000 ppm**	**3000 ppm**
Glucose (mmol/L) ± SD	12.4 ± 2.1	11 ± 1.4	11.9 ± 2.5	10.5 ± 2.2	10.0 ± 0.5	9.3 ± 0.8	9.6 ± 0.7	8.7 ± 0.9*
% difference		*−11*	*−4*	*−15*		*−8*	*−4*	*−13*
Creatine (µmol/L) ± SD	26 ± 3	27 ± 3	28 ± 2	30 ± 2	26 ± 3	29 ± 3	27 ± 2	30 ± 2*
% difference		*+4*	*+8*	*+15*		*+12*	*+4*	*+15*
Organ weight (g) ± SD								
Prostate								
Absolute	1.35 ± 0.08	1.29 ± 0.20	1.29 ± 0.17	1.05 ± 0.08*	NA	NA	NA	NA
% difference		*–5*	*–4*	*–22*	NA	NA	NA	NA
Covariance with body weight	1.36 ± 0.07	1.29 ± 0.07	1.30 ± 0.07	1.04 ± 0.08*	NA	NA	NA	NA
% difference		*−5*	*−5*	*−23*	NA	NA	NA	NA
Histopathology								
Harderian gland								
Number examined	5	0	0	5	5	5	5	5
Degeneration/ atrophy, diffuse, minimal/mild	0	-	-	0	0	0	1	2
Increased mitosis, minimal	0	-	-	0	0	0	0	1
Thyroid								
Number examined	5	5	5	5	5	5	5	5
Follicular cell hypertrophy, diffuse minimal	0	0	4*	4*	0	0	4*	4*
Toxicokinetic parameters of metabolite SYN547305 (*n* = 5 mice/sex per group)								
Day 2								
C_{max}		1290	6280	13 200		1580	4090	7760
AUC $_{(0-t)}$		14 600	72 500	108 000		17 400	34 000	81 900
Day 9								
C_{max}		1100	7280	14 400		1340	6560	12000
AUC $_{(0-t)}$		11 800	81 200	177 000		17 700	71 300	141 000
Day 28								
C_{max}		1120	5920	10 900		2070	6500	12700
AUC $_{(0-t)}$		12 700	67 800	133 000		22 100	63 800	126 000

NA: not applicable; SD: Standard deviation PT: Prothrombin time; Source: Adapted from Shearer, 2014

MCV: Mean corpuscular volume; WBC: White blood cells; APTT: Avtivated partial thromboplastin time;

ALP: Alkaline phosphatase; ALT: Alanine transaminase; CPK: Creatine phosphokinase

C_{max}: Maximum concentration;

$AUC_{(0-t)}$: The area under the concentration–time curve from the start of dose administration to the time after dosing at which the last quantifiable concentration was observed (ng.h/mL)

* $p < 0.05$, ** $p < 0.01$, statistically significant difference from control group mean (Dunnett's)

The Meeting noted that a number of the control blood samples subject to bioanalysis were contaminated with spiropidion-enol metabolite (SYN547305). Nevertheless The Meeting concluded that the contamination did not invalidate the results or interpretation of study′s toxicological findings.

Due to decreased cholesterol in males and triglycerides females at the lowest dose tested, no NOAEL could be identified. The LOAEL of the study was 500 ppm (equal to 44 mg/kg bw per day) (Shearer, 2014).

Study 2

Spiropidion (SYN546330; purity 98.4%) was administered for 90 daysto approximately seven-week-old Han Wistar rats (Crl:WI(Han), four groups of 10/sex per group at a dietary concentration of 0, 100, 500, 1500 (females) or 2500 ppm (males) (equal to 0, 6.2, 31.5 and 159 mg/kg bw per day in males, 0, 7.0, 36.1 and 110 mg/kg bw per day in females). All animals were checked twice each day for viability. Once each week starting pretrial, all animals received a detailed clinical examination (appearance, movement and behaviour patterns, skin and hair condition, eyes and mucous membranes, respiration and excreta). Body weight was measured twice weekly during pretrial and weekly during the treatment period. Food consumption was measured once a week during pretrial and treatment period. An visual inspection of water consumption was performed throughout the study. Ophthalmoscopic examination was conducted once during the pretrial period and all control and high-dose animals were examined during week 13 of treatment. All animals received a detailed FOB assessment (including motor activity) during week 12 of treatment. An adequate number of haematological, coagulation, clinical chemistry and urine parameters were evaluated. Additional blood samples were collected on days 2, and 91 from all animals, to be used for whole blood bioanalysis and toxicokinetic evaluation (C_{max} and $AUC_{(0-t)}$) for spiropidion and metabolite SYN547305. All surviving animals were euthanized after 90 days of treatment and subjected to a detailed necropsy examination. The following organs were weighed: brain, epididymides, adrenals, pituitary, prostate, heart, kidneys, liver, thymus, ovaries/testes, spleen and uterus/cervix, thyroid with parathyroid. An adequate number of tissues was sampled for microscopic examination. Histopathology was conducted on all tissues collected from control and high-dose animals. Additionally, all gross lesions and the thyroid gland from both sexes were processed from intermediate and high-dose animals.

Achieved concentrations of the compound in the diet and its homogeneity were assessed to be acceptable.

Spiropidion was not detected in samples taken from the control diet.

No treatment-related mortality or clinical signs of toxicity were observed.

Statistically significant treatment-related decreases in body weight gain (up to –20%) and body weight (up to –9%) were noted in males at 2500 ppm and females at 1500 ppm (up to –8%) over the duration of treatment. In males at 500 ppm, a treatment-related decrease in body weight was reported (approximately –8% from control), achieving statistical significance from days 7–77 in males. These lower mean body weights were considered to be a reflection of the lower initial mean body weight for the group (215 g compared with 224 g for control on day 0), and of a slight effect on body weight gain (consistently ca 11% lower than for controls) achieving statistical significance in the interval 0–77 days. In females at 500 ppm no significant effect on body weight was observed. Lower body weight gains, achieving statistical significance, were noted in both sexes in the intervals 0–28, 0–70 and 0–77 days. As in males at 500 ppm, the slight effect of treatment on body weight gain (approximately 12% lower than control values) in the absence of mean body weight changes was considered not to be of toxicological significance. A statistically significant decrease in food consumption was noted in males at 2500 ppm and females at 1500 ppm over the first four weeks of treatment, but consumption returned to levels comparable with the controls thereafter until study termination. Animals at 100 ppm and 500 ppm showed food consumption values similar to controls except for weeks 1 and 2 of treatment. Food utilization was significantly depressed in the first four weeks for both sexes at the high dose, which in females at 1500 ppm resulted in a statistically significant food utilization decrease over the whole exposure period.

No treatment-related effects on water consumption, ophthalmoscopic examination or motor activity were observed. A statistically significant decrease in foot splay was reported for males at 2500 ppm and females at 1500 ppm when compared to controls. Since values were similar to those during pretreatment these changes were considered incidental and of no toxicological significance. Males at

2500 ppm showed a statistically significant decrease in hindleg grip strength. Given the high variability of this parameter (historical control data mean: 641; range, 231–1316) and the lack of evidence from other parameters, this change was considered to be of no toxicological significance.

The white blood cell count, neutrophils, lymphocytes and basophils were significantly higher in males at 2500 ppm when compared with controls. Due to its limited magnitude basophils change was considered to be of low toxicological importance. A statistically significant increase in prothrombin time (PT) was reported in males at 2500 ppm. Considering that the change in PT at 500 ppm was similar to that observed at 2500 ppm, and that there were no changes in the rat chronic study up to 500 ppm, these changes were considered unlikely to be treatment-related and of low toxicological relevance.

A statistically significant decrease in ALP was reported for females at all dose levels, with no clear dose–response relationship. A statistically significant increase in ALT was reported in males at 2500 ppm and in females at 500 ppm and 1500 ppm. A statistically significant increase in AST was seen in males at 2500 ppm. It was noted that changes of ALP in females at all dose levels, and of ALT in females at 500 ppm, were within normal biological variation; these were therefore considered not to be adverse. A dose-related decrease in cholesterol was reported in both sexes, achieving statistical significance at the mid and high dose where reductions by ca 30% and 50%, respectively were seen in both sexes. A statistically significant and dose-related decrease in triglycerides of 60% was reported in males at 2500 ppm, and of approximately 50% in females at 500 ppm and 1500 ppm. Total proteins and albumin were decreased to a statistically significant extent in males at 500 ppm and 2500 ppm and in females at 1500 ppm. However, the limited reductions in total protein and albumin were considered, in themselves, to be of doubtful toxicological significance. A statistically significant and dose-related increase in inorganic phosphate was reported in males at all dose levels. Although changes appear to be treatment-related, the individual values in all dosed groups were well within the historical control data (HCD) of the laboratory (mean: 1.44,range: 0.94–2.25). It was noted that no changes in inorganic phosphate were observed in the chronic/carcinogenicity study on rats in either sex given spiropidion at doses up to 500 ppm for 53 weeks. In the absence of histopathological correlates the toxicological significance of inorganic phosphate values is doubtful. A statistically significant decrease in calcium was reported in females at 1500 ppm. Given the small size of the change and its lack of histopathological correlates calcium changes were considered to be of no toxicological significance.

Absolute and covariate adrenal gland weights were lower (statistically significant) by approximately 15% in males at 500 and 2500 ppm. A statistically significant absolute and covariate spleen weight when compared with controls by approximately 15% was reported for females at 500 or 1500 ppm. Since no changes in adrenal or spleen weights nor correlated gross or histopathological findings were observed in the chronic/carcinogenicity rat study, at doses up to 500 ppm, the reported changes were considered of low toxicological relevance. Absolute kidney weight was lower in males (statistically significant) at 2500 ppm when compared with controls. However this was not evident after covariate analysis and was therefore considered to be incidental. No gross or histopathological correlates were reported for adrenals, spleen or kidney.

No test substance-related gross findings were noted.

Treatment-related findings from microscopy consisted of minimal to mild diffuse follicular cell hypertrophy and colloid contraction in the thyroid glands of males at 2500 ppm and females at 1500 ppm.

Other microscopic findings were recorded in the lung (minimal to mild inflammatory cell infiltration) and in the mandibular lymph node (minimal to mild erythrocytosis/erythrophagocytosis) in both sexes at the high dose. The latter findings are commonly observed in this strain and age of rat, and/or they were found with similar incidence and severity in control and treated animals; they were therefore considered unrelated to treatment.

Bioanalysis showed that spiropidion (SYN546330) was present in quantifiable levels (17.7–67.2 ng/mL) in three female controls at one timepoint on day 2. All other control samples were below the LOQ. The presence in controls was considered unlikely to be due to the animals being exposed to the test substance since spiropidion is very unstable and converts to its metabolite (SYN547305) very easily in vivo. However, none of the control samples were found to contain quantifiable levels of metabolite. The actual cause of the contamination remains unknown.

Toxicokinetic evaluation showed that on day 2, systemic exposure to metabolite SYN547305 increased subproportionally between the low and high doses in both sexes following continuous dietary administration. Following continuous dietary administration, values of C_{max} were generally greater on day 2 than day 91 at the low and intermediate doses in both sexes. At the high dose, mean C_{max} was comparable between sample days. At low and intermediate doses the $AUC_{(0-t)}$ was generally comparable between sample days in both sexes. However, the $AUC_{(0-t)}$ on day 91 compared to day 2 in the high-dose group in both sexes. On day 91 systemic exposure had increased approximately proportionally to dose.

Table 16. Key findings of the 90-day dietary toxicity study in rat

Parameters	**Males**				**Females**			
	0	**100 ppm**	**500 ppm**	**2500 ppm**	**0**	**100 ppm**	**500 ppm**	**1500 ppm**
Body weight (g) ± SD								
Day 0	224 ± 10	213 ± 8	215 ± 12	220 ± 13	153 ± 10	157 ± 10	154 ± 11	151 ± 9
3	234 ± 10	222 ± 9	224 ± 14	215 ± 12**	159 ± 13	163 ± 8	160 ± 9	154 ± 10
7	252 ± 10	240 ± 10	239 ± 12*	232 ± 14**	169 ± 14	174 ± 8	169 ± 9	162 ± 11
14	282 ± 9	267 ± 13	267 ± 16*	255 ± 17**	187 ± 17	186 ± 9	184 ± 12	174 ± 12
28	326 ± 11	314 ± 17	307 ± 19*	291 ± 21**	213 ± 16	214 ± 11	204 ± 14	195 ± 15*
56	370 ± 18	355 ± 19	347 ± 23*	332 ± 23**	229 ± 21	230 ± 12	219 ± 17	211 ± 18
91	400 ± 24	385 ± 23	377 ± 25	364 ± 26**	240 ± 25	240 ± 10	230 ± 18	221 ± 15
% difference days 0–91		*−4*	*−6*	*−9*		*0*	*−4*	*−8*
Body weight gain (g) ± SD								
Days 0–3	10 ± 3	9 ± 3	9 ± 4	−5 ± 3**	7 ± 5	6 ± 4	6 ± 4	3 ± 5
0–7	28 ± 4	27 ± 5	24 ± 5	12 ± 5**	17 ± 5	17 ± 3	15 ± 5	12 ± 8
0–14	59 ± 8	54 ± 9	52 ± 8	35 ± 7**	34 ± 8	29 ± 7	30 ± 6	24 ± 6**
0–28	102 ± 14	101 ± 13	92 ± 10	71 ± 13**	60 ± 9	56 ± 4	51 ± 8*	45 ± 8**
0–56	146 ± 19	141 ± 16	132 ± 13	112 ± 16**	76 ± 13	73 ± 6	66 ± 11	61 ± 10**
0–77	171 ± 22	165 ± 20	151 ± 14*	134 ± 17*	85 ± 16	80 ± 6	73 ± 12*	68 ± 8**
0–91	176 ± 25	172 ± 23	162 ± 17	144 ± 19**	88 ± 19	82 ± 6	77 ± 14	70 ± 9*
% difference days 0–91		*−2*	*−8*	*−18*		*−7*	*−13*	*−20*

Food consumption (g/animal/day) ± SD								
Week 1	22.9 ± 0.8	20.7 ± 0.3**	21.3 ± 1.3*	18.3 ± 0.6**	15.7 ± 1.1	15.3 ± 0.5	14.8 ± 0.8	13.2 ± 0.8**
2	24.5 ± 1.6	21.5 ± 0.9**	23.3 ± 1.8	21.1 ± 0.8**	17.3 ± 0.8	15.9 ± 1.6	16.9 ± 0.8	16.0 ± 1.0
4	22.3 ± 1.3	21.7 ± 0.7	20.8 ± 0.6	19.8 ± 1.3**	16.5 ± 0.6	16.1 ± 0.7	15.6 ± 0.9	15.2 ± 1.0
7	19.3 ± 1.6	17.9 ± 1.0	17.9 ± 0.9	18.1 ± 1.4	14.4 ± 1.1	14.7 ± 0.4	14.2 ± 0.7	13.6 ± 0.9
13	18.9 ± 1.4	18.2 ± 1.0	18.6 ± 1.1	19.1 ± 1.3	13.5 ± 1.0	13.6 ± 0.4	13.6 ± 0.5	± 0.4 ±
Mean weeks 1–4	23.4	21.6	22.0	20.1	16.7	16.3	16.2	15.2
% difference		*−8*	*−6*	*−14*		*−2*	*−3*	*−9*
Mean weeks 1–13	20.6	19.4	19.5	19.0	15.2	14.9	14.8	14.4
% difference		*−6*	*−5*	*−8*		*−2*	*−3*	*−5*
Food utilization (g body weight gain/g food consumed) ± SD								
Week								
1–4	15.6 ± 1.0	16.7 ± 1.2	14.9 ± 0.9	12.6 ± 1.6**	12.9 ± 1.2	12.4 ± 0.3	11.2 ± 1.3	10.4 ± 1.3**
% difference		*+7*	*−4*	*−19*		*−4*	*−13*	*−19*
1–13	9.4 ± 0.3	9.7 ± 0.9	9.1 ± 0.7	8.3 ± 0.7	6.3 ± 0.6	6.1 ± 0.2	5.7 ± 0.6	5.4 ± 0.4*
% difference		*+3*	*−3*	*−12*		*−3*	*−10*	*−14*
Functional observations								
Foot splay (mm) ± SD								
Pretrial	80 ± 21	80 ± 18	77 ± 19	76 ± 19	78 ± 16	76 ± 15	78 ± 22	79 ± 19
Week 12	99 ± 27	103 ± 22	90 ± 28	72 ± 13*	96 ± 21	88 ± 14	82 ± 19	64 ± 20*
Hind grip (g) ± SD								
Pretrial	262 ± 54	229 ± 34	230 ± 41	289 ± 58	242 ± 25	260 ± 27	262 ± 35	265 ± 42
Week 12	529 ± 71	485 ± 62	478 ± 106	438 ± 51*	434 ± 109	411 ± 92	402 ± 101	347 ± 79
Haematology ($\times 10^9$/L) ± SD								
White blood cell count	6.42 ± 1.9	6.34 ± 2.2	6.86 ± 1.3	9.19 ± 1.2**	4.50 ± 1.0	5.13 ± 1.4	5.35 ± 1.1	5.27 ± 0.9
% difference		*−1.2*	*+6.9*	*+43.1*		*+14.0*	*+18.9*	*+17.1*
Neutrophils	0.98 ± 0.4	1.16 ± 0.5	1.02 ± 0.4	1.64 ± 0.4**	0.57 ± 0.2	0.63 ± 0.1	0.73 ± 0.3	0.65 ± 0.2
% difference		*+18.4*	*+4.1*	*+67.3*		*+10.5*	*+28.1*	*+14.0*
Lymphocytes	5.12 ± 1.5	4.85 ± 1.7	5.55 ± 1.2	7.16 ± 0.9**	3.64 ± 0.9	4.22 ± 1.2	4.35 ± 0.9	4.39 ± 0.8
% difference		*−5.3*	*+8.4*	*+39.8*		*+15.9*	*+19.5*	*+20.6*

Basophils	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.04 ± 0.02*	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.00
% diff		*0.0*	*0.0*	*+100.0*		*0.0*	*−50.0*	*0.0*
PT (s) ± SD	24.1 ± 2.2	25.2 ± 2.3	26.7 ± 2.5	26.9 ± 2.5*	25.2 ± 1.1	25.3 ± 1.5	25.2 ± 1.7	24.9 ± 2.1
% difference		*+4.5*	*+10.7*	*+11.6*		*0.0*	*0.0*	*−2*
Clinical biochemistry								
ALP (U/L) ± SD	82 ± 17	70 ± 15	72 ± 11	75 ± 22	43 ± 11	34 ± 4*	26 ± 7**	26 ± 5**
% difference		*−14.6*	*−12.2*	*−8.5*		*−20.9*	*−39.5*	*−39.5*
ALT (U/L) ± SD	43 ± 10	41 ± 6	41 ± 7	78 ± 26**	37 ± 6	37 ± 8	49 ± 11*	63 ± 15**
% difference		*−4.7*	*−4.7*	*+81.4*		*0.0*	*+32.4*	*+70.3*
AST (U/L) ± SD	63 ± 6	64 ± 6	63 ± 11	100 ± 52*	61 ± 6	58 ± 21	73 ± 36	63 ± 7
% difference		*+1.6*	*0.0*	*+58.7*		*−4.9*	*+19.7*	*+3.3*
Cholesterol (mmol/L) ± SD	1.8 ± 0.3	1.5 ± 0.2	1.2 ± 0.2**	1.0 ± 0.2**	1.5 ± 0.3	1.4 ± 0.3	1.0 ± 0.1**	0.8 ± 0.2**
% difference		*−16.7*	*−33.3*	*−44.4*		*−6.7*	*−33.3*	*−46.7*
Triglycerides (mmol/L) ± SD	1.69 ± 0.5	1.35 ± 0.3	1.46 ± 0.3	0.67 ± 0.1**	1.45 ± 06	1.31 ± 0.4	0.78 ± 0.4**	0.69 ± 0.5**
% difference		*−20.1*	*−13.6*	*−60.4*		*−9.7*	*−46.2*	*−52.4*
Total protein (g/L) ± SD	61 ± 1	60 ± 2	58 ± 2*	57 ± 1**	65 ± 4	66 ± 3	64 ± 2	61 ± 2**
% difference		*−1.6*	*−4.9*	*−6.6*		*+1.5*	*−1.5*	*−6.2*
Albumin (g/L) ± SD	41 ± 1	41 ± 1	39 ± 2**	38 ± 1**	48 ± 3	47 ± 3	46 ± 2	44 ± 2**
% difference		*0.0*	*−4.9*	*−7.3*		*−2.1*	*−4.2*	*−8.3*
Phosp (mmol/L) ± SD	1.17 ± 0.2	1.37 ± 0.1*	1.43 ± 0.1**	1.57 ± 0.2**	1.23 ± 0.2	1.29 ± 0.3	1.34 ± 0.2	1.23 ± 0.2
% difference		*+17*	*+22*	*+34*		*+5*	*+9*	*0*
Calcium (mmol/L) ± SD	2.65 ± 0.06	2.62 ± 0.04	2.64 ± 0.07	2.69 ± 0.07	2.70 ± 4	2.70 ± 2	2.69 ± 3	2.62 ± 3*
% difference		−1	−1	+1		0	−1	−3
Organ weight								
Adrenals								
Absolute (g) ± SD	0.055 ± 0.007	0.051 ± 0.006	0.046 ± 0.007**	0.046 ± 0.004**	0.071 ± 0.012	0.068 ± 0.015	0.066 ± 0.007	0.062 ± 0.007
% difference		*−8.3*	*−16.5*	*−17.7*		*−3.3*	*−7.2*	*−12.3*
Covariance with BW ± SD	0.055 ± 0.002	0.051 ± 0.002	0.046 ± 0.002*	0.046 ± 0.002*	0.069 ± 0.004	0.068 ± 0.004	0.066 ± 0.003	0.06 ± 0.004
% difference		*−7.3*	*−15.5*	*−15.9*		*−3.0*	*−5.3*	*−8.8*
Relative body weight (%) ± SD	0.014 ± 0.002	0.013 ± 0.002	0.012 ± 0.002	0.013 ± 0.002	0.029 ± 0.005	0.029 ± 0.006	0.029 ± 0.004	0.028 ± 0.002
% difference		*−3.5*	*−11.6*	*−9.4*		*−3.1*	*−3.0*	*−5.6*

Spleen								
Absolute (g) ± SD	0.60 ± 0.08	0.55 ± 0.06	0.54 ± 0.06	0.56 ± 0.04	0.48 ± 0.09	0.44 ± 0.05	0.41 ± 0.05*	0.39 ± 0.03**
% difference		*−8.3*	*−10.0*	*−6.7*		*−8.3*	*−14.6*	*−18.8*
Covariance with bw ± SD	0.58 ± 0.02	0.55 ± 0.02	0.54 ± 0.02	0.58 ± 0.02	0.47 ± 0.02	0.43 ± 0.02	0.41 ± 0.02*	0.40 ± 0.02*
% difference		*−5.2*	*−6.9*	*0.0*		*−8.5*	*−12.8*	*−14.9*
Relative body weight (%) ± SD	0.15 ± 0.02	0.15 ± 0.02	0.14 ± 0.02	0.16 ± 0.01	0.20 ± 0.03	0.19 ± 0.02	0.18 ± 0.03	0.18 ± 0.02
% difference		*−4.6*	*−5.3*	*+2.0*		*−7.8*	*−11.8*	*−12.3*
Kidney								
Absolute (g) ± SD	2.15 ± 0.15	2.05 ± 0.15	2.03 ± 0.21	1.94 ± 0.17*	1.48 ± 0.15	1.46 ± 0.05	1.44 ± 0.11	1.35 ± 0.07*
% difference		*−4.7*	*−5.6*	*−9.8*		*−1.4*	*−2.7*	*−8.8*
Covariance with bw ± SD	2.07 ± 0.04	2.04 ± 0.04	2.04 ± 0.04	2.02 ± 0.04	1.45 ± 0.03	1.43 ± 0.03	1.44 ± 0.03	1.39 ± 0.03
% difference		*−1.4*	*−1.4*	*−2.4*		*−1.4*	*−0.7*	*−4.1*
Relative body weight (%) ± SD	0.54 ± 0.03	0.54 ± 0.04	0.54 ± 0.03	0.54 ± 0.04	0.63 ± 0.07	0.61 ± 0.02	0.63 ± 0.03	0.62 ± 0.03
% difference		*−0.7*	*−0.6*	*−0.9*		*−1.6*	*+0.5*	*−1.1*
Histopathology								
Thyroid								
Number examined	10	0	10	10	10	0	10	10
Follicular cell hypertrophy, diffuse:								
total	0	NE	0	4	0	NE	0	7**
minimal	0	NE	0	1	0	NE	0	4
mild	0	NE	0	3	0	NE	0	3
Contraction, colloid:								
total	0	NE	0	8***	0	NE	0	4
minimal	0	NE	0	3	0	NE	0	1
mild	0	NE	0	5*	0	NE	0	3

Toxicokinetic parameters of metabolite SYN547305 (*n* = 10 rats/sex pergroup)

Day 2							
C_{max}		289	1280	2320	328	1460	3270
AUC $_{(0-t)}$		2030	8680	13 100	2360	10 700	22 700
Day 91							
C_{max}		123	542	2370	163	894	2560
AUC $_{(0-t)}$		2400	10 700	50 300	3230	16 600	53 600

bw: Body weight; SD: Standard deviation; PT: Prothrombin time; Source: adapted from Shearer, 2018c

ALP: Alkaline phosphatase; ALT: Alanine transaminase; AST: Aspartate transaminase;

C_{max}: Maximum concentration; NE: Not examined;

$AUC_{(0-t)}$: The area under the concentration–time curve from the start of dose administration to the time after dosing at which the last quantifiable concentration was observed (ng.h/mL)

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, statistically significant difference from control group mean (Dunnett's)

The Meeting noted that a number of control blood samples subject to bioanalysis were contaminated with spiropidion. Nevertheless the Meeting concluded that this contamination did not invalidate the results or interpretation of study′s toxicological findings.

The NOAEL was 100 ppm (equal to 6.2 mg/kg bw per day) based on reduced cholesterol in both sexes, triglycerides in females and minor changes of other clinical chemistry parameters at 500 ppm (equal to 31.5 mg/kg bw per day) (Shearer, 2018c).

Dog

Study1

Spiropidion (SYN546330; purity 98.3%) was administered via gelatine capsules to beagle dogs, approximately five months old, four groups of three animals/sex per group, at dose levels of 0, 10, 30 and 100↓65 mg/kg bw per day for 28 days. Animals that received 100 mg/kg bw per day were given up to two administrations before dosing was stopped due to adverse clinical observations (seizures). After a 26-day washout period the surviving animals recommenced treatment at 65 mg/kg bw per day, however dosing was again stopped after up to four days due to adverse clinical observations of a similar nature to those seen at 100 mg/kg. All animals were monitored regularly for viability and signs of ill health or reaction to treatment. Body weight and food consumption were measured and recorded at predetermined intervals from pretrial until the completion of treatment. Blood and urine samples were collected from all animals during pretrial and on day 28 (control and high-dose groups only) for haematology, coagulation, clinical chemistry and urinalysis parameter evaluations. Blood samples were also collected from all animals for bioanalytical and toxicokinetic parameter (T_{max}, C_{max} and $AUC_{(0-t)}$) analyses for spiropidion and metabolite SYN547305 on days 1 and 28 of the study. All animals receiving 0, 10 or 30 mg/kg bw per day were euthanised after at least 28 days of treatment and subjected to a detailed necropsy examination. Animals receiving 100↓65 mg/kg bw per day were euthanised following the treatment-free period after being dosed at 65 mg/kg bw per day for four days, and were also subjected to a detailed necropsy examination. Selected organs were weighed and a full list of tissues from all animals were preserved and processed for histopathologically examination by the study pathologist.

One male (animal 12M) and two females (animal 22F and 24F) that received 100 mg/kg bw per day or 100↓65 mg/kg bw per day were euthanized due to severe clinical observations including salivation, unsteadiness on feet, ataxia, subdued appearance, twitching and abnormal breathing. These signs were considered to be treatment-related. Clinical signs of subdued behaviour unsteadiness on feet, uncoordination and lack of awareness of surroundings were also observed in the surviving animals receiving 100↓65 mg/kg bw per day. The above clinical signs appeared after one of two doses at 100 mg/kg bw per day, or two or four doses at 65 mg/kg bw per day.

In female 22F the following findings were recorded at necropsy: dark discoloration of the lung, duodenum, colon (foci), lymph nodes (pancreatic and mesenteric), pancreas and stomach; stomach dilation and raised area (pylorus). Erythrocytosis/erythrophagocytosis were observed in the lymph nodes upon histopathological examination.

In female 24F the following findings were recorded at necropsy: accumulation of blood in the thoracic cavity, froth in the trachea, distension of the stomach, pale foci in the pylorus, and dark discoloration of the skin, lung, bronchial lymph node and duodenum. Pulmonary haemorrhage and dermal vasculitis were noted upon histopathological evaluation.

In male 12M the following findings were recorded at necropsy: gross dark discoloration was observed in the lung and lymph nodes (mandibular and bronchial). This corresponded to pulmonary agonal congestion and lymph node erythrocytosis/erythrophagocytosis seen upon histopathological examination.

There were no clinical signs, changes in body weights, food consumption, haematology, coagulation, clinical chemistry, gross or microscopic histopathological findings that were attributable to treatment at dose levels of 10 or 30 mg/kg bw per day.

Bioanalysis showed that in nearly all samples spiropidion was not quantifiable. Metabolite SYN547305 was quantifiable (LOQ: 5 ng/mL) in all male controls and one female control animal at most or all time points. The concentration found in control animal blood samples represented a maximum of 2% of the blood concentration measured in animals given 10 mg/kg bw per day. The contamination was

considered not to have affected the integrity or outcome of the study.

The T_{max} values in animals at 10 and 30 mg/kg bw per day were noted to be shorter on day 28 than on day 1. Values of C_{max} and $AUC_{(0-t)}$ in animals at 10 and 30 mg/kg bw per day were higher on day 28 than on day 1.

Table 17. Toxicokinetic parameters of metabolite SYN547305 (three dogs/sex per group)

	Dose (mg/kg bw per day)							
	Males				Females			
	10	**30**	**65**	**100**	**10**	**30**	**65**	**100**
Day 1								
T_{max} (s)	12	18	8	8	24	24	18	18
C_{max} (ng/mL)	3340	9670	19 400	29 900	2410	7980	27 400	28 600
$AUC_{(0-t)}$ (ng h/mL)	55 700	169 000	362 000	509 000	42 300	144 000	420 000	515 000
Day 28								
T_{max} (s)	1	18	8	8	18	1	18	18
C_{max} (ng/mL)	7030	11 300	NE	NE	8760	11 300	NE	NE
$AUC_{(0-t)}$ (ng h/mL)	130 000	238 000	NE	NE	148 000	222 000	NE	NE

NE: not examined; T_{max}: Time to peak concentration Source: adapted from Shearer, 2018d

C_{max}: Maximum concentration

$AUC_{(0-t)}$: The area under the concentration–time curve from the start of dose administration to the time after dosing at which the last quantifiable concentration was observed (ng.h/mL)

The Meeting noted that a number of control blood samples subject to bioanalysis were contaminated with spiropidion-enol metabolite (SYN547305). Nevertheless the Meeting concluded that this contamination did not invalidate the results or interpretation of study′s toxicological findings.

The NOAEL was 30 mg/kg bw per day, based on clinical signs and mortality at 100 mg/kg bw per day, later reduced to 65 mg/kg bw per day (Shearer, 2018d).

Study2

Spiropidion (SYN546330; purity 98.4%) was administered for 90 days to beagle dogs approximately seven months of age via gelatine capsules (four groups of four animals/sex per group) at dose levels of 0, 5, 15 or 30 mg/kg bw per day. The animals were monitored regularly for viability and signs of ill health or reaction to treatment. Body weights and food consumption were measured and recorded at predetermined intervals from pretrial up until the completion of treatment. Ophthalmoscopy examinations were conducted before the start and at the end of dosing. Blood and urine samples were collected throughout the study and an adequate number of haematology, coagulation and clinical chemistry and urinalysis parameters were evaluated. Blood samples were also collected from all animals for bioanalytical and toxicokinetic parameters (T_{max}, C_{max} and $AUC_{(0-t)}$) for spiropidion and metabolite SYN547305 on days 1, 27, 55 and once during week 13 of the study and were analysed. All surviving animals were killed at earliest after 13 weeks of treatment, and subjected to a detailed necropsy examination, with selected organs being weighed (brain, epididymis, adrenal, pituitary, prostate thyroid and parathyroid, heart, kidneys, liver, lung, ovaries, spleen, testes, thymus and uterus with cervix). Tissues from all control and high-dose animals were subjected to comprehensive histopathological evaluation. In addition, gross lesions from the low and intermediate groups, and adrenal glands from the intermediate group and females in the low group, were also evaluated.

One female at 30 mg/kg bw per day was killed on day 13 due to initial clinical signs (3.5 hours post dose) of unawareness of surroundings, body tremors, no coordination of hind limbs and subdued behaviour; later this female was found lying on the pen floor, unresponsive to stimulation. Following gross and histopathological examination there were no findings to indicate the cause of the animal′s clinical condition. Based on similar clinical signs being observed on a previous 28-day study (Shearer, 2018d) at higher dose levels (65↓100 mg/kg bw per day), these clinical signs were considered

to be treatment-related.

No treatment-related effects on body weight, body weight gain or food consumption were observed.

Ophthalmoscopy examinations reported retina/choroid moderate depigmentation in both eyes of one male at 30 mg/kg bw per day at week 6 and 13 of observation.

Haematological investigation showed a statistically significant decrease compared with controls in APTT for females at 30 mg/kg bw per day. As APTT level was similar to that in the one-year concurrent control, APTT change in females at the high dose was considered of no toxicological significance.

No treatment-related effects were observed in biochemical or urine parameters.

Adrenal gland weights, both absolute and as a covariate to body weight, were higher in males at all dose levels when compared with controls, but with no dose–response relationship. The same parameters in females at all doses achieved statistical significance at 30 mg/kg bw per day. The adrenal weights of all treated animals were within the historical control data range. Increased incidence when compared to controls of minimal vacuolation in the zona granulosa of adrenal glands was reported in females at 30 mg/kg bw per day. An increased incidence of mild to moderate vacuolation in the zona vasiculata was reported in the females at 15 and 30 mg/kg bw per day when compared to controls. There was some correlation between vacuolation in the adrenal gland and higher adrenal weights in females. The highest adrenal weights were recorded in the two animals (one male and one female) at 30 mg/kg bw per day, which had mild or moderate vacuolation of the adrenal zona fasiculata. However, correlation in the case of other animals was lacking. Overall, given the lack of consistency between the 90-day and one year dog study, adrenal findings were considered incidental to treatment.

Pituitary weights, both absolute and as a covariate to body weight, were higher in all female groups with an unclear dose–response relationship at 5 and 15 mg/kg bw per day; since the individual values for these groups fell within the range of the control values this finding was incidental to treatment. The increase observed at 30 mg/kg bw per day may be ascribable to differences in sexual maturity as observed for ovarian weight changes, and given the lack of histopathological correlates it was considered of no toxicological relevance.

Ovarian weights, both absolute and as a covariate to body weight, were higher compared to controls at 15 and 30 mg/kg bw per day. Given that females at 15 and 30 mg/kg bw per day with ovarian weights outside the individual control values revealed corpora lutea (correlated with necropsy finding) the effects on ovarian weight can be ascribed to differences in sexual maturity; for this reason they were not considered to be treatment-related.

Bioanalysis showed that there were no quantifiable samples of spiropidion following on day 1 or week 13. Quantifiable levels of metabolite SYN547305 were reported in 17/160 control blood samples (males #2 and #3, and/or females #17 and #20) at up to three time points on days 27, 55 or 91 or any combination of these days, with the exception of male #3, where quantifiable levels were found in all but one blood sample. The concentrations found were between 7.83 ng/mL and 22.7 ng/mL, with a LOQ of 5 ng/mL. The highest concentration found amounts to $<1\%$ of the blood concentration in animals given the lowest dose of spiropidion, 5 mg/kg bw per day. No obvious evidence from the animal room or bioanalytical data indicated that any contamination occurred during blood collection, sample preparation or analysis. It was concluded that this contamination did not affect the outcome or integrity of the study.

On day 1 times to peak blood concentration (T_{max}) were observed at 24 h for all dose groups, reflecting the ongoing increase in blood of the metabolite SYN547305 for the duration of sampling. On day 90 T_{max} was greater at increasing doses in males. In females time to peak blood concentration was shorter for the low and high dose compared to the mid dose. Exposure to metabolite SYN547305 (C_{max} and $AUC_{(0-t)}$) increased with increasing dose on day 1. This increase in exposure was proportional between the 5 and 30 mg/kg bw per day doses in males. However, there was no appreciable increase in exposure estimates between the 5 and 15 mg/kg bw per day doses in males. In females, exposure was supraproportional with increasing dose. Following repeat dosing, systemic exposure was greater at week 13 than on day 1. Systemic exposure was comparable in males and females on day 1 and at week 13 in the 5 and 30 mg/kg bw per day dose groups. In the 15 mg/kg bw per day dose group, exposure estimates were greater in females than in males.

Table 18. Key findings of the 90-day toxicity study in dog

	Dose (mg/kg bw per day)									
	Males					Females				
Parameters	**0**	**5**	**15**	**30**	**HCD**	**0**	**5**	**15**	**30**	**HCD**
Haematology										
Activated partial prothrombin time, APTT ± SD										
pretrial	16 ± 1.5	16.8 ± 1.4	14.9 ± 1.5	16.5 ± 1.0		16.8 ± 1.8	16.0 ± 0.5	17.8 ± 1.1	15.9 ± 1.8	
week 6	15.2 ± 1.0	14.3 ± 1.3	14.1 ± 0.7	15.1 ± 0.5		15.4 ± 1.0	15.0 ± 0.4	15.7 ± 1.0	13.1 ± 2.0	
week 13	14.7 ± 0.9	14.7 ± 1.2	14.0 ± 0.8	14.7 ± 0.3		14.4 ± 1.2	14.0 ± 0.4	14.9 ± 1.2	11.5 ± 0.4**	
Organ weights										
Adrenals										
Absolute (g)	1.14 ± 0.11	1.53 ± 0.32	1.35 ± 0.15	1.56 ± 0.33	1.16; 0.78 to 1.60	1.01 ± 0.09	1.16 ± 0.12	1.11 ± 0.09	1.42 ± 0.20**	1.29; 0.75 to 1.83
% difference		*+34*	*+18*	*+37*			*+15*	*+10*	*+41*	
Covariance to bw	1.15 ± 0.11	1.55 ± 0.11	1.34 ± 0.11	1.54 ± 0.11		1.01 ± 0.07	1.16 ± 0.07	1.11 ± 0.07	1.41 ± 0.08**	
% difference		*+35*	*+17*	*+34*			*+15*	*+10*	*+40*	
Pituitary										
Absolute (g)	0.061 ± 0.006	0.061 ± 0.009	0.055 ± 0.009	0.062 ± 0.017		0.051 ± 0.018	0.064 ± 0.024	0.058 ± 0.012	0.076 ± 0.003	
% difference		*0*	*−10*	*+2*			*+25*	*+14*	*+49*	
Covariance to bw	0.061 ± 0.005	0.062 ± 0.005	0.055 ± 0.005	0.061 ± 0.005		0.051 ± 0.009	0.064 ± 0.009	0.058 ± 0.009	0.075 ± 0.010	
% difference		*+2*	*−10*	*0*			*+25*	*+14*	*+47*	
Ovaries										
Absolute (g)	NA	NA	NA	NA		1.37 ± 0.57	1.17 ± 0.83	2.1 ± 0.93	2.54 ± 2.93*	
% difference	NA	NA	NA	NA			*−15*	*+53*	*+85*	
Covariance to bw	NA	NA	NA	NA		1.36 ± 0.75	1.15 ± 0.76	2.18 ± 0.75	2.57 ± 0.88	
% difference	NA	NA	NA	NA			*−15*	*+60*	*+89*	
Histopathology										
Examined (*n*)	4	0	4	4		4	4	4	3	
Adrenal glands										
Vacuolation, zona glomerulosa										
total	0	NE	0	0	2/44	0	0	0	1	1/44
minimal	0	NE	0	0		0	0	0	1	
Vacuolation, zona fasiculata										
total	0	NE	0	2	0/44	1	1	3	1	3/44
minimal	0	NE	0	1		0	1	1	0	
mild	0	NE	0	1		1	0	2	0	
moderate	0	NE	0	0		0	0	0	1	

	Dose (mg/kg bw per day)									
	Males					Females				
Parameters	**0**	**5**	**15**	**30**	**HCD**	**0**	**5**	**15**	**30**	**HCD**
Toxicokinetic parameters of metabolite SYN547305 (*n* = 4 dogs/sex per group)										
Day 1										
T_{max} (s)	24	24	24			24	24	24		
C_{max} (ng/mL)	1 810	1 840	12 500			1 270	4 580	11 100		
$AUC_{(0-t)}$ (ng h/mL)	26 100	28 100	164 000			19 100	70 400	165 000		
Day 90										
T_{max} (s)	0.5	1.5	13			0.5	18	0.5		
C_{max} (ng/mL)	5 450	8 720	28 400			4 220	14 100	16 600		
$AUC_{(0-t)}$ (ng h/mL)	87 300	148 000	426 000			67 900	250 000	286 000		

NE: not examined; NA: Not applicable; bw: Body weight Source: adapted from Shearer, 2018e

HCD: Historical control data from 13 studies (2002–2016) on 44 males and 43 females; SD: Standard deviation;

C_{max}: Maximum concentration; T_{max}: Time to peak concentration;

$AUC_{(0-t)}$: The area under the concentration–time curve from the start of dose administration to the time after dosing at which the last quantifiable concentration was observed (ng.h/mL)

* $p < 0.05$, ** $p < 0.01$, statistically significant difference from control group mean (Dunnett's)

The Meeting noted that a number of the control blood samples subject to bioanalysis were contaminated with spiropidion-enol metabolite (SYN547305). Nevertheless the Meeting concluded that the contamination did not invalidate the results or interpretation of study′s toxicological findings.

The NOAEL of the study was 15 mg/kg bw per day, based on mortality, clinical signs in females and bilateral retina/choroid depigmentation at 30 mg/kg bw per day (Shearer, 2018e).

Study3

Spiropidion (SYN546330; purity 98.4%) was administered for one year to approximately seven-month-old beagle dogs, four animals/sex per group, via gelatine capsules at a dose of 0, 3, 10 or 30 mg/kg bw per day. Due to adverse clinical observations and mortalities (one female on day 7 and one male on day 14), dosing at 30 mg/kg bw per day was stopped on day 15 of the study. Subsequently an additional two groups, of four dogs/sex per group were given spiropidion via gelatine capsules at dose levels of 0 or 1 mg/kg bw per day for at least one year. The animals were monitored regularly for viability and signs of ill health or reaction to treatment. Body weight and food consumption were measured and recorded at predetermined intervals from pretrial until the completion of treatment. Ophthalmoscopy examinations were conducted before the start and at the end of dosing. Blood and urine samples were collected at predetermined intervals from pretrial until the completion of treatment, and an adequate number of haematology, coagulation and clinical chemistry and urinalysis parameters were evaluated. Blood samples were also collected from all animals on day 15 and used to measure spiropidion and metabolite SYN547305 blood concentrations. All surviving animals were killed after at least one year of treatment and subjected to a detailed necropsy examination, with selected organs being weighed (brain, epididymis, adrenal, pituitary, prostate thyroid and parathyroid, heart, kidneys, liver, lung, ovaries, spleen, testes, thymus and uterus with cervix). Tissues from all animals were subjected to a comprehensive histopathological evaluation.

One male (4004M) and one female (4502F) at 30 mg/kg bw per day were euthanized early in the study (female on day 7, male on day 14) due to adverse clinical signs: animal lying on its side, uncoordinated movements, salivation, decreased activity, hypersensitivity, tremors and subdued behaviour. There were no gross or microscopic findings noted that might account for the decline in clinical condition. However, the early euthanasia of these animals was considered to be associated with treatment. There were no other mortalities or clinical signs ascribable to treatment.

Body weight in females at 10 mg/kg bw per day was approximately 6% lower than for controls throughout the treatment period. The lowest body weight was recorded between days 175 and 189, when it was 8% lower than in controls. It was noted that all individual body weight values were within the control range values. Body weight gain and food consumption in females at 10 mg/kg bw per day were also generally lower than controls, with means of approximately 30% and 20% lower, respectively, which occasionally achieved statistical significance. The lowest body weight gain was 50% of control, in one period, between days –1 and day 189. Since body weight gain and food consumption changes did not affect body weight, these changes were considered of no toxicological relevance. No treatment-related effects on body weight, body weight gain or food consumption were observed in males at any dose, nor in females at 1 or 3 mg/kg bw per day.

No treatment-related effects were observed at ophthalmoscopic examination.

There were no treatment-related changes in haematological or coagulation parameters. Reported changes were considered not to be treatment-related since were small compared to controls or similar to pretrial values and lacked a dose–effect relationship or temporal concordance.

Clinical biochemistry analysis showed an apparent change in inorganic phosphate in males and females at 3 and 10 mg/kg bw per day on week 52. However, given the opposite nature of changes between sexes (increase in males, decrease in females), the lack of a clear dose–response relationship or any histopathological correlates, these changes were not considered treatment-related.

No treated-related effects were observed in urine parameters.

Liver weights were decreased at 3 and 10 mg/kg bw per day when compared to concurrent controls. Lower liver weights here were ascribable to particularly high concurrent control weights for the liver. Relative liver weights at 3 and 10 mg/kg bw per day were noted to be similar to each other and to the other control group (0B). Therefore the liver weights changes seen were not considered to be treatment-related. Increases in thymus weight were reported in males at 3 and 10 mg/kg bw per day, achieving statistical significance at 10 mg/kg bw per day as absolute weights and covariate to body weight. Higher thymus weights were also reported in females at 3 and 10 mg/kg bw per day, but with an unclear dose–response relationship. This was considered to be the result of an of animals with age-related thymic involution which were over-represented in the control group, as was evident from microscopic examination. Decreased testis weights (covariate and relative to body weight) were noted in animals at 3 and 10 mg/kg bw per day, with decreases as large as ca 20% at 10 mg/kg bw be per day, when compared to control group 0A.

Histopathological evaluation showed minimal mononuclear cell infiltration in epididymides in one animal at 1 mg/kg bw per day, two animals at 3 mg/kg bw per day and three animals at 10 mg/kg bw per day. For one animal at the high dose, in addition to this effect, bilateral moderate tubular degeneration and mild inflammation of mixed cells in the testis along with a decrease of spermatozoa cellularity in the epididymides were reported. It was noted that testis tubular degeneration was also reported in one animal of the 90-day concurrent control group. Testis weight changes and microscopic findings in the testis and epididimydes were considered unrelated to treatment as they occured with similar incidence in control and treated animals, and/or they are of nature commonly observed in this strain and age of dogs.

Bioanalysis was conducted to determine the concentrations of spiropidion and metabolite SYN547305. Spiropidion was not detected in any blood sample. Metabolite SYN547305 was detected at concentrations above the LOQ in all samples except in the control group. The data were used only to aid dose level selection for the additional group of animals.

Table 19a. Key findings of the one-year toxicity study in dog – males

	Dose (mg/kg bw per day)					
	Males					
	0A	**0B**	**1**	**3**	**10**	**30**
Body weight (kg) ± SD						
Day −1	7.9 ± 1.4	9.0 ± 0.5	8.9 ± 0.4	8.3 ± 0.8	8.3 ± 0.3	8.5 ± 1.2
Day 365	9.3 ± 1.0	11.8 ± 0.8	12.6 ± 1.4	10.2 ± 0.8	10.3 ± 0.5	-
Body weight gain (kg) ± SD						
Day –1 to 365	1.4 ± 0.7	2.8 ± 1.2	3.7 ± 1.0	1.9 ± 1.0	1.9 ± 0.3	-
Clinical biochemistry						
Phosphate (mmol/L) ± SD						
Pretrial	1.97 ± 0.15	2.27 ± 0.08	2.31 ± 0.11	2.00 ± 0.10	1.92 ± 0.14	1.94 ± 0.14
week 3	1.80 ± 0.06	NE	NE	1.89 ± 0.04	2.05 ± 0.17*	2.03 ± 0.15
week 13	1.61 ± 0.09	1.67 ± 0.11	1.79 ± 0.12	1.66 ± 0.13	1.65 ± 0.08	-
week 26	1.29 ± 0.10	1.43 ± 0.15	1.41 ± 0.25	1.25 ± 0.08	1.27 ± 0.07	-
week 52	1.09 ± 0.03	1.29 ± 0.07	1.29 ± 0.14	1.11 ± 0.07	1.10 ± 0.05	-
Organs weights (mean ± SD)						
Liver						
Absolute (g)	361.7 ± 45.8	341.1 ± 24.7	329.5 ± 43.4	310.7 ± 31.3	312.1 ± 52.1	
% difference				*−14*	*−14*	
Covariance to bw	373.9 ± 24.9	347.6 ± 14.4	323.0 ± 14.4	305.8 ± 22.4	304.8 ± 23.1	
% difference				*−18*	*−18*	
Relative to bw (%)	3.95 ± 0.5	2.91 ± 0.2	2.65 ± 0.2	3.10 ± 0.4	3.05 ± 0.4	
% difference				*−22*	*−24*	
Thymus						
Absolute (g)	2.39 ± 0.71	5.6 ± 0.99	7.5 ± 1.80	4.17 ± 1.31	5.59 ± 1.03*	
% difference				*+74*	*+134*	
Covariance to bw	2.08 ± 0.6	5.4 ± 0.7	7.6 ± 0.7	4.29 ± 0.5	5.77 ± 0.6*	
% difference				*+106*	*+177*	
Relative to bw (%)	0.026 ± 0.009	0.048 ± 0.010	0.061 ± 0.021	0.042 ± 0.015	0.054 ± 0.009	
% difference				*+62*	*+108*	
Testis						
Absolute (g)	15.2 ± 2.31	19.5 ± 0.12	18.9 ± 2.43	15.2 ± 4.53	14.2 ± 2.43	
% difference				*0*	*−7*	
Covariance to bw	16.9 ± 1.5	19.4 ± 0.9	19.0 ± 0.9	14.5 ± 1.4	13.2 ± 1.4	
% difference				*−14*	*−22*	
Relative to bw (%)	0.16 ± 0.025	0.16 ± 0.013	0.15 ± 0.032	0.14 ± 0.034	0.13 ± 0.019	
% difference				*−13*	*−19*	
Histopathology						
Thymus						
Number examined	4	4	4	4	4	
Involution						
Minimal	1	1	0	0	0	

	Dose (mg/kg bw per day)					
	Males					
	0A	**0B**	**1**	**3**	**10**	**30**
Mild	2	0	0	0	1	
Moderate	1	0	0	0	0	
Total	4	1	0	0	1	
Epididimydes						
Number examined	4	4	4	4	4	
Mononuclear cell infiltration						
Minimal	1	0	1	2	3	
	Bilateral multifocal		Bilateral multifocal	1 bilateral focal, 1 unilateral multifocal	2 bilateral, multifocal, 1 unilateral, multifocal	
Decreased cellularity spermatozoa						
Marked	0	0	0	0	1	
Testis						
Number examined	4	4	4	4	3	
Tubular degeneration						
Bilateral, moderate	0	0	0	0	1	
Inflammation, mixed cell						
Mild	0	0	0	0	1	

SD: Standard deviation; bw: Body weight; Source: adapted from Shearer, 2018f

* $p < 0.05$, ** $p < 0.01$, statistically significant difference from control group mean (Dunnett's)

Table 19b. Key findings of the one-year toxicity study in dog – females

	Doses (mg/kg bw per day)					
	Females					
	0A	**0B**	**1**	**3**	**10**	**30**
Body weight gain (kg) ± SD						
Day −1	7.0 ± 0.8	8.1 ± 1.2	7.8 ± 0.6	7.1 ± 0.3	7.2 ± 0.6	7.2 ± 0.4
Day 365	9.1 ± 1.5	11.6 ± 2.0	11.0 ± 1.0	8.9 ± 1.1	8.6 ± 0.4	-
Body weight gain (kg) ± SD						
Day –1 to 365	2.1 ± 0.9	3.5 ± 1.3	3.3 ± 0.7	1.8 ± 0.9	1.4 ± 0.2	-
Clinical biochemistry						
Phosphate (mmol/L) ± SD						
Pretrial	1.83 ± 0.15	2.35 ± 0.31	2.15 ± 0.05	1.71 ± 0.11	1.71 ± 0.15	-
week 3	1.81 ± 0.14	NE	NE	1.85 ± 0.20	1.73 ± 0.13	1.90 ± 0.07
week 13	1.59 ± 0.06	1.70 ± 0.14	1.69 ± 0.11	1.45 ± 0.16	1.36 ± 0.09*	-
week 26	1.29 ± 0.13	1.28 ± 0.11	1.32 ± 0.04	1.30 ± 0.08	1.21 ± 0.17	-
week 52	1.22 ± 0.23	1.18 ± 0.07	1.29 ± 0.05*	0.99 ± 0.11	1.06 ± 0.07	-

	Doses (mg/kg bw per day)					
	Females					
	0A	0B	1	3	10	30
Organs weights (mean ± SD)						
Liver						
Absolute (g)	247.4 ± 28.2	323.8 ± 100.9	299.7 ± 36.3	250.7 ± 40.7	223.9 ± 11.5	
% difference				*+1*	*−9*	
Covariance to bw	245.5 ± 15.4	311.2 ± 31.6	312.2 ± 31.6	250.6 ± 15.1	225.8 ± 15.4	
% difference				*+2*	*−8*	
Relative to bw (%)	2.74 ± 0.2	2.78 ± 0.5	2.78 ± 0.4	2.89 ± 0.7	2.64 ± 0.2	
% difference				*+5*	*−4*	
Thymus						
Absolute (g)	3.03 ± 0.81	5.89 ± 3.29	5.09 ± 1.25	6.77 ± 3.65	5.25 ± 4.03	
% difference				*+123*	*+73*	
Covariance to bw	2.60 ± 1.5	5.29 ± 0.3	5.69 ± 0.3	6.80 ± 1.5	5.70 ± 1.5	
% difference				*+162*	*+119*	
Relative to bw (%)	0.033 ± 0.008	0.048 ± 0.020	0.046 ± 0.007	0.074 ± 0.031	0.061 ± 0.046	
% difference				*+124*	*+85*	
Histopathology						
Thymus						
Number examined	4	4	4	4	4	
Involution						
Minimal	0	0	0	0	1	
Mild	0	0	0	0	1	
Moderate	0	0	0	0	0	
Total	0	0	0	0	2	

SD: Standard deviation; bw: Body weight; Source: adapted from Shearer, 2018f

* $p < 0.05$, ** $p < 0.01$, statistically significant difference from control group mean (Dunnett's)

The NOAEL for the study was 10 mg/kg bw per day, based on mortality and clinical observations in two animals at 30 mg/kg bw per day (Shearer, 2018f).

(b) Dermal application

In a study of dermal toxicity spiropidion (purity 98.4%) was applied repeatedly for 28 consecutive days to the skin of RccHanTM WIST rats (10/sex per group) at dose levels of 100, 300 or 1000 mg/kg bw per day for six hours/day under a semi-occlusive dressing. Animal were treated five days per week, except for the week preceding necropsy, when a daily treatment regime was followed. During the study, clinical condition, detailed physical examination, sensory reactivity, grip strength, motor activity, body weight, food consumption, ophthalmic examinations, haematology (peripheral blood) and blood chemistry were all studied. An adequate number of organs were weighed, and macropathology and histopathology evaluations made.

One male at 300 mg/kg bw per day was killed prematurely on day 28 because of poor clinical condition (underactive/decreased activity, piloerection, abnormal coldness to the touch, flattened posture and irregular/deep breathing). The cause of this animal's clinical condition was not attributed to treatment but was due to an ascending inflammation in the urinary tract and accessory sex glands (seminal vesicles and prostate). The appearance and behaviour of the animals were unaffected by treatment and no test item-related signs were recorded in association with the dosing procedure.

Males at 1000 mg/kg bw per day showed lower body weight gains by approximately 20% than controls, but this was not statistically significant. In females body weight gain was supressed by approximately 14% and 30% at 300 and 1000 mg/kg bw per day, respectively, but not with statistical significance. The effect reported in females at 300 mg/kg bw per day was not considered treatment-related as terminal body weights (207 g to 239 g at week 4) of all females which received this dose level were within the concurrent control range (195 g to 244 g). The group mean average appeared lower due to the large interanimal variability in body weight gain observed in the control animals (between 22 g and 58 g).

Food consumption was found to be approximately 13% (statistically significant) lower than controls in males at 1000 mg/kg bw per day during the first eight days of treatment, and 8% lower during the overall treatment period (days 1–29).

No treatment-related effects were observed for sensory activity, grip strength or motor activity. Ophthalmoscopic examination did not reveal any treatment-related effects.

Statistically significant increases in haematocrit (Ht) and haemoglobin (Hb) were observed in males at 300 and 1000 mg/kg bw per day. Considering the modest extent of the changes and the absence of a clear dose–response relationship these changes were considered of no toxicological significance. In addition, all group mean values were within the background data control mean ranges (0.429–0.475 L/L, 14.6–16.3 g/dL and 8.31–8.96 $\times 10^{12}$/L, respectively) with the exception of the mean Hb value (16.4 g/dL) for males fed 1000 mg/kg bw per day. A statistically significant increase in erythrocyte count was reported in males at 300 mg/kg bw per day, however since the change was minimal and there was no dose–response relationship this finding was considered incidental to treatment. A slight decrease in MCV was reported in females at 100 and 1000 mg/kg bw per day, which achieved statistical significance at 1000 mg/kg bw per day. Considering the lack of a dose–response relationship, and that the mean value was within the background control range (52.5–55.8 fL) this change was considered incidental to treatment. Statistically significant prolongation of prothrombin time was reported in males at 300 and 1000 mg/kg bw per day. The group mean values at these dose levels were above the background control mean range (20.4–23.8 s), but a substantial proportion of the individual values (7/10 at 300 mg/kg bw per day) were within the concurrent control range (20.7–25.0 s). A statistically significant decrease in APTT was observed in females at 1000 mg/kg bw per day. The group mean value (15.7 s) was below the background control mean range (16.7–20.1 s).

Clinical chemistry analysis results indicated a dose-related and statistically significant decrease in total cholesterol concentration at all dose levels in males, and at 300 and 1000 mg/kg bw per day in females, compared with the controls. The mean values for males at 300 mg/kg bw per day and for both sexes at 1000 mg/kg bw per day were below the background control mean range (1.73–2.39 mmol/L for males and 1.66–2.20 mmol/L for females). All other group mean values for cholesterol were within these ranges with the exception of the control male value (2.42 mmol/L) which was marginally above the respective range. A slight but statistically significant reduction in plasma albumin concentration was evident in males at 300 and 1000 mg/kg bw per day compared with the controls; at 1000 mg/kg bw per day this was also associated with a reduced albumin : globulin ratio; the group mean values for these parameters at these dose levels were below their respective mean background control ranges (36–39 g/L for albumin and 1.27–1.43 for albumin : globulin ratio).

There was a minor, but statistically significant, reduction in albumin : globulin ratio in females at all doses. All mean values for control and treated female groups were below the background control mean range (1.48–1.57). It was noted that these changes were not accompanied by changes in total proteins or albumin at any dose level, therefore they were considered to be of low toxicological significance. A slight increase in AST activity was observed in males at all doses, achieving statistical significance at 1000 mg/kg bw per day. A slight decrease in ALP activity was observed in females at 300 and 1000 mg/kg bw per day, achieving statistical significance only at 300 mg/kg bw per day. Given the small size of changes to these enzyme activities and their lack of a dose–response relationship the changes were attributed to normal biological variation. A statistically significant decrease in sodium was reported for females at all dose levels, however considering the size of the change, its lack of a dose–response relationship, and that the majority of the individual values for all doses were within the concurrent control ranges, the differences were attributed to normal biological variation.

A slight, but statistically significant decrease was reported in absolute heart weight in males at

300 and1000 mg/kg bw per day, and in females at 100 and 1000 mg/kg bw/day, with no dose–response relationship in either sex. All group mean values for control and treated groups were within the background control mean ranges (0.878–1.018 g and 0.648–0.758 g for males and females respectively), with the exception of the control female value (0.772 g) which was marginally above its respective range. A slight but statistically significant decrease in absolute and adjusted liver weights was evident at all dose levels in males. Given the small size of the change, the absence of a dose–response relationship, and that all control and mean absolute values for treated groups were within the background control mean range (9.353–13.038 g), these changes were considered within normal biological variability.

There were no treatment-related macroscopic findings. Histopathological investigation showed a statistically significant increase in the incidence of minimal follicular cell hypertrophy of the thyroid in females at 1000 mg/kg bw per day which was characterised by colloid depletion, obliteration of the follicular space or partial collapse of follicles lined by hypertrophic thyroid follicular epithelium.

The NOAEL for this study was 300 mg/kg bw per day, based on reduced body weight gain in both sexes and minimal follicular cell hypertrophy in the female thyroid at 1000mg/kg bw per day (Hughes, 2018).

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

Spiropidion (purity 98.4%) was administered to four groups of 50 male and 50 female CD-1 mice (6–7 weeks old) at a dietary concentration of 0, 50, 250 or 500 ppm (equal to 0, 6.4, 31.8 or 65.4 mg/kg bw per day for males, 0, 7.0, 36.8 or 72.8 mg/kg bw per day for females) for 80 weeks. All animals were checked in their cages twice daily for general health/mortality and moribundity. All animals were removed from their cages and received a detailed clinical examination (size, appearance, position and duration of any masses) on a weekly basis commencing at the start of week –1. Body weights, food consumption, test item consumption and food utilization were recorded during pretreatment, weekly at weeks 1–14 and once every two weeks during weeks 16–80. Water consumption was qualitatively monitored by visual inspection of the water bottles on a regular basis throughout the study. Blood samples were collected from all animals at weeks 52/53 for blood smear evaluation, and at termination for evaluation of total white cell count and differential white cell count. At termination all animals were subject to a complete necropsy examination. The following organs were weighed: brain, epididymides, adrenal glands, seminal vesicles with prostate and coagulating glands, heart, kidneys, liver with gall bladder, ovaries, spleen, testes, thymus, uterus with cervix and oviducts. All tissues were microscopically analysed, except bone marrow smears, oviducts, pharynx and tongue.

No treatment-related clinical effects or mortality were observed. Females suffered reduced mortality at 250 ppm, an effect that achieved statistical significance.

There was a higher incidence of palpable masses observed in all treated male groups as compared with controls. However, due to the lack of a dose–response relationship, the relatively small difference, and that no treatment-related histological findings were observed, occurrence of the masses was considered incidental. There was no difference in the number of masses occurring in female treated animals and in their control.

Body weight and body weight gain was generally considered to be lower throughout the treatment period in both sexes at 250 and 500 ppm when compared with controls. At the end of the treatment period, body weight gain was lower in males and females at 500 ppm by approximately 18% for both sexes; at that point males and females fed 250 ppm were 23% and 11% lower, respectively when compared with controls. No effects on food consumption were observed, while food utilization was found to be lower in males (statistically significant) at 250 ppm and 500 ppm over weeks 1-13, in both sexes at 500 ppm, and in females at 250 ppm over weeks 1–4. Food utilisation compared with controls was lower to a statistically significant extent over weeks 1–13 in males at 250 ppm and 500 ppm. Additionally, food utilisation was lower over weeks 1-4 in animals at 500 ppm and in females at 250 ppm.

No treatment related effects on water consumption were observed.

A decrease in absolute weight of the epididimydes was reported in males at 250 and 500 ppm

with an unclear dose response, achieving statistical significance only at 250 ppm. Since no changes in the weight of epididimydes relative to body weight or covariance to body weight were observed, this change is likely to be a consequence of lower body weight in these animals. A decrease in the weight of adrenal glands, not statistically significant, was reported in females at all dose levels, with unclear dose response. All individual values in all dosed groups were within the control range values (absolute: 0.005–0.2 g). In the absence of histopathological effects, these changes were considered of no toxicological relevance. A significant decrease of prostate weights (not statistically significant) was reported in animals at all dose levels, but with no clear dose–response relationship. It was noted that almost all individual values in all dose groups were within the control range (absolute: 0.65–11.1 g). In the absence of histopathological effects these changes were considered of no toxicological relevance. A statistically significant decrease in absolute kidney weight was reported in males at 250 and 500 ppm, and in females at 500 ppm. Given the small size of these changes and that no effect was observed in weight relative to body weight or covariate to body weight, they are likely to be a consequence of lower body weight in these animals. Increased testis weight was reported at all dose levels, however, the lack of a clear dose–response relationship, the small size of the changes and the absence of histopathological correlates suggested that these changes were of no toxicological relevance. A dose-related increase in ovarian weights was reported at all dose levels, achieving statistical significance for the absolute weight in animals at 500 ppm. The extent of ovary weights increase was of approximately 50%, 200% and 600% at 50, 250 and 500 ppm respectively, compared with controls, . It was noted that the ovary weight increases were ascribable to 1, 3 and 3 females at 50, 250 and 500 ppm, respectively. These animals had absolute ovary weights that were four-fold (50 ppm), between three- and seven-fold (250 ppm), and from seven- to 16-fold (500 ppm) higher than the upper limit of the control range. A decrease in uterus weight (not statisticallly significant) was observed at 250 and 500 ppm, to approximately 50% of controls but with no clear dose response. Since all individual values were within the control range values (absolute: 0.13–6.9 g) and no histopathological treatment-related effects were observed, changes in uterus weight were considered to be of no toxicological significance.

Pathological macroscopic evaluation revealed an increase in the incidence of dark/dark red discoloration of the mesenteric lymph nodes in males at all doses and in females at 250 and 500 ppm. This colour change correlated microscopically with the presence of erythrocytes and/or erythrophagocytosis (most frequently), angiectasis, lymphoma or histiocytic sarcoma.

Microscopic analysis revealed an increased incidence and severity of erythrophagocytosis in the mesenteric lymph nodes, statistically significant at all doses in males and in females at 250 and 500 ppm, but not achieving statistical significance compared to controls. The significance of an increased incidence of mesenteric lymph node erythrophagocytosis in this study was unknown due to the potential for this change to occur from agonal or procedural processes (euthanasia or tissue dissection) and due to the absence of concurrent regional tissue haemorrhage, hemosiderin or vascular or cardiac treatment-related effects. Without evidence of a deleterious effect due to this change on the morphology or function of the lymph node it was considered not to be adverse.

An increased incidence and severity of calculi in the gallbladder was reported in males at all doses, achieving statistical significance at 500 ppm, and in females at 250 and 500 ppm without dose response or any statistical significance compared to controls. The calculi were not observed macroscopically and were not associated with inflammatory or epithelial changes. The increased incidence and severity observed were considered to be test substance-related, however the finding was considered not to be adverse due to the absence of accompanying inflammatory/epithelial changes or evidence of a deleterious effect on gallbladder function (for example a blockage of bile flow due to biliary obstruction).

There were no neoplastic or proliferative findings attributable to treatment. All neoplastic and proliferative findings observed were considered incidental and of the nature commonly observed in this strain and age of mice, and/or were of similar incidence and severity in controls and treated animals.

Table 20. Key findings of the two-year toxicity study in mice

	Dose							
	Males				Females			
Observation	**0 ppm**	**50 ppm**	**250 ppm**	**500 ppm**	**0 ppm**	**50 ppm**	**250 ppm**	**500 ppm**
Mortality								
Killed	19	15	15	16	15	12	9	11
Died	4	4	3	0	3	8	0	6
Total	23	19	18	16	18	20	9*	17
Survival (%)	54	62	64	68	64	60	82	66
Palpable masses								
Mice examined	50	50	50	50	50	50	50	50
Incidence	11	16	19	15	4	3	6	2
Body weight (g) ± SD								
Week 1	31.0 ± 2.6	30.8 ± 2.7	30.9 ± 2.0	30.7 ± 2.3	23.2 ± 1.6	23.4 ± 1.6	23.2 ± 1.6	23.1 ± 1.5
4	35.5 ± 2.9	34.8 ± 3.2	34.7 ± 2.4	33.7 ± 2.6**	26.9 ± 2.2	26.7 ± 2.1	26.0 ± 2.0	25.7 ± 2.1*
8	39.1 ± 3.4	38.7 ± 3.8	38.2 ± 3.2	37.0 ± 2.9**	29.7 ± 3.0	29.9 ± 3.0	29.2 ± 3.0	29.1 ± 2.8
16	44.2 ± 4.8	43.4 ± 4.9	42.0 ± 4.2	40.9 ± 4.0**	33.7 ± 4.5	33.8 ± 4.5	32.1 ± 3.8	32.2 ± 3.4
32	48.4 ± 6.3	47.8 ± 7.0	46.0 ± 5.7	44.3 ± 5.3**	37.6 ± 6.2	38.2 ± 6.3	35.6 ± 5.7	35.8 ± 5.4
48	51.6 ± 6.5	50.7 ± 7.6	48.6 ± 6.7	47.3 ± 6.1**	41.7 ± 7.6	43.3 ± 7.8	39.7 ± 7.6	37.3 ± 5.6*
64	53.3 ± 7.0	52.0 ± 7.8	49.5 ± 6.1*	49.3 ± 6.4*	44.0 ± 7.8	46.3 ± 8.9	41.2 ± 8.7	39.7 ± 6.4*
80	55.8 ± 6.5	53.0 ± 9.2	49.9 ± 6.1**	51.0 ± 6.3*	45.1 ± 9.4	48.2 ± 10.1	42.7 ± 8.9	40.7 ± 7.2
% difference: week 80		*−5*	*−11*	*−9*		*7*	*−5*	*−10*
Body weight gain (g) ± SD								
Days 1–2	1.7 ± 1.0	1.5 ± 1.0	1.0 ± 1.1**	0.8 ± 1.0**	1.8 ± 1.0	1.2 ± 1.0**	1.1 ± 1.1**	0.7 ± *0.7*
1–4	4.4 ± 1.2	4.0 ± 1.4	3.8 ± 1.3*	2.9 ± 1.2**	3.8 ± 1.4	3.3 ± 1.6	2.8 ± 1.4**	2.6 ± 1.5**
1–8	8.1 ± 2.2	7.9 ± 2.0	7.3 ± 2.1	6.3 ± 2.0**	6.6 ± 2.2	6.5 ± 2.3	5.9 ± 2.2	5.9 ± 2.2
1–16	13.1 ± 3.7	12.6 ± 3.2	11.2 ± 3.1**	10.2 ± 3.2**	10.5 ± 3.6	10.4 ± 3.8	8.9 ± 3.0*	9.1 ± 3.0
1–32	17.5 ± 5.3	17.0 ± 5.6	15.2 ± 4.9	13.6 ± 4.5**	14.5 ± 5.4	14.7 ± 5.6	12.4 ± 5.0	12.6 ± 4.9
1–48	20.7 ± 5.8	19.9 ± 6.5	17.8 ± 6.0	16.5 ± 5.3**	18.5 ± 7.0	19.9 ± 7.2	16.4 ± 6.8	14.2 ± 5.2**
1–64	22.2 ± 6.5	21.5 ± 7.0	18.6 ± 5.8**	18.5 ± 6.0**	20.7 ± 7.2	22.9 ± 8.3	17.9 ± 7.8	16.7 ± 6.1*
1–80	24.5 ± 6.0	22.6 ± 8.6	18.9 ± 6.3**	20.1 ± 5.6*	21.8 ± 8.9	24.7 ± 9.6	19.5 ± 8.1	17.8 ± 6.7
% difference: weeks 1–48		*−4*	*−14*	*−20*	–	*+8*	*−11*	*−23*
% difference: weeks 1–80		*−8.0*	*−23*	*−18*	–	*+13*	*−11*	*−19*

	Dose							
	Males				Females			
Observation	**0 ppm**	**50 ppm**	**250 ppm**	**500 ppm**	**0 ppm**	**50 ppm**	**250 ppm**	**500 ppm**
Food utilization (weight gain per 100g/food consumption)								
Weeks 1–4	3.33 ± 0.9	3.08 ± 1.1	2.93 ± 1.0	2.31 ± 1.0**	3.96 ± 1.2	3.50 ± 0.8	2.86 ± 0.6**	3.10 ± 0.8*
Weeks 1–13	2.33 ± 0.6	2.23 ± 0.6	2.02 ± 0.5*	1.84 ± 0.6**	2.21 ± 0.4	2.13 ± 0.5	1.89 ± 0.4	2.01 ± 0.5
Organ weights								
Epididimydes								
Absolute (g) ± SD	0.136 ± 0.03	0.129 ± 0.02	0.123 ± 0.02*	0.124 ± 0.02	NA	NA	NA	NA
% difference		*−5*	*−10*	*−9*	NA	NA	NA	NA
Covariance to bw ± SD	0.134 ± 0.004	0.129 ± 0.004	0.124 ± 0.004	0.125 ± 0.003	NA	NA	NA	NA
% difference		*−4*	*−7*	*−7*	NA	NA	NA	NA
Relative to bw (%) ± SD	0.244 ± 0.04	0.25 ± 0.05	0.24 ± 0.04	0.24 ± 0.04	NA	NA	NA	NA
% difference		*2*	*−2*	*−2*	NA	NA	NA	NA
Adrenals								
Absolute (g) ± SD	0.004 3 ± 0.002	0.004 3 ± 0.002	0.004 1 ± 0.002	0.003 9 ± 0.001	0.014 ± 0.03	0.009 9 ± 0.003	0.009 7 ± 0.003	0.009 1 ± 0.003
% difference		*0*	*−5*	*−9*		*−29*	*−31*	*−35*
Covariance to bw ± SD	0.0042 ±0.000 3	0.0043 ±0.000 3	0.0041 ±0.000 3	0.0039 ±0.000 3	0.015 ± 0.003	0.011 ± 0.003	0.0094 ± 0.003	0.008 ± 0.003
% difference		*2*	*−2*	*−7*		*−27*	*−37*	*−47*
Relative to bw (%) ± SD	0.0077 ± 0.003	0.0083 ± 0.003	0.0082 ± 0.004	0.0075 ± 0.003	0.039 ± 0.11	0.022 ± 0.01	0.023 ± 0.01	0.023 ± 0.01
% difference		*8*	*6*	*−3*		*−44*	*−41*	*−41*
Prostate								
Absolute (g) ± SD	1.51 ± 1.0	1.29 ± 0.8	1.21 ± 0.9	1.17 ± 0.9	NA	NA	NA	NA
% difference		*−15*	*−20*	*−23*	NA	NA	NA	NA
Covariance to bw ± SD	1.39 ± 0.18	1.28 ± 0.16	1.30 ± 0.16	1.19 ± 0.16	NA	NA	NA	NA
% difference		*−8*	*−6*	*−14*	NA	NA	NA	NA
Relative to bw (%) ± SD	2.74 ± 2.1	2.39 ± 1.2	2.41 ± 1.9	2.20 ± 1.7	NA	NA	NA	NA
% difference		*−13*	*−12*	*−20*	NA	NA	NA	NA
Kidney								
Absolute (g) ± SD	0.836 ± 0.1	0.818 ± 0.1	0.750 ± 0.1*	0.750 ± 0.1*	0.482 ± 0.08	0.492 ± 0.05	0.489 ± 0.09	0.437 ± 0.07*
% difference		*−2*	*−10*	*−10*		*2*	*1*	*−9*
Covariance to bw ± SD	0.81 ± 0.02	0.81 ± 0.02	0.76 ± 0.02	0.75 ± 0.02	0.479 ± 0.01	0.483 ± 0.01	0.491 ± 0.01	0.446 ± 0.01
% difference		*0*	*−6*	*−7*		*1*	*3*	*−7*

	Dose							
	Males				Females			
Observation	**0 ppm**	**50 ppm**	**250 ppm**	**500 ppm**	**0 ppm**	**50 ppm**	**250 ppm**	**500 ppm**
Relative to bw (%) ± SD	1.50 ± 0.2	1.57 ± 0.3	1.51 ± 0.3	1.47 ± 0.2	1.09 ± 0.2	1.08 ± 0.2	1.19 ± 0.3	1.12 ± 0.2
% difference		*+5*	*+1*	*−2*		*−1*	*+9*	*3*
Testis								
Absolute (g) ± SD	0.225 ± 0.05	0.254 ± 0.04*	0.243 ± 0.04	0.247 ± 0.04	NA	NA	NA	NA
% difference		*+13*	*+8*	*+10*	NA	NA	NA	NA
Covariance to bw ± SD	0.22 ± 0.008	0.25 ± 0.008*	0.246 ± 0.007	0.248 ± 0.007*	NA	NA	NA	NA
% difference		*+14*	*+12*	*+13*	NA	NA	NA	NA
Relative to bw (%) ± SD	0.4 ± 0.09	0.49 ± 0.10	0.49 ± 0.10	0.48 ± 0.09	NA	NA	NA	NA
% difference		*+23*	*+23*	*+20*	NA	NA	NA	NA
Ovary								
Absolute (g) ± SD	NA	NA	NA	NA	0.039 ± 0.054	0,064 ± 0.146	0.118 ± 0.250	0.272 ± 0.651*
% difference	NA	NA	NA	NA		*+64*	*+203*	*+597*
Covariance to bw ± SD	NA	NA	NA	NA	0.048 ± 0.062	0.085 ± 0.063	0.114 ± 0.056	0.249 ± 0.063
% difference	NA	NA	NA	NA		*+77*	*+138*	*+419*
Relative to bw (%) ± SD	NA	NA	NA	NA	0.099 ± 0.14	0.15 ± 0.39	0,29 ± 0.67	0.78 ± 1.87
% difference	NA	NA	NA	NA		*+52*	*+193*	*+688*
Uterus								
Absolute (g) ± SD	NA	NA	NA	NA	1.17 ± 1.6	1.19 ± 2.2	0.63 ± 0.5	0.62 ± 0.7
% difference	NA	NA	NA	NA		*+2*	*−46*	*−47*
Covariance to bw ± SD	NA	NA	NA	NA	1.22 ± 0.2	1.33 ± 0.2	0.59 ± 0.2	0.48 ± 0.2
% difference	NA	NA	NA	NA		*+9*	*−52*	*−61*
Relative to bw (%) ± SD	NA	NA	NA	NA	2.88 ± 3.9	2.84 ± 5.3	1.60 ± 1.6	1.70 ± 2.2
% difference	NA	NA	NA	NA		*−1*	*−44*	*−41*
Macroscopic findings								
Lymph node: mesenteric								
Number examined	50	50	50	50	50	50	50	50
Dark/dark red discoloration	3	6	8	9	4	4	10	8
Histopathology								
Lymph node: mesenteric								
Number examined	49	50	48	50	49	48	50	49
Angiectasis	1	4	3	1	3	5	3	4

	Dose							
	Males				Females			
Observation	**0 ppm**	**50 ppm**	**250 ppm**	**500 ppm**	**0 ppm**	**50 ppm**	**250 ppm**	**500 ppm**
Erythrophagocytosis:								
Minimal	2	2	8	5	3	1	2	4
Mild	4	11	1	7	3	4	6	10
Moderate	0	3	5	6	1	1	2	0
Marked	0	0	0	1	0	0	0	0
Total	6	16*	14*	19**	7	6	10	14
Gallbladder								
Number examined	49	47	48	45	48	44	50	47
Calculi								
Minimal	1	0	2	2	0	0	1	1
Mild	0	1	0	3	0	0	0	0
Moderate	0	1	3	3	0	0	1	0
Total	1	2	5	8*	0	0	2	1

NA: Not applicable; SD: Standard deviation; bw: Body weight; *Source: adapted from Shearer, 2018g*

* $p < 0.05$, ** $p < 0.01$, statistically significant difference from control group mean (Dunnett's)

The NOAEL for the study was 50 ppm (equal to 6.4 mg/kg bw per day) based on lower body weight and reduced body weight gain at 250 ppm (Shearer, 2018g).

Rat

Spiropidion (purity 98.4%) was administered for 104 weeks to four groups of 52 male and 52 female Crl: WI (Han) rats (6–7 weeks old) at dietary concentrations of 0, 50, 100 or 300 ppm (females)/500 ppm (males). This was equal to 0, 2.4, 4.7 and 24.0 mg/kg bw per day for males and 0, 3.1, 6.1 and 18.7 mg/kg bw per day for females. An additional four groups of 12 animals per sex were administered spiropidion for 52 weeks at a dietary concentration of 0, 50, 100 or 300 ppm (females)/500 ppm (males), which was equal to 0, 2.8, 5.5 and 27.6 mg/kg bw per day for males and 0, 3.5, 6.8 and 20.5 mg/kg bw per day for females. All animals were checked in their cages twice daily for general health/mortality and moribundity. All animals were removed from their cages and received a detailed clinical examination (size, appearance, position and duration of any masses) on a weekly basis commencing at the start of week –1. Body weights, food consumption and test item consumption were recorded during pretreatment, weekly during weeks 1–14 and once every two weeks during weeks 16–104. Food utilization was determined during weeks 1–14. Water consumption was qualitatively monitored by visual inspection of the water bottles on a regular basis throughout the study. Ophthalmoscopy examinations were carried out during pretreatment, at week 50 and weeks 103/104. Detailed functional observations and functional tests (including motor activity) were carried out during pretreatment, at week 12/13, week 25/26 and week 51/52. Blood samples were collected from all animals at weeks 14, 27 and 53 and an adequate number of haematological and biochemical parameters evaluated. Blood smear evaluation was conducted on surviving animals at weeks 52/53 and 78/80. Urine analysis was conducted on samples collected at weeks 13, 26, 51. At termination all animals were subject to a complete necropsy examination. The following organs were weighed: brain, epididymides, adrenal glands, seminal vesicles with prostate and coagulating glands, heart, kidneys, liver, ovaries, spleen, testes, thymus, uterus with cervix and oviducts. All tissues were analysed microscopically, except bone marrow smears, oviducts and nasal cavity.

There were no treatment-related mortalities or clinical signs of toxicity in the chronic or the carcinogenicity cohorts. In the carcinogenicity cohort the number of animals with at least one palpable masse was equivalent in males and their controls. In females, the number was higher at all dose levels when compared to controls, but with no dose relationship and therefore this was not considered to be treatment-related.

Body weight and body weight gain were lower compared with controls (6% and 10%, respectively) from day 8 (week 2) throughout the treatment period in males given 500 ppm. Statistical significance was achieved in body weight from day 22 (week 3) to day 400 (week 58) and in supression of body weight gain from day 8 (week 1) to day 484 (week 70) at 500 ppm. At a dose of 100 ppm, body weight gain was 7% lower than controls from day 1 to 22. It was also noted that the differences from control became smaller towards the end of the treatment period. Food utilisation was lower compared to controls, and with statistical significance, during the periods weeks 1–4, 5–8 and 9–13 in males given 500 ppm. Overall food utilisation (weeks 1–13) in males given 500 ppm was similar to that of the control group.

No treatment-related effects were observed for water consumption or noted as a result of ophthalmoscopic examinations.

There were no treatment-related findings in the quantitative functional observations. Occasionally statistically significant changes were reported, however, due to the lack of any dose relationship and the lack of consistency over the time points, these findings were considered to be incidental. There were no treatment-related differences in the motor activity assessment. Occasionally statistically significant changes were reported, however, due to the lack of any dose relationship, no consistency through the observation period or across time points, and since they resulted in no change to the overall assessment these changes were considered incidental.

Haematology analysis showed a statistically significant slight increase in fibrinogen compared with controls at weeks 14, 27 and 53 in males given 500 ppm. However, due to many of the individual values being within the concurrent control range and a lack of any associated changes in other coagulation parameters, it was considered of no toxicological significance. No other treatment-related changes in haematological parameters were reported. Occasional statistically significant changes were reported, but these were considered to be incidental and unrelated to treatment due to their small magnitude and/or directional difference, their lack of change over time, and of any dose–effect relationship, as well as an absence of correlating histopathology findings.

Clinical chemistry assessment showed a statistically significant decrease in cholesterol at weeks 4, 27 and 53 in males at 500 ppm. Albumin was lower compared with controls (statistically significant) at week 14 (500 ppm only), and weeks 27 and 53 in males at 100 or 500 ppm. This was reflected in lower total protein levels (statistically significant) at week 27 in males at 100 ppm, and at week 53 in males at 500 ppm, but with no clear dose–response relationship at the highest dose. However, due to the small magnitude of albumin and total protein changes these findings were considered of no toxicological significance. An increase of AST activity was reported in males at 500 ppm at weeks 27 and 53 (34% and 58% of the control, respectively) achieving statistical significance only at week 27. Occasional statistically significant changes were reported for other clinical chemistry parameters, however, due to their size and/or direction of difference, the lack of consistent change over time, lack of any clear dose–response relationship, and absence of correlating histopathology findings these occasional differences were considered incidental and unrelated to treatment.

Urine analysis revealed lower specific gravities compared to controls (statistically significant) at week 26 in males dosed at 50 or 500 ppm, and at weeks 26 and 51 in females given 300 ppm. However, due to the small differences seen, the lack of agreement between sexes and the lack of any correlating histopathology findings these differences in specific gravity were considered incidental.

No treatment-related effects on organ weights were noted in the chronic toxicity cohort. In the carcinogenicity cohort, ovarian weights were decreased by approximately 80% at all dose levels when compared to controls, but with no statistical significance . It was noted that the individual values in all dose groups were within the particularly wide control range (absolute weights, 0.03–8.9 g) and that one female in the control group had a particularly high ovarian weight. In the absence of a dose–response relationship and lacking histopathological correlates, ovary weight changes were considered to be of no toxicological significance.

A statistically insignificant increase in uterus weights was reported in all dosed groups, with no clear dose–response relationship. Almost all individual values from all dosed groups were within the control range (absolute weight, 0.46–1.7 g), except one female at 50 ppm and two females, one each at 100 and 300 ppm, the uterine weights of which were higher than the upper limit of the control range.

Given the lack of dose–response relationship or any histopathological correlates, the observed uterus weight changes were considered of no toxicological significance.

No test substance-related gross findings were noted in the chronic cohort. A statistically insignificant increase in the incidence of bile duct enlargement was reported in males at 500 ppm. This change correlated with the presence of luminal dilatation observed by microscopy.

No test substance-related histopathology findings were noted in the chronic cohort. In the carcinogenicity cohort, a statistically insignificant increase in the incidence of luminal dilatation and inflammation of the bile duct was observed in males at 500 ppm when compared to controls. This change correlated with the bile duct enlargement that was observed macroscopically. A statistically insignificant increas in the incidence of kidney mineralization (pelvis) was reported in males at 100 and 500 ppm, when compared to controls. In the absence of treatment-related necrosis, degeneration or inflammation, the increased incidence of mineralization was not considered adverse.

The following neoplasms were reported in males or females at the high-dose levels (except for Leydig cell adenomas) with their corresponding controls showing no occurrence of these tumours. None of these tumours were statistically significant in either pair-wise comparison or trend tests.

- Multiple fibroadenomas in the mammary gland were reported in two females at 300 ppm. It was noted that the total of mammary gland fibroadenomas was increased in females at all dose levels, but with no dose–effect relationship. The Meeting concluded that fibroadenoma in the mammary gland of females was not treatment-related.
- An increased incidence of malignant ependymoma was reported in animals at 500 ppm (two males). Because there are no adequate diagnostic criteria by which neuroectodermal neoplasms in rats can be distinguished, it was considered appropriate to combine neuroectodermal tumours in this case where a mixed patterns of differentiation was apparent. The combination of total incidences of malignant astrocytoma and ependymoma was 1, 0, 1 and 2 for the control, 50, 100 and 500 ppm groups, respectively. The increased incidence at the high dose was considered equivocal.

An increase in Leydig cell adenomas were reported at 100 ppm (two animals) and at 500 ppm (two animals), with no obvious dose response. No treatment-related preneoplastic lesions were reported. The incidences of this finding were higher than the laboratory background mean, but was within its range. The Meeting noted that in the toxicity database there was no evidence of hypothalamus-pituitary–gonadal axis perturbation and that this tumour is generally considered of low human relevance.

Table 21. Key findings of the two-year toxicity study in rats

	Dose (ppm)							
	Males				Females			
	0	**50**	**100**	**500**	**0**	**50**	**100**	**300**
Mortality (carcinogenicity cohort)								
Killed	14	13	20	16	9	12	11	11
Died	5	3	3	2	0	4	3	1
Total	19	16	23	18	9	16	14	12
Survival (%)	63	69	56	65	83	69	73	77
Palpable masses								
Number examined	52	52	52	52	52	52	52	52
	13	19	16	11	10	18	17	15
Body weight (g) ± SD								
Week 1	177 ± 14	177 ± 14	177 ± 13	176 ± 14	138 ± 10	139 ± 11	138 ± 12	139 ± 11
8	219 ± 16	220 ± 17	218 ± 16	214 ± 16	158 ± 12	159 ± 11	157 ± 14	158 ± 12

	Dose (ppm)							
	Males				Females			
	0	50	100	500	0	50	100	300
22	281 ± 20	279 ± 22	275 ± 20	271 ± 22*	185 ± 14	188 ± 14	185 ± 16	186 ± 15
92	409 ± 35	408 ± 41	399 ± 33	392 ± 38*	237 ± 20	241 ± 18	237 ± 19	237 ± 19
176	471 ± 42	469 ± 47	458 ± 40	447 ± 46**	257 ± 23	259 ± 19	255 ± 22	257 ± 22
274	525 ± 49	525 ± 58	509 ± 45	494 ± 53**	282 ± 35	282 ± 25	279 ± 32	278 ± 30
358	562 ± 59	564 ± 68	548 ± 52	532 ± 63*	298 ± 43	301 ± 33	293 ± 38	294 ± 37
456	587 ± 68	579 ± 77	564 ± 54	555 ± 72	302 ± 40	318 ± 43	297 ± 39	302 ± 47
540	617 ± 75	616 ± 95	596 ± 63	582 ± 86	328 ± 44	348 ± 52	322 ± 48	324 ± 55
638	623 ± 82	635 ± 110	613 ± 73	616 ± 97	354 ± 54	366 ± 49	352 ± 59	355 ± 60
722	643 ± 77	646 ± 106	632 ± 85	628 ± 95	368 ± 57	389 ± 53	367 ± 67	371 ± 58
Body weight gain (g) ± SD								
Days 1–8	42 ± 4	43 ± 5	41 ± 5	38 ± 5**	21 ± 5	20 ± 4	20 ± 5	19 ± 4
% difference	-	*+2.4*	*−2.4*	*−9.5*	-	*−4.8*	*−8*	*−9.5*
1–22	105 ± 11	102 ± 12	98 ± 11**	95 ± 13**	48 ± 8	49 ± 8	47 ± 8	46 ± 8
% difference	-	*−2.9*	*−6.7*	*−9.5*	-	*+2.1*	*−2.1*	*−4.2*
1–92	232 ± 29	231 ± 34	221 ± 26	215 ± 32**	99 ± 16	102 ± 14	99 ± 14	97 ± 13
% difference	-	*−0.4*	*−4.7*	*−7.3*	-	*+3.0*	*0.0*	*−2.0*
1–176	295 ± 37	293 ± 41	280 ± 33	270 ± 40**	119 ± 19	121 ± 16	117 ± 17	118 ± 17
% difference	-	*−0.7*	*−5.1*	*−8.5*	-	*+1.7*	*−1.7*	*−0.8*
1–274	348 ± 43	348 ± 51	332 ± 38	318 ± 48**	145 ± 32	144 ± 21	141 ± 27	139 ± 25
% difference	-	*0.0*	*−4.6*	*−8.6*	-	*−0.7*	*−2.8*	*−4.1*
1–358	385 ± 54	387 ± 62	371 ± 46	356 ± 58**	160 ± 40	163 ± 31	155 ± 33	155 ± 33
% difference	-	*+0.5*	*−3.6*	*−7.5*	-	*+1.9*	*−3.1*	*−3.1*
1–456	414 ± 61	406 ± 73	389 ± 50	382 ± 68*	168 ± 37	183 ± 41	163 ± 34	166 ± 45
% difference	-	*−1.9*	*−6.0*	*−7.7*	-	*+8.9*	*−3.0*	*−1.2*
1–540	445 ± 68	443 ± 92	420 ± 59	410 ± 83	194 ± 42	212 ± 51	187 ± 44	188 ± 52
% difference	-	*−0.4*	*−5.6*	*−7.9*	-	*+9.3*	*−3.6*	*−3.1*
1–638	451 ± 77	461 ± 107	438 ± 72	444 ± 93	220 ± 53	230 ± 48	217 ± 54	218 ± 58
% difference	-	*+2.2*	*−2.9*	*−1.6*	-	*+4.5*	*−1.4*	*−0.9*

	Dose (ppm)							
	Males				Females			
	0	50	100	500	0	50	100	300
1–722	472 ± 71	472 ± 101	458 ± 83	454 ± 92	234 ± 56	252 ± 54	233 ± 62	234 ± 56
% diff	-	*0.0*	*−3.0*	*−3.8*	-	*+7.7*	*−0.4*	*0.0*
Food utilization (weight gain per 100g/food consumption) ± SD								
Weeks 1–4	20.4 ± 1.2	20.1 ± 0.7	19.9 ± 0.9	19.2 ± 1.0**	13.4 ± 0.7	13.4 ± 1.0	13.6 ± 1.0	13.4 ± 0.8
5–8	10.5 ± 0.9	10.1 ± 0.7	10.1 ± 0.7	9.5 ± 0.7**	6.2 ± 0.7	5.8 ± 0.6	5.8 ± 0.6	6.0 ± 1.0
9–13	5.2 ± 0.9	5.7 ± 0.7	5.5 ± 0.7	6.0 ± 0.5*	2.0 ± 0.8	2.3 ± 0.7	2.3 ± 0.7	2.1 ± 0.9
1–13	11.6 ± 0.6	11.5 ± 0.4	11.4 ± 0.6	11.2 ± 0.5	6.6 ± 0.5	6.7 ± 0.5	6.8 ± 0.3	6.7 ± 0.4
% difference weeks 1–13		*−0.9*	*−1.7*	*−3.4*	–	*+1.5*	*+3.0*	*+1.5*
Haematology								
Fibrinogen (mg/dL) ± SD								
Week 14	189 ± 17	192 ± 24	195 ± 19	211 ± 19*	131 ± 23	131 ± 21	138 ± 16	143 ± 15
% difference	-	*+1.6*	*+3.2*	*+11.6*	-	*0.0*	*+5.3*	*+9.2*
Week 27	198 ± 22	189 ± 17	200 ± 21	221 ± 23*	127 ± 18	128 ± 16	137 ± 14	141 ± 15
% difference	-	*−4.5*	*+1.0*	*+11.6*	-	*+0.8*	*+7.9*	*+11.0*
Week 53	189 ± 16	182 ± 14	193 ± 16	208 ± 18*	134 ± 23	128 ± 40	131 ± 13	133 ± 19
% difference	-	*−3.7*	*+2.1*	*+10.1*	-	*−4.5*	*−2.2*	*−0.7*
Clinical chemistry								
Cholesterol (mmol/L) ± SD								
Week 14	1.5 ± 0.2	1.8 ± 0.3**	1.5 ± 0.2	1.2 ± 0.2**	1.3 ± 0.2	1.3 ± 0.2	1.4 ± 0.2	1.5 ± 0.2
% difference	-	*+20.0*	*0.0*	*−20.0*	-	*0.0*	*+7.7*	*+15.4*
Week 27	1.7 ± 0.3	1.8 ± 0.2	1.5 ± 0.3	1.3 ± 0.2**	1.5 ± 0.5	1.4 ± 0.3	1.5 ± 0.3	1.5 ± 0.3
% difference	-	*+5.9*	*−11.8*	*−23.5*	-	*−6.7*	*0.0*	*0.0*
Week 53	2.1 ± 0.4	2.1 ± 0.4	2.0 ± 0.3	1.5 ± 0.2**	1.7 ± 0.3	1.8 ± 0.3	1.9 ± 0.5	1.9 ± 0.5
% difference	-	*0.0*	*−4.8*	*−28.6*	-	*+5.9*	*+11.8*	*+11.8*
Albumin (g/L) ± SD								
Week 14	43 ± 2	42 ± 1	42 ± 1	41 ± 2*	48 ± 2	49 ± 3	50 ± 2	49 ± 2
% difference	-	*−2.3*	*−2.3*	*−4.7*	-	*+2.1*	*+4.2*	*+2.1*
Week 27	44 ± 1	43 ± 1	42 ± 1**	42 ± 1**	51 ± 3	50 ± 2	50 ± 2	48 ± 2*
% difference	-	*−2.3*	*−4.5*	*−4.5*	-	*−2.0*	*−2.0*	*−5.9*
Week 53	43 ± 2	42 ± 1	42 ± 1*	41 ± 1**	49 ± 2	50 ± 3	50 ± 3	49 ± 3
% % difference	-	*−2.3*	*−2.3*	*−4.7*	-	*+2.0*	*+2.0*	*0.0*

	Dose (ppm)							
	Males				Females			
	0	50	100	500	0	50	100	300
Total protein (g/L) ± SD								
Week 14	62 ± 2	62 ± 2	61 ± 2	60 ± 3	64 ± 2	64 ± 2	65 ± 2	65 ± 3
% difference	-	*0.0*	*−1.6*	*−3.2*	-	*0.0*	*+1.6*	*+1.6*
Week 27	63 ± 2	61 ± 2	60 ± 3**	61 ± 2	66 ± 4	64 ± 3	64 ± 3	63 ± 2
% difference	-	−3.2	−4.8	−3.2	-	−3.0	−3.0	−4.5
Week 53	64 ± 2	62 ± 3*	63 ± 2	62 ± 2*	66 ± 3	66 ± 3	66 ± 3	65 ± 3
% difference	-	*−3.1*	*−1.6*	*−3.1*	-	*0.0*	*0.0*	*−1.5*
Aspartate transaminase (U/L) ± SD								
Week 14	60 ± 5	60 ± 9	60 ± 13	65 ± 18	64 ± 13	57 ± 13	57 ± 6	66 ± 21
% difference	-	*0*	*0*	*+8*	-	*−11*	*−11*	*+3*
Week 27	58 ± 8	53 ± 3	62 ± 16	78 ± 32*	69 ± 21	69 ± 25	62 ± 13	88 ± 62
% difference		*−9*	*+7*	*+34*		*0*	*−10*	*+28*
Week 53	71 ± 16	64 ± 16	61 ± 16	112 ± 89	90 ± 45	86 ± 29	80 ± 23	95 ± 38
% difference	-	*−10*	*−14*	*+58*	-	*−4*	*−11*	*+6*
Urinalysis								
Specific gravity ± SD								
Week 13	1.031 ± 0.01	1.036 ± 0.01	1.033 ± 0.02	1.025 ± 0.01	1.028 ± 0.01	1.029 ± 0.01	1.023 ± 0.01	1.032 ± 0.01
Week 26	1.038 ± 0.01	1.026 ± 0.01*	1.028 ± 0.01	1.027 ± 0.01*	1.025 ± 0.01	1.020 ± 0.01	1.025 ± 0.01	1.038 ± 0.01**
Week 51	1.031 ± 0.01	1.040 ± 0.02	1.034 ± 0.02	1.032 ± 0.01	1.025 ± 0.01	1.029 ± 0.01	1.032 ± 0.01	1.039 ± 0.02*
Organ weights (carcinogenicity cohort)								
Ovary								
Absolute (g) ± SD	NA	NA	NA	NA	0.449 ± 1.48	0.100 ± 0.03	0.093 ± 0.03	0.094 ± 0.03
% difference	NA	NA	NA	NA	-	*−78*	*−79*	*−79*
Covariance to bw ± SD	NA	NA	NA	NA	0.452 ± 0.11	0.092 ± 0.13	0.096 ± 0.12	0.094 ± 0.12
% difference	NA	NA	NA	NA	-	*−80*	*−79*	*−79*
Relative to bw (%) ± SD	NA	NA	NA	NA	0.12 ± 0.39	0.026 ± 0.11	0.026 ± 0.009	0.025 ± 0.008
% difference	NA	NA	NA	NA	-	*−78*	*−78*	*−79*
Uterus								
Absolute (g) ± SD	NA	NA	NA	NA	0.85 ± 0.35	1.22 ± 1.86	1.03 ± 0.70	1.30 ± 1.92
% difference	NA	NA	NA	NA	-	*+42*	*+20*	*+51*
Covariance to bw ± SD	NA	NA	NA	NA	0.84 ± 0.21	1.25 ± 0.23	1.03 ± 0.22	1.30 ± 0.23
% difference	NA	NA	NA	NA	-	*+49*	*+23*	*+55*
Relative to bw (%) ± SD	NA	NA	NA	NA	0.25 ± 0.13	0.33 ± 0.52	0.30 ± 0.24	0.36 ± 0.49

	Dose (ppm)							
	Males				Females			
	0	50	100	500	0	50	100	300
% difference	NA	NA	NA	NA	-	*+32*	*+20*	*+44*
Macroscopic findings (carcinogenicity cohort)								
Bile duct: Number examined	1	1	2	8	1	0	2	2
Enlargement	1	1	2	7	1	-	0	1
Histopathology findings (carcinogenicity cohort)								
Bile duct								
Number examined	1	1	2	8	1	0	1	2
Dilatation, lumen								
Minimal	0	1	1	2	1	0	1	1
Mild	0	0	1	3	0	0	0	1
Moderate	0	0	0	3	0	0	0	0
Marked	1	0	0	0	0	0	0	0
Total	1	1	2	8	1	0	1	2
Inflammation								
Minimal	0	0	0	3	0		1	0
Mild	1	0	0	0	0		0	0
Moderate	0	0	0	1	0		0	0
Total	1	0	0	4	0		1	0
Kidney								
Number examined	51	49	51	51	52	50	49	51
Mineralization, (pelvis)	9	10	12	15	39	28	26*	38
Neoplastic findings								
Brain								
Number examined	64	64	64	64	64	64	64	64
Ependymoma: malignant	0	0	0	2	0	0	0	0
Astrocytoma: malignant	1	0	1	0	0	0	0	0
Total	1	0	1	2	0	0	0	0
Mammary gland								
Number examined	47	51	45	45	52	52	51	52
Fibroadenoma	0	1	0	0	4	7	5	4
Fibroadenoma: multiple	0	0	0	0	0	1	1	2
Total number [%]	0	1	0	0	4 [7.7]	8 [15.4]	6 [11.8]	6 [11.5]
HCD female mammary total fibroadenoma: mean = 12.4%; range = 0–19.2%								
Testis								
Number examined	63	64	64	64	NA	NA	NA	NA
Hyperplasia								
Mesothelial	2	1	0	0	NA	NA	NA	NA

	Dose (ppm)							
	Males				Females			
	0	50	100	500	0	50	100	300
Leydig cells	0	0	1	0	NA	NA	NA	NA
Leydig cell adenoma [%]	0	0	2 [3.1]	2 [3.1]	NA	NA	NA	NA
HCD: Leydig cell adenomas: mean = 1.5%; range = 0 to 3.8%								

bw: Body weight; NA: Not applicable SD: Standard deviation Source: adapted from Shearer, 2018h

HCD: Hisorical control data; generated between 2012 and 2017, including 7 studies; 466 males and 466 females

** $p < 0.05$, ** $p < 0.01$, statistically significant difference from control group mean (Dunnett's)

The NOAEL for non-neoplastic lesions was 100 ppm (equal to 4.7 mg/kg bw per day), based on increased incidence of luminal dilatation and inflammation in the bile duct observed in males at 500 ppm (equal to 24.0 mg/kg bw per day). No adverse effects were observed in females up to the highest dose.

The NOAEL for carcinogenicity was 50 ppm (equal to 2.4 mg/kg bw per day) based on an equivocal increase in testicular interstitial cell adenomas at 100 ppm (equal to 4.7 mg/kg bw per day). Equivocal malignant ependymomas in males were seen at the highest dose level (Shearer, 2018h).

2.4 Genotoxicity

Table 22. Results of genotoxicity studies performed with spiropidion

Type of study	Organism/Cells	Dose range tested	Purity	Result	Reference
		In vitro			
Ames test	*Salmonella typhimurium* (TA100, TA98, TA1535 and TA1537) *Escherichia coli* (WP2 and WP2*uvrA*)	**Experiment 1** Incorporation test ±S9: 1.5–5000 µg/plate **Experiment 2** Pre-incubaion test ±S9: 5–5000 µg/plate	95.6%	With and without S9 activation: negative	Thompson, 2018
Ames test	*Salmonella typhimurium* (TA100, TA98, TA1535 and TA1537) *Escherichia coli* (WP2, WP2*uvrA*)	**Experiment 1** Incorporation test ±S9: 3–5000 µg/plate **Experiment 2** Pre-incubaion test ±S9: 33–5000 µg/plate	98.4%	With and without S9 activation: negative	Sokolowski 2014
Chromosome aberration assay	Human lymphocytes	**−S9:** 149.7–2457.1 µg/mL **+S9:** 262–802.3 µg/mL	98.4%	With and without S9 activation: positive	Bohnenberger, 2015

Forward mutation assay	Mouse lymphoma L5178Y cells, *TK* locus	**Experiment I** −S9 8.4–100.5 µg/mL **Experiment II** −S9 15–70 µg/mL **Experiment III** −S9 60–80 µg/mL **Experiment I** +S9 16.8–134 µg/mL **Experiment II** +S9 30–120 µg/mL	98.4%	With and without S9 activation: negative	Wollny, 2015
Micronucleus	Human lymphocytes	**Experiment I** −S9 41.6–223 µg/mL **Experiment II** −S9 74.6–400 µg/mL **Experiment I** +S9 41.6–223 µg/mL	95.6%	With and without S9 activation: negative	Naumann, 2018
		In vivo			
Micronucleus assay	Rat (Wistar) (gavage)	125–500 mg/kg bw	95.6%	Negative	Dunton, 2018
Micronucleus assay	Rat (Wistar) (gavage)	125–500 mg/kg bw	98.4%	Negative	Dunton, 2015
Chromosome aberration assay	Rat (Wistar) (gavage)	Males: 500–2000 mg/kg bw Females: 187.5–750 mg/kg bw	98.4%	Negative	Whitwell, 2018

(a) In vitro

Spiropidion (purity 95.6%) was tested for mutagenic potential in the plate incorporation test (Experiment 1) and the pre-incubation test (Experiment 2) on *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100, and the *Escherichia coli* strains WP2 *uvrA* pKM101 and WP2 pKM101, at concentrations up to 5000 µg/plate in the absence and presence of S9 mix. No meaningful cytotoxicity was observed at any concentration, either in the presence or absence of metabolic activation (S9 mix), in either experiment. No precipitation occurred up to the highest concentration used. There was no significant concentration-related increase in the number of revertant colonies, with or without S9 mix. Positive controls showed a marked increase in the number of revertant colonies. Spiropidion was not mutagenic under the test conditions used (Thomson, 2018).

In a second mutagenicity test spiropidion (purity 98.4%) was tested in a plate incorporation test (experiment I) and in a pre-incubation test (experiment II) using the *Salmonella typhimurium* strains TA1535, TA1537, TA98, and TA100, and the *Escherichia coli* strains WP2 *uvrA* pKM101 and WP2 pKM101, at concentrations up to 5000 µg/plate in the absence and presence of S9 mix. No meaningful cytotoxicity was observed at any concentration, either in the presence or absence of metabolic activation (S9 mix), in either experiment. No precipitation occurred up to the highest concentration used. There was no significant concentration-related increase in the number of revertant colonies, with or without S9 mix. Positive controls showed a marked increase in the number of revertant colonies. Spiropidion was not mutagenic under the test conditions used (Sokolowski, 2014).

Spiropidion (purity 98.4%) was assessed for it potential to induce structural chromosomal aberrations in cultured human lymphocytes in a single experiment (four hour exposure) with two parallel cultures for each experimental group at concentrations of 149.7, 262.0, 1404.1 and 2457.1 µg/mL in the absence of S9 mix, and at concentrations of 262.0, 458.5 and 802.3 µg/mL in the presence of S9 mix. Per culture at least 100 metaphases were evaluated for structural chromosomal aberrations. Precipitation of the

test substance in the culture medium was observed at the end of treatment in the absence of S9 mix at 262.0 µg/mL and above and in the presence of S9 mix at 458.5 µg/mL and above.

The results obtained indicated statistically significant and biologically relevant increases (exceeding the range of the laboratory historical solvent control data) in chromosomal aberrations in human lymphocytes cultured in vitro at the two highest concentrations tested (1404.1 and 2457.1 µg/mL) in the absence of S9 metabolism and in its presence (802.3 and 458.5 µg/mL). It should be noted here that precipitation of the test item was observed at all of the three concentrations selected for the scoring of aberrations (2457.1, 1404.1 and 262.0 µg/mL) in the absence of S9 metabolism and in its presence at (802.3 and 458.5 µg/mL). In this respect, even if the cytotoxicity occurs above the lowest insoluble concentration, it is advisable to test at only one concentration producing turbidity (or with a visible precipitate) as also recommended by the relevant current OECD Guideline 475, because artifactual effects may result from the precipitate. In addition, a limited number of metaphases per culture (100 instead of 150) were scored thus reducing the statistical power of the evaluation. The highest concentration level selected for scoring in the absence of S9 metabolism (2457.1 µg/mL) clearly exceeded the maximum concentration of 2000 µg/mL recommended by the relevant test guideline. On this basis the Meeting considered the positive outcome observed as unreliable. Appropriate mutagens were used as positive controls. They induced statistically significant increases in cells with structural chromosome aberrations.

It was concluded that the positive outcome observed is not reliable (Bohnenberger, 2015).

Spiropidion (purity 98.4%) was tested for its potential to induce mutations at the mouse lymphoma thymidine kinase (*TK*) locus using the cell line L5178Y. The assay was performed in three independent experiments, using two parallel cultures each. Experiments I and II were performed with and without liver microsomal activation and a treatment period of four hours. Experiment III was solely performed without metabolic activation and a treatment period of four hours. The maximum concentration in the main experiments was limited by cytotoxic effects. Experiment I was evaluated using five concentrations ranging from 8.4 to 100.5 µg/mL without metabolic activation, and from 16.8 to 134 µg/mL with metabolic activation. Experiment II was evaluated using four concentrations ranging from 15.0 to 70 µg/mL without metabolic activation, and five concentrations ranging from 30.0 to 120.0 µg/mL with metabolic activation. Experiment III was evaluated using five concentrations ranging from 60.0 to 80.0 µg/mL. DMSO served as vehicle.

Relevant cytotoxic effects indicated by a relative cloning efficiency 1 (survival) or relative total growth of less than 50% in both cultures occurred in Experiment I at 33.5 µg/mL and above without metabolic activation and at 100.5 µg/mL and above with metabolic activation. In Experiment II cytotoxic effects were noted at 60.0 µg/mL and above without metabolic activation and at 100 µg/mL and above with metabolic activation. In Experiment III, solely performed without metabolic activation, relevant cytotoxic effects were noted at 70 µg/mL and above.

No substantial and reproducible increase in the mutation frequency was noted in the main experiments with or without metabolic activation. The threshold of a global evaluation factor (GEF) of 126 above the mutation frequency of the solvent control was solely exceeded at the maximum concentration of Experiment II, culture I with metabolic activation (242 compared to 211 colonies per 106 cells). However, the increase was not reproduced in the parallel culture under identical conditions. Furthermore, the increase occurred at a relative total growth of just 4.9 and thus below the recommended limit of 10%. However, the data are covered under the International Workgroup on Genotoxicity Testing (IWGT) exception criteria and thus are reported since the relative total growth (RTG) showed no cytotoxic level at the next lowest concentration of 100 µg/mL. A statistically significant dose-dependent trend of the mutation frequency was solely detected in the first culture of the Experiment II without metabolic activation. Since the mutation frequency did not exceed the GEF as indicated above, the statistical result is considered as a biologically irrelevant fluctuation. Positive and negative controls gave the expected results.

It was concluded that spiropidion is considered to be non-mutagenic according to this mouse lymphoma assay (Wollny, 2015).

Spiropidion (purity 95.6%) dissolved in tetrahydrofuran was assessed for its potential to induce micronuclei in human lymphocytes in vitro in two independent experiments. In each experimental group two parallel cultures were analysed. Per culture 1000 binucleated cells were evaluated for cytogenetic

damage. The highest concentration tested was identified as the lowest concentration that showed phase separation at the end of treatment. In the first experiment (pretest for toxicity), cells were exposed to four concentrations ranging from 41.6 to 223 µg/mL for four hours with and without metabolic activation. In the second experiment cells were exposed for 20 hoursto four concentrations ranging from 74.6 to 400 µg/mL without metabolic activation. Micronucleus formation was evaluated during the postexposure period and thus a cytokinesis blocker, cytochalasin B (4 µg/mL) was added.

No relevant influence on the osmolarity and pH was observed. In this study no biologically relevant increase in the number of micronucleate cells was observed after treatment with the test substance. The mean percentage of the micronuclei in all treated conditions was within the 95% of control limit and none of the values were increased to a statistically significant degree when compared to the vehicle control. Demecolcin (100 ng/mL), mitomycin C (0.8 µg/mL) or cyclophosphamide (15.0 µg/mL) were used as positive controls and showed statistically significant increases in binucleated cells with micronuclei.

In this case inappropriate exposure to cytochalasin B following treatment with the test item at both the short (four hour) and long (20 hour) treatment times was considered to compromise the reliability of the study (Naumann, 2018).

(b) In vivo

Spiropidion (purity 98.4%) was tested for its ability to induce micronucleus formation in polychromatic erythrocytes in the bone marrow of young adult Crl:WI(Han) rats. Four groups, each of six male rats were treated at dose levels of 0, 125, 250 or 500 mg/kg bw per day on two successive days, approximately 24 hours apart, at a dose volume of 10 mL/kg bw (in 0.5% w/v aqueous CMC, acidified to pH 5). A positive control group, also of six male rats, was given a single 15 mg/kg bw oral (5 mL/kg bw) dose of cyclophosphamide (CPA). Dose levels were identified after a preliminary toxicity assay in which three males were dosed by gavage at 500 and 800 mg/kg bw per day and three females at 320 and 500 mg/kg bw per day on two successive days approximately 24 hours apart. Based on the results of this phase, the maximum tolerated dose (MTD) was considered to be 500 mg/kg bw per day in males and 320 mg/kg bw per day in females. According to the laboratory criteria, as there were no substantial intersex differences in toxicity (a difference in MTD of three-fold or greater), the main study was conducted in males only, with the high dose selected as 500 mg/kg bw per day. Two thousand polychromatic erythrocytes (PCEs), including micronucleated PCEs (MN-PCEs), were counted for each animal. The numbers of normochromatic erythrocytes (NCEs) and micronucleated NCEs (MN-NCEs) were also recorded for the first 1000 cells scored.

Clinical signs were observed in two animals at 500 mg/kg bw per day after they were placed in the warming chamber, prior to the terminal bleed. These signs included decreased activity, unsteady gait, laboured breathing, incoordination and piloerection in one animal, which was euthanised before blood sampling due to clinical condition. Abnormal sensitivity to touch or disturbance was observed in the second animal.

There were no statistically significant increases in micronucleus frequency in male rats treated at any dose level when compared to the negative control group. All individual numbers of micronuclei were within the range of historical controls.

No bone marrow toxicity was observed with respect to the PCE : NCE ratio. However, exposure was confirmed by the presence of metabolite SYN547305 (a major spiropidion metabolite) in both blood and plasma samples of all dosed groups.

It was concluded that spiropidion was not clastogenic or aneugenic following an oral gavage dose up to the MTD of 500 mg/kg bw per day in male rats (Dunton, 2015).

In a second micronucleus assay, spiropidion (purity 95.6%) was administered by gavage to four groups, each of six male rats at dose levels of 0, 125, 250 or 500 mg/kg bw per day on two successive days, approximately 24 hours apart, at a dose volume of 10 mL/kg bw (0.5% w/v aqueous CMC, acidified to pH 5). A positive control group, also of six male rats, was given a single oral dose of 15 mg/kg bw (in 5 mL/kg bw) CPA. The dose levels were selected based on a previous study in which the maximum tolerated dose was determined to be 500 mg/kg bw per day in male rats. As there were no substantial intersex differences in toxicity, the study was conducted in males only. Four thousand PCEs, including

micronucleated MN-PCEs, were counted for each animal. The numbers of NCEs and MN-NCEs were also recorded for the first 1000 cells scored.

Clinical signs were observed at 500 mg/kg bw per day, which included decreased activity, intermittent twitching and unsteady gait. Two animals had convulsions when placed into the warming chamber before blood sampling. No body weight changes were observed.

Exposure was confirmed by the presence of metabolite SYN547305 (a major spiropidion metabolite) in both blood and plasma samples taken 1, 4 and 24 hours after the second dosing of the group given 500 mg/kg bw per day. No bone marrow toxicity was observed with respect to the PCE : NCE ratio.

There were no statistically significant increases in micronucleus frequency in animals given any dose level of spiropidion compared with the negative control group. The positive control showed a statistically significant increase in the number of micronucleated cells compared with the concurrent control group.

It was concluded that spiropidion was not clastogenic or aneugenic following an oral gavage dose up to the MTD of 500 mg/kg bw per day in male rats (Dunton, 2018).

Significant shortcomings were observed in the above in vivo bone marrow micronucleus tests in rats (Dunton 2015, 2018) since a single sex (male) was analysed, despite a marked differential toxicity between sexes being noticed in the preliminary dose range-finding experiments. In addition, the maximum dose level selected for the male animals (800 mg/kg bw) was much lower than the maximum dose level selected in a similar study (2000 mg/kg bw) using the same strain of animals and identical purity of test item (Whitwell, 2018). On this basis, both studies are of limited validity.

Spiropidion (purity 98.4%) was tested for its ability to induce chromosome aberrations in the bone marrow of rats. Groups of male and female rats (six/group per sex) were given a single, oral gavage administration of either the vehicle control (0.5% w/v CMC) or spiropidion at 500, 1000 or 2000 mg/kg bw for males, 187.5, 375 or 750 mg/kg bw for females. Dose levels were determined in a preliminary range-finding experiment in which severe clinical signs were observed in females at 1000 mg/kg bw. In addition, three male and three female rats received a single oral gavage administration of positive control (cyclophosphamide, 25 mg/kg bw). Animals were necropsied and bone marrow sampled 16 hours (all groups) or 42 hours (vehicle and high dose only) after dose administration. Bone marrow smears were prepared, and the slides scored for chromosome aberrations. Mitotic index was measured in at least 1000 cells per animal (including animals from the positive control group) to assess any evidence of toxicity. Where possible, 200 metaphases from each animal were analysed for chromosome aberrations. Any cell observed during this search with more than 42 chromosomes, that was polyploidy or was endoreduplicated was noted and recorded separately (hyperdiploid cells were not scored).

In male animals no clinical signs were observed for any animal dosed with vehicle, or at 500 mg/kg bw. At 1000 mg/kg bw one of six males exhibited anogenital soiling just prior to necropsy. At 2000 mg/kg bw (16 hour sampling) one of six males exhibited reduced activity and laboured respiration and a second animal exhibited anogenital soiling. These signs were apparent just prior to necropsy. At 2000 mg/kg bw (42 hour sampling) one of six animals exhibited piloerection, reduced activity and hunched posture (signs were apparent on day 2 at equivalent times to day 1, that is at 4, 6 and 8 hours post dose, and at necropsy).

In females no clinical signs were observed for any animal dosed with vehicle, or at 187.5 mg/kg bw. At 375 mg/kg bw 1/6 females exhibited reduced activity and twitching (8 hours post day 1 dose). At 750 mg/kg bw (16 hour sample time) one of six animals exhibited reduced activity and piloerection (4, 6 and 8 hours post dose on day 1). At the 42 hour sampling time one of six animals exhibited reduced activity two hours post dose, with further signs of piloerection observed prior to necropsy. A second animal exhibited reduced activity and twitching prior to necropsy. A further animal exhibited twitching, vocalisation, piloerection and rapid respiration on day 2 (equivalent to immediately post dose on day 1). Group mean percentage body weight changes for all male and female dose groups were below 10%.

At the 16 hour sampling time no evidence of cytotoxicity (as measured by mitotic inhibition [% MIH]) was observed in male animals. However, at the 42 hour sampling, cytotoxicity was 45% at the 2000 mg/kg bw dose, indicating evidence of bone marrow toxicity (and exposure).

In females cytotoxicity mitotic inhibition was 25%, 14% and 54% at 187.5, 375 and 750 mg/kg bw respectively at the 16 hour sampling time, and 40% at 750 mg/kg bw at the 42 hour sampling time. These results provided evidence of bone marrow toxicity (and exposure).

Male and female animals in all dosed groups exhibited frequencies of cells with structural chromosome aberrations (excluding gaps) that were similar to those observed in concurrent vehicle controls. Individual aberration frequencies for all dosed animals were similar to those observed in the vehicle control group, which were themselves consistent with historical vehicle control data ranges. Statistically significant increases were also observed in the in vivo chromosomal aberration assay at the highest dose level (750 mg/kg bw) and extended (42 hours) sampling time, but in the female animals only. The increase however was weak and limited to a single animal that exhibited an elevated frequency of aberrant cells (2.5%) which consisted essentially of chromatid breaks, a type of aberration usually also found in the negative control animals. The other five of this six animal group exhibited aberrant cell frequencies similar to those observed in the concurrent vehicle control. On this basis the isolated increase was not considered of biological importance. It is further noted that in female animals at the same dose level (750 mg/kg bw) with the shorter sampling time, the previous finding was not reproduced by similar cytotoxic conditions (54% mitotic inhibition index).

Frequencies of cells with numerical aberrations were, for the majority of animals (all dose groups), similar to or lower than those observed in the concurrent vehicle control and generally fell within historical control ranges. The exception was the male low-dose group (500 mg/kg bw; sampled at 16 hours) where small increases in polyploid cells were observed in the majority of animals. However, as similar levels were observed in sporadic vehicle control animals at this time point, the increases were not test article-related and were considered of no biological importance.

It was concluded that spiropidion did not induce chromosome aberrations in the bone marrow cells of male or female Crl:WI(Han) rats when tested at up to either 2000 mg/kg bw (males) or 750 mg/kg bw (females) (Whitwell, 2018).

2.5 Reproductive toxicity

(a) Multigeneration studies

Rat

In a two-generation reproductive toxicity study spiropidion (purity 98.4%) was administered to (Crl:WI(Han) rats, 24 animals/sex per group at dietary concentrations of 0, 50, 100 or 500 ppm (males)/300 ppm (females) (equal to 0, 3, 6 or 31 mg/kg bw per day for males, 0, 4, 8 or 24 mg/kg bw per day, for females, pre-pairing,). The diet was freely available for at least 10 weeks before pairing for mating and during pairing, gestation and lactation until necropsy. Four groups of 24 males and 24 females were selected from the weaned parental (F0) generation litters to form the F1generation; these animals were dosed, via the diet at the same dietary concentrations as the F0 generation. For 21 days before the start of the pairing period for both the F0 and F1 generation females, vaginal smears were taken daily by lavage and examined by light microscopy and the stage of the estrous cycle was determined by the type of cell present. Each female was paired with one male from the same test group for a period of up to 14 days. During the pairing period vaginal smears were taken daily, by lavage, until mating was confirmed by sperm being found in the smear. Animals were examined twice daily for mortality and morbidity. Each animal was given a detailed clinical examination once a week. All animals were examined for effects on general condition, body weight and food intake. The stage of the estrous cycle was recorded for 21 days before pairing for F0 and F1 females, and during the pairing period vaginal smears were taken daily until sperm were found in the smear. The females were allowed to litter and rear their offspring to weaning. The day of sexual development was recorded for all selected F1 generation animals. All F1 generation females were examined daily from day 25 of age for vaginal opening. All F1 generation males were examined daily from day 35 of age for balanopreputial separation. Quantitative evaluation of follicles was performed on the F1 generation only. Sperm motility and concentration were assessed for all F0 and F1 males at scheduled necropsy. Sperm morphological abnormalities and resistant testicular spermatid count were assessed for each control and high-dose male. Mating, fertility, gestation, post-implantation loss, viability, live birth, lactation and survival indices were calculated for F0 and F1 generations. The F0 and F1 males were subjected

to macroscopic necropsy once successful littering had been completed. The testes and epididymides were removed and weighed and sperm evaluation conducted. The F0 and F1 generation females were killed and necropsy carried out on day 21 of lactation. A macroscopic necropsy was performed, and the number of implantation scars recorded. For the F0 and F1 males and females, the following selection of organs was weighed: adrenal glands, brain, epididymides, kidneys, liver, ovaries, pituitary, prostate and seminal vesicles, spleen, testis, thyroids and uterus. A microscopic examination was conducted on selected organs (gross lesions, epididymides, ovaries, prostate and seminal vesicles, testes, thyroid, uterus and vagina) from all control and high-dose animals (except for thyroid that was examined in all females), any premature decedents, non-pregnant females, males failing to sire a pregnancy and females where the litter had died. Unselected F1 pups and all F2 animals were killed at 21 days of age. A gross macroscopic necropsy was performed on all pups; for one male and one female pup per litter, brain, spleen and thymus weights were recorded. No microscopic examination was conducted on pups.

There were no clinical signs or mortalities related to treatment in the F0 generation. One female at 300 ppm was found dead on lactation day (LD) 14. No earlier clinical signs were observed nor any were there any microscopic findings that could be used to ascertain the cause of death. Therefore, as this death was an isolated event it was considered not to be related to treatment.

No body weight or body weight gain effects that appeared treatment-related were reported for the F0 generation males. Body weights for F0 generation females were similar to control values except for a statistically significant decrease in body weight gain of approximately 20% compared to controls in females at 300 ppm at gestation interval 0–7 days; however, body weight gain was similar to control values in subsequent intervals. There were no treatment-related effects on food consumption in F0 generation males or females at any dose level.

There were no treatment-related effects on either the mean number of estrous cycles or the mean length of each cycle for the F0 generation.

In the F0 generation a statistically significant decrease in precoital interval was reported in females at 300 ppm. All animals mated within four days. Considering that the precoital interval at 300 ppm was the same as for the control of the F1 generation, this finding was considered incidental and not related to treatment.

In the F0 generation there were no treatment-related effects on fertility, mating performance, gestation, parturition, pregnancy or litter data.

A statistically significant decrease of 8% in absolute liver weight was reported in females at 100 and 300 ppm compared to controls. A statistically significant decrease of 6% in absolute kidney weight was reported for females at 300 ppm compared to controls. Given the small size of the changes and the lack of significant corresponding changes in the adjusted weight to body weight, or relative to body weight, these changes were considered of no toxicological relevance. A statistically significant increase of 18% in absolute thyroid weight compared to controls was reported in females at 300 ppm; no changes were observed however in the corresponding adjusted or "relative to body weight" values. Histopathological investigation showed an increased incidence in minimal thyroid follicular epithelium hypertrophy. There were no macroscopic treatment-related effects observed in the F0 generation.

There were no differences in sperm motility or concentration for males of the F0 generation, when compared with controls. Sperm morphology was unaffected by treatment and homogenization-resistant testicular spermatid counts were similar for all groups.

No treatment-related clinical signs or mortality were observed in the F1a offspring.

In the F1 generation a statistically significant decrease in body weight (ca 9% compared to controls) was reported for females at 300 ppm at LD 14, however at LD 21 body weight was similar to controls. No changes in body weight gain were observed in either sexe.

There were treatment-related findings at necropsy nor organ weights changes observed in F1a animals.

There were no clinical signs, mortalities, changes in body weight or body weight gain, or food consumption related to treatment in adults of either sex from the F1 generation. There were no treatment-related effects on sexual maturation in either sex; the day of attainment of balano-preputial

separation or vaginal opening was similar to that in controls. There were no treatment-related effects on either the mean number of estrous cycles or the mean length of each cycle for the F1 generation. No treatment-related effects on fertility, mating performance, gestation, parturition, pregnancy or litter data were observed for the F1 generation.

No treatment-related effects on organ weight or macroscopic findings were observed in adults of either sex in the F1 generation.

An increased incidence of minimal thyroid follicular epithelium hypertrophy was reported in F0 females at 300 ppm.

There were no findings in the F2 offspring for mortality, clinical signs, body weights/body weight gains, necropsy or organ weights that were treatment-related.

Table 23. Key findings of the two-generation reproductive toxicity study in rats

	Dietary dose (ppm)							
	Males				Females			
Finding	**0**	**50**	**100**	**500**	**0**	**50**	**100**	**300**
	F0 parental animals							
Dose (mg/kg bw per day) ± SD								
Weeks 1 to 17	0	3.04 ± 0.7	5.98 ± 1.4	30.62 ± 6.7	-	-	-	-
Premating (weeks 1–10)	-	-	-	-	0	3.94 ± 0.6	8.11 ± 1.0	24.06 ± 3.3
Gestation	-	-	-	-	0	4.10 ± 0	8.97 ± 0.2	25.18 ± 0.9
Lactation	-	-	-	-	0	9.23 ± 2.2	18.47 ± 3.7	53.23 ± 11.8
Body weight gain (g) ± SD								
Gestation								
Days 0–7	-	-	-	-	23.7 ± 6.0	20.7 ± 6.0	21.2 ± 6.9	18.7 ± 7.1*
Days 0–14	-	-	-	-	49.2 ± 8.8	46.0 ± 7.5	48.2 ± 9.3	45.0 ± 10.0
Days 0–20	-	-	-	-	111.6 ± 15.3	104.8 ± 13.1	110.9 ± 15.0	110.6 ± 16.7
Pre-coital interval; mated females (day)	-	-	-	-	3.8	2.8	2.9	2.6*
Organ weights								
Liver								
Absolute (g) ± SD	12.97 ± 1.2	12.22 ± 1.4	13.30 ± 1.2	12.76 ± 1.6	13.31 ± 1.4	12.85 ± 1.2	12.28 ± 1.4*	12.28 ± 1.3*
Covariance to bw	13.02	12.67	12.98	12.59	13.18	12.77	12.31*	12.45
Relative to bw (%) ± SD	3.05 ± 0.2	2.97 ± 0.2	3.04 ± 0.2	2.94 ± 0.2	4.84 ± 0.4	4.70 ± 0.4	4.53 ± 0.5	4.60 ± 0.4
Kidney								
Absolute (g) ± SD	2.72 ± 0.3	2.60 ± 0.3	2.68 ± 0.3	2.67 ± 0.3	2.16 ± 0.2	2.10 ± 0.2	2.06 ± 0.2	2.04 ± 0.2*
Covariance to bw	2.72	2.69	2.62	2.64	2.16	2.10	2.06	2.04
Relative to bw (%) ± SD	0.63 ± 0.04	0.63 ± 0.04	0.61 ± 0.04	0.62 ± 0.04	0.78 ± 0.05	0.76 ± 0.04	0.76 ± 0.04	0.76 ± 0.05

	Dietary dose (ppm)							
	Males				Females			
Finding	**0**	**50**	**100**	**500**	**0**	**50**	**100**	**300**
Thyroid								
Absolute (g) ± SD	0.024 ± 0.005	0.021 ± 0.003	0.021 ± 0.003*	0.024 ± 0.008	0.017 ± 0.003	0.019 ± 0.004	0.018 ± 0.004	0.020 ± 0.002*
Covariance to bw	0.024	0.022	0.020*	0.024	0.017	0.019	0.018	0.019
Relative to bw (%) ± SD	0.005 ± 0.001	0.005 ± 0.001	0.004 ± 0.001	0.005 ± 0.002	0.006 ± 15	0.007 ± 19	0.006 ± 16	0.007 ± 19
Histopathology								
Thyroid								
Number examined	24	-	-	24	24	24	24	24
Hypertrophy, follicular epithelium; minimal	0	-	-	0	0	0	0	9
				F1 pup data				
Body weight (g) ± SD								
Lactation day 1	6.64 ± 0.6	6.79 ± 0.6	6.58 ± 0.8	6.55 ± 0.7	6.28 ± 0.5	6.40 ± 0.6	6.29 ± 0.8	6.34 ± 0.7
Lactation day 14	31.6 ± 4.0	32.6 ± 3.7	30.5 ± 4.6	30.7 ± 4.1	32.0 ± 3.4	32.1 ± 2.9	31.2 ± 3.1	29.4 ± 4.9*
Lactation day 21	50.1 ± 6.8	51.5 ± 5.7	48.0 ± 6.5	49.0 ± 4.3	49.0 ± 5.1	49.8 ± 4.4	47.7 ± 4.5	46.9 ± 4.9
				F1 parental animals				
Dose (mg/kg bw per day) ± SD								
Weeks 1 to 17	0	3.8 ± 1.7	7.8 ± 3.5	38.5 ± 17.1	-	-	-	-
Premating (weeks 1–10)	-	-	-	-	0	4.9 ± 1.4	9.6 ± 2.8	28.4 ± 8.5
Gestation	-	-	-	-	0	4.0 ± 0.2	7.8 ± 0.2	23.2 ± 0.5
Lactation	-	-	-	-	0	9.3 ± 2.0	19.3 ± 4.0	57.7 ± 11.2
Precoital interval; mated females (days)					2.6	3.1	2.5	3.1
Histopathology								
Thyroid								
Number examined	24	2	1	24	24	24	24	24
Hypertrophy, follicular epithelium; minimal	0	0	0	0	0	0	0	5

bw: Body weight; SD: Standard deviation; Source: adapted from King, 2018

* $p < 0.05$, ** $p < 0.01$, statistically significant difference from control group mean (Dunnett's)

The NOAEL for parental toxicity was 100 ppm (equal to 7.8 mg/kg bw per day), based on increased incidence of thyroid follicular hypertrophy in females of both generations at 300 ppm (equal to 23 mg/kg bw per day).

The NOAEL for offspring toxicity was 300 ppm (equal to 23 mg/kg bw per day) the highest dose tested.

The NOAEL for reproductive toxicity was 300 ppm (equal to 23 mg/kg bw per day) the highest dose tested (King, 2018).

(b) Developmental toxicity

Rats

In a non-GLP and guideline tolerability study, spiropidion (purity 98.4%) was administered by gavage to five groups of two non-pregnant Crl:WI(Han) rats at a dose level of 0, 200, 150, 150 or 75 mg/kg bw per day, using a dose volume was 10 mL/kg bw of 0.5% w/v aqueous CMC, acidified to pH 5. Groups were identified as 1, 2, 3, 4 and 5 in ascending dose order. Dosing of the groups was staggered, continuing with the next dose level after at least three days administration of the preceding dose level. Dosing began with groups 1 and 2, followed by groups 3, 4 and 5 in that order. Group 1 animals were dosed with vehicle for seven consecutive days and then retained without further dosing until being re-allocated to group 5. In groups 2–5 animals were dosed once daily for seven consecutive days until the day before necropsy. Body weights and food intake were recorded at the start of treatment and daily thereafter, up to and including the day of necropsy. Blood samples for toxicokinetic evaluation were taken from all animals in groups 1 to 3 and group 5 on day 1 and day 7 of dosing, samples taken at seven time points between predose and 24 hours after dosing. Terminal blood samples were taken from group 4 only. Upon completion of blood sampling, all animals were subjected to a macroscopic necropsy examination.

There were no deaths at any dose level and no clinical signs at 75 or 150 mg/kg bw per day. Signs of agitation were observed over the second half of the treatment period at 200 mg/kg bw per day. Body weight losses were observed for all animals at 150 mg/kg bw per day (between 1% and 7% of initial weight) and 200 mg/kg bw per day (between 4% and 8% of initial weight) and for one animal at 75 mg/kg bw per day (the other animal at 75 mg/kg bw per day maintained weight). Food intake was slightly lower than controls at 75 and 150 mg/kg bw per day, but at 200 mg/kg bw per day food intake was approximately half that of the controls. There were no macroscopic postmortem findings at any dose level.

Bioanalysis showed that spiropidion was detected in a negligible number of samples at a low concentration; concentrations of the remaining samples were below the LOQ. None of the plasma samples analysed from control animals were found to contain the metabolite SYN547305.

Between 75 and 150 mg/kg bw per day, peak exposure (C_{max}) to metabolite SYN547305 increased proportionally and increases in total systemic exposure (AUC_{0-24}) were supraproportional on day 1. On day 7, however, increases in C_{max} were subproportional and increases in AUC_{0-24} were proportional. Between 75 and 200 mg/kg bw per day, AUC_{0-24} increased subproportionally in relation to the increase in dose on both days 1 and 7. Increases in C_{max} were subproportional on day 1 and no increase was seen on day 7. Both C_{max} and AUC_{0-24} were higher at 150 mg/kg bw per day than at 200 mg/kg bw per day. Total systemic exposure at all doses was lower on day 7 than day 1 which was reflected in accumulation ratios of less than 1, indicating there was no potential for accumulation.

Oral gavage administration of spiropidion to non-pregnant Crl:WI(Han) rats at a dose level of 200 mg/kg bw per day for seven days was not well tolerated, resulting in clinical signs, low food intake and body weight losses, suggesting that this dose exceeds the maximum tolerated dose.

At 75 and 150 mg/kg bw per day there were slight reductions in food intake and some slight body weight losses (Hackford, 2016a).

In a non-GLP and guideline dose range-finding study, spiropidion (purity 98.4%) was administered from gestation day (GD) 6 to GD 19 by gavage to five groups of ten time-mated Crl:WI(Han) rats at a dose level of 0, 25, 75 or 150 mg/kg bw per day, and a dose volume of 10 mL/kg bw in 0.5% w/v aqueous CMC, acidified to pH 5. Following assessment of the study data, and as there were no clear signs of maternal toxicity at 75 mg/kg bw per day, three additional groups of 10 females were dosed at 0 (concurrent controls), 100 or 125 mg/kg bw per day in order to meet the study objective. Females given

125 mg/kg bw per day were terminated prematurely on GD 8 or 9 due to body weight loss and reduced food consumption from the start of the dosing period, and the animals given the test compound at 100 mg/kg bw per day were dosed once daily from GD 6 to GD 19 inclusive. Females given 150 mg/kg bw per day were terminated prematurely on GD 18 or 19, due to body weight loss and significantly lower food consumption between GDs 6 and 9. The following were assessed during the course of the study: clinical signs, body weight and food consumption. Females terminated prematurely at 150 mg/kg bw per day underwent gross macroscopic necropsy only and at 125 mg/kg bw per day animals were terminated without examination. On GD 20 the remaining females were terminated, a blood sample was obtained for proof of absorption and the live fetuses were removed from the uterus, weighed, their sex determined and they were examined for external and visceral abnormalities. Placenta and gravid uterus weights were also recorded.

One female at 150 mg/kg bw per day had brown hair staining from GD 7 to 10. A single occurrence of piloerection and agitation was observed in one female at 125 mg/kg bw per day on GD 7. One female at 75 mg/kg bw per day had brown discharge from the vulva on GD 15; the animal was pregnant at scheduled necropsy. There were no other clinical observations for females given 25, 75 or 100 mg/kg bw per day.

Females given 100 mg/kg bw per day had significantly reduced cumulative body weight gains throughout the study until GD 19, however, on GD 20 mean body weight was comparable with controls. Body weight gain adjusted for gravid uterus weight was similar to controls. At 75 mg/kg bw per day, cumulative body weight gains were lower than controls during GDs 6–13 (as much as –70%) achieving statistical significance on some occasions during this period. Subsequently after GD 14, cumulative body weight gains became similar to the controls and were greater than the controls by GD 20, resulting in a statistically significant higher body weight gain when adjusted for gravid uterus weight. No treatment-related effects on body weight were observed in these animals.

Overall group mean food intake was slightly lower (statistically significant) than controls at 100 mg/kg bw per day due to reduced food intake over the majority of the gestation period during which statistical significance was achieved from GD 9–18. At 75 mg/kg bw per day food intake was slightly lower than for the controls over the majority of the gestation period and attained statistical significance during GDs 6–9. However, higher food intake was apparent over GDs 18–20 (statistically significant), resulting in an overall mean food intake which was similar to controls. There was no effect on food intake at 25 mg/kg bw per day with values similar to the controls.

There were 10, 10, 10, 10 and 9 females pregnant (live fetuses on GD 20) at 0, 25, 75 and 100 mg/kg bw per day respectively. There was no effect of treatment on the uterine or implantation parameters. At 25 mg/kg bw per day statistical significance was achieved for a higher number of corpora lutea and incidence of pre-implantation loss, however as implantation in the rat occurs either on GD 5 or 6 (before treatment), these results were considered to be due to the small group size and not related to treatment. A statistically significant reduction in the percentage of males was observed at 100 mg/kg bw per day. However no change to the percentage of male fetuses was repeated in the main developmental study (Pottle, 2017) up to 100 mg/kg bw per day, so the reduced proportion of male fetuses seen in this study was considered incidental to treatment, probably ascribable to the small group size.

At 100 mg/kg bw per day, group mean fetal and placental weights were lower than for controls to a statistically significant extent. By contrast group mean fetal weight was higher at 75 mg/kg bw per day compared with the controls, also statistically significant. These values were within the HCD ranges and were therefore considered likely to be due to the small group sizes and not related to treatment. In addition, no changes in fetal or placental weight were observed in the main developmental study (Pottle, 2017) up to 100 mg/kg bw per day. There were no effects on group mean fetal parameters for females given 25 mg/kg bw per day when compared with the controls.

Major fetal abnormalities were noted in one control fetus, and in one fetus at 25 mg/kg bw per day. The malformations observed in the fetus at 25 mg/kg bw per day were aortic and pulmonary arch transposition of the great vessels and right-sided descending aorta. There were no major fetal abnormalities observed at 75 or 100 mg/kg bw per day.

At 100 mg/kg bw per day, there was a slightly higher incidence of a minor abnormality, specifically one or more accessory liver lobes (eight fetuses from three litters). The litter incidence was

however within the HCD range and was similar to the incidence in the concurrent control of the main developmental toxicity study (Pottle, 2017), therefore, it was considered not to be treatment-related.

There were no increases in the incidence of minor or variant fetal abnormalities at 25 or 75 mg/kg bw per day.

Bioanalysis showed no quantifiable concentration of spiropidion in any of the blood samples analysed. Metabolite SYN547305 was detected in all samples from the animals given spiropidion. None of the blood samples analysed from control animals were found to contain either spiropidion or metabolite SYN547305.

Table 24. Key findings of the preliminary developmental toxicity study in rats

	Dose level (mg/kg bw per day)						
Finding	**0**	**25**	**75**	**150**	**0**	**100**	**125**
Body weight gain (g) ± SD							
Gestation days interval							
GDs 0–6	19.9 ± 7.7	21.4 ± 2.6	21.2 ± 12	24.2 ± 7.1	18.4 ± 7.3	16.3 ± 6.9	18.0 ± 6.6
GDs 6–9	9.7 ± 4.3	5.8 ± 4.8	3.0 ± 7.0*	–2.4 ± 5.7**	10.2 ± 4.6	2.0 ± 3.9**	–3.6 ± 12.6**
GDs 6–12	21.6 ± 7.3	21.6 ± 4.4	15.3 ± 10.4	11.6 ± 6.9**	26.2 ± 6.6	15.0 ± 3.8**	-
GDs 6–19	69.5 ± 13.9	74.9 ± 8.1	72.5 ± 18.1	70.4 ± 11.3	86.0 ± 6.8	76.6 ± 6.9*	-
GDs 6–20	67.7 ± 15.5	74.9 ± 11.0	82.1 ± 15.6	-	88.1 ± 7.9	86.0 ± 7.8	-
Food consumption (g/animal per day) ± SD							
Gestation days interval							
GDs 6–9	23.8 ± 2.1	21.6 ± 2.6	20.2 ± 2.2*	18.4 ± 4.2**	20.3 ± 3.0	17.0 ± 2.7	13.4 ± 4.0**
GDs 9–12	22.1 ± 3.6	22.0 ± 2.1	20.6 ± 3.2	19.7 ± 3.5	24.3 ± 2.2	20.5 ± 2.0**	-
GDs 12–15	24.3 ± 2.9	24.3 ± 2.8	23.8 ± 2.3	23.6 ± 4.1	24.9 ± 1.3	22.1 ± 1.8**	-
GDs 15–18	26.5 ± 4.1	26.6 ± 1.5	25.7 ± 2.2	25.0 ± 2.6	26.7 ± 1.9	24.0 ± 2.7*	-
GDs 18–20	15.9 ± 4.8	16.5 ± 4.6	21.6 ± 3.9*	-	21.1 ± 3.9	22.7 ± 3.0	-
GDs 6–20	23.01 ± 2.1	22.61 ± 1.9	22.41 ± 1.9	-	23.63 ± 1.4	21.15 ± 1.7**	
Caesarian observations							
Mean number of live fetuses/dam ± SD	11.4 ± 1.3	11.9 ± 1.2	11.2 ± 1.6		11.0 ± 1.5	11.7 ± 1.2	
Mean male fetuses (%)	51.9	48.9	43.7		54.0	39.2*	
Mean litter weight (g)	39.02	40.63	40.43		41.09	41.30	
Mean fetal weight (g)	3.42	3.42	3.63*		3.74	3.53*	
(HCD data for mean fetal weight: range min–max, 3.37–3.94 g)							
Mean fetal weight – male (g)	3.45	3.50	3.71*		3.86	3.61*	3.71
Mean fetal weight – female (g)	3.37	3.35	3.56*		3.63	3.47	

	Dose level (mg/kg bw per day)						
Finding	**0**	**25**	**75**	**150**	**0**	**100**	**125**
Mean placental weight (g)	0.49	0.47	0.48		0.51	0.48*	0.52
(HCD data for mean placental weight: range min–max, 0.47–0.69 g)							
Fetal abnormalities							
Number of fetuses examined	114	119	112		110	105	
Number of litters examined	10	10	10		10	9	
Minor abnormalities							
Liver one or more lobes: accessory lobe present							
Number of fetuses (%)	0	0	0		1 (1.3)	8 (7.1)	
(HCD data for fetuses: range min–max, 0–10)							
Number of litters (%)	0	0	0		1 (10)	3 (33.3)	
(HCD data for litters: range min–max, 0–5)							
Blood concentration of metabolite SYN547305 ng/mL ± SD; 10 females per group							
	≤LOQ	162 ± 53.1	275 ± 176			308 ± 196	

GD: Gestation day; SD: standard deviation; Source: adapted from Hackford, 2016b

LOQ: Limit of quantitation = 5 ng/mL;

HCD: Laboratory historical control data from 32 dose range-finding studies conducted from 2011 to 2016, on 2360 fetuses from 277 litters

* $p < 0.05$, ** $p < 0.01$, statistically significant difference from control group mean (Dunnett's)

The NOAEL for maternal toxicity was 25 mg/kg bw per day based on initial reduction of body weight gain and food consumption at 75 mg/kg bw per day.

The NOAEL for developmental toxicity was 100 mg/kg bw per day, the highest dose tested (Hackford, 2016b).

In the main developmental toxicity study spiropidion (purity 98.4%) was administered by gavage to 22 time-mated Crl:WI(Han) rats at dose levels of 0, 10, 30 or 100 mg/kg bw per day once daily, at a dose volume of 10 mL/kg (in 0.5 % w/v CMC, acidified to pH ≤ 5), from GD 6 to GD 19, inclusive. Animals were examined twice daily for mortality and morbidity and were given a detailed clinical examination daily. Body weights were recorded daily from GD 5 to GD 20 inclusive, and the amount of food consumed by each animal was recorded over GDs 6–9, 9–12, 12–15, 15–18, and 18–20. On GD 20, the females were terminated and uterine examinations carried out. The live fetuses were removed from the uterus, weighed, their sex determined, and they were examined for external and visceral abnormalities. Placenta and gravid uterus weights were also recorded.

There were no deaths or treatment-related clinical observations.

At 100 mg/kg bw per day, from GD 6 to 9, there was an initial group mean body weight loss. From GD 10 to 14, mean body weight gains were observed but statistically were significantly lower than those of the control group; body weight gains continued to be lower than controls for the remainder of the dosing period. In addition, there was a slight reduction (–3 %) in mean terminal body weight compared with the controls, when adjusted for the weight of the gravid uterus. There was no effect on body weight or body weight gain at 30 or 10 mg/kg bw per day with values being similar to the controls.

There was an initial statistically significant reduction in food consumption compared with the controls over the period GDs 6–12 at 100 mg/kg bw per day. There was an overall statistically significant reduction in mean food consumption over the dosing period, compared with that of the control group. No treatment-related effect on food intake was observed at 30 or 10 mg/kg bw per day.

There were no relevant maternal necropsy findings.

Four females (two from the control group, one given 30 mg/kg bw per day and one given 100 mg/kg bw per day) were not pregnant, and two females (one given 10 mg/kg bw per day and one given 30 mg/kg bw per day) had total early intrauterine deaths. Females 9 and 20 (controls), 31 (10 mg/kg bw per day) and 59 (30 mg/kg bw per day) were confirmed as being in fact at GD 21, therefore their data were excluded from the group means. This left a total of 18, 20, 19 and 21 females in the groups given 0, 10, 30 and 100 mg/kg bw per day respectively, with live fetuses on GD 20. Pregnancy data were similar in all groups, with no treatment-related effect on the mean numbers of corpora lutea or implantations, the incidence of pre- or post-implantation loss or on the number of live fetuses.

At all dose levels, mean fetal and litter weights were comparable with those of the control group. Mean placental weights were similar in the treated groups and with the controls and there was no treatment-related effect on fetal sex ratio.

Fetal examination did not reveal treatment-related major abnormalities. Following visceral examination, a statistically significant increase in incidence, using trend analysis, was reported in the number of litters with fetuses showing the minor abnormality of accessory liver lobe(s) at 100 mg/kg bw per day. From examination after visceral fixation no statistical significance was achieved.

Following skeletal and cartilage examination there were statistically significant increases, using trend analysis, in the number of litters with fetuses or number of fetuses at 100 mg/kg bw per day that showed the following variations: hole in the centre of xiphoid cartilage, forelimb ossified phalanges or incomplete ossification of the 5th sternum sternebra. A statistically significant increase (trend test) in the incidence of litters including fetuses with bifurcated sternum xiphoid cartilage was observed at 30 and 100 mg/kg bw per day.

The incidences of all the above fetal abnormalities were within the laboratory historical control data. The Meeting concluded that the observed visceral and skeletal fetal findings were not adverse.

Table 25. Key findings of the main developmental toxicity study in rats

	Dose level (mg/kg bw per day)				HCD	
Finding	**0**	**10**	**30**	**100**	**Mean**	**min–max**
Body weight gain (g) ± SD						
Gestation days interval						
GDs 6–9 *(difference from control)*	4.8 ± 5.1	6.8 ± 7.4	7.2 ± 5.4	−1.0 ± 7.1* (↓121%)		
GDs 6–12 *(difference from control)*	20.9 ± 6.6	24.5 ± 7.3	23.4 ± 6.2	13.4 ± 6.8** (↓36%)		
GDs 6–15	36.6 ± 8.8	41.3 ± 8.2	37.9 ± 7.8	32.5 ± 7.8		
GDs 6–18	65.8 ± 11.8	73.0 ± 11.0	68.9 ± 12.3	63.0 ± 7.9		
GDs 6–20 *(difference from control)*	93.0 ± 13.5	99.3 ± 13.5 (↑6.8%)	94.9 ± 16.0 (↑2.0%)	88.4 ± 9.4 (↓4.9%)		
GDs 6–20 adjusted for uterus wt *(difference from control)*	30.31 ± 8.8	35.83 ± 11.1 (↑18.2%)	32.62 ± 32.6 (↑7.6%)	25.16 ± 5.7 (↓17.0%)		
Food consumption (g/day) ± SD						
Days 6–9 *(difference from control)*	18.4 ± 2.8	17.7 ± 2.5	18.0 ± 5.7	15.8 ± 2.5** (↓14%)		
Days 9–12 *(difference from control)*	22.1 ± 2.2	22.0 ± 2.8	21.8 ± 1.8	19.4 ± 1.3** (↓12%)		
Days 12–15	23.2 ± 2.6	22.6 ± 2.9	22.6 ± 2.7	22.2 ± 3.2		
Days 15–18	24.7 ± 3.2	25.1 ± 3.0	24.9 ± 3.5	23.9 ± 2.4		
Days 18–20	24.6 ± 2.9	23.9 ± 3.5	24.8 ± 3.2	23.5 ± 2.3		
Days 6–20 *(difference from control)*	22.45 ± 2.0	22.15 ± 2.2 (↓1.3%)	22.27 ± 2.7 (↓0.8%)	20.78 ± 1.5* (↓7.4%)		

	Dose level (mg/kg bw per day)				HCD	
Finding	**0**	**10**	**30**	**100**	**Mean**	**min–max**
Caesarian observations						
Animals mated	20	21	21	22		
Animals pregnant	18	21	20	21		
Fetal abnormalities (minor)						
Visceral						
Number of fetuses examined	91	107	97	114		
Number of litters examined	18	20	19	21		
Liver – one or more lobe: accessory lobe present						
Fresh visceral examination						
Number of fetuses [%]	5 [4.4]	6 [5.3]	5 [5.1]	13 [11.6] J	3.6	0.8–8.0
Number of litters [%]	4 [22.2]	6 [30.0]	5 [26.3]	9 [42.9]#	22.8	4.5–47.4
Fixed visceral examination						
Number of fetuses [%]	3 [3.0]	5 [4.6]	6 [6.5]	11 [9.5]J	4.3	0.9–9.0
Number of litters [%]	3 [16.7]	4 [20.0]	6 [31.6]	9 [42.9]	20	5–45
Skeletal (variants)						
Number of fetuses examined	96	107	97	114		
Number of litters examined	18	20	19	21		
Forelimb – one or more digit: phalanges ossified						
Number of fetuses [%]	26 [25.1]	24 [20.8]	33 [34.1]	57 [48.8] JJ	26.6	10.4–54.5
Number of litters [%]	10 [55.6]	10 [50.0]	15 [78.9]	17 [81.0]#	58.8	30–85.7
Sternum – xiphoid cartilage: bifurcated						
Number of fetuses [%]	17 [16.2]	15 [13.9]	24 [24.1]	33 [28.7]J	15.1	3.4–34.0
Number of litters [%]	8 [44.4]	9 [45.0]	14 [73.7]#	16 [76.2]#	57.1	22.7–85.7
Sternum – xiphoid cartilage: hole in centre						
Number of fetuses [%]	22 [23.4]	44 [41.5]	28 [28.4]	50 [45.4]J	45.9	31.4–78.4
Number of litters [%]	13 [72.2]	17 [85.0]	13 [68.4]	18 [85.7]	91.0	71.4–100
Sternum – 5th sternebra: incomplete ossification						
Number of fetuses [%]	2 [1.7]	3 [2.7]	6 [6.0]	7 [6.1]	5.7	1.0–16.4
Number of litters [%]	2 [11.1]	2 [10.0]	4 [21.1]	6 [28.6]#	26.8	5.0–55.0

GD: Gestation day; Source: adapted from Pottle, 2017

* $p < 0.05$; ** $p < 0.01$ statistically significant difference from control group mean (Dunnett's test)

J $p < 0.05$; JJ $p < 0.01$ Jonckheere test, one-sided; # $p < 0.05$ Cochran–Armitage test, one-sided

HCD: Historical control data from 26 studies conducted at the same test facility between March 2013 and November 2016; examined 3563 fetuses from 541 litters

The NOAEL for maternal toxicity was 30 mg/kg bw per day, based on body weight loss, reduced body weight gain and food consumption at 100 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity was 100 mg/kg bw per day, the highest dose tested (Pottle, 2017).

Rabbit

Study 1

In a non-GLP, guideline tolerability study spiropidion (purity 98.4%) was administered by gavage to four groups of two nonpregnant New Zealand White rabbits for seven days. Animals in the control group were dosed with the vehicle only for seven days and subsequently retained undosed until day 21. From day 22 of the study, these animals were at 85 mg/kg bw per day for seven days. Another two groups of animals were dosed at 250 and 50 mg/kg bw per day for seven days. Since treatment-related changes were evident at 250 mg/kg bw per day an additional group of animals was dosed at 125 mg/kg bw per day for seven days. A constant dose volume of 10 mL/kg was employed (0.5% w/v aqueous CMC, acidified to pH 5). Animals were observed daily from arrival. Body weights and food intake were recorded daily from five days before dosing up to, and including the day of necropsy. Blood samples for toxicokinetic evaluation were taken from all treated animals on day 1 and day 7 of dosing. Upon completion of blood sampling, all animals were subject to a macroscopic necropsy examination.

There was no mortality during the study.

Marked body weight losses (up to 17%) were observed at 250 and 125 mg/kg bw per day. These losses were accompanied by negligible food intake. At 85 mg/kg bw per day, slight body weight losses and reduced food intake were seen. Small and reduced faeces were observed for both animals at 250 and 125 mg/kg bw per day throughout the treatment period. Reduced faeces were also observed on a single occasion for one animal given 85 mg/kg bw per day. The occurrence of these faecal observations was associated with periods of reduced food intake.

There was no effect on food intake at 50 mg/kg bw per day and one animal maintained its body weight whilst the other lost a small amount of body weight over the treatment period.

Spiropidion was detected in a negligible number of samples at a low concentration; concentrations of the remaining samples were below the LOQ and therefore toxicokinetic parameters could not therefore be derived. Toxicokinetic parameters were derived for the metabolite SYN546305.

On day 1, C_{max} and AUC_{0-24} were subproportional to the increase in dose between 50 and 125 or 250 mg/kg bw per day, and no change was observed between 50 and 85 mg/kg bw per day. On day 7, there was no appreciable change in C_{max} with increasing dose. Total systemic exposure, however, increased proportionally with dose between 50 and 85 or 125 mg/kg bw per day, and subproportionally between 50 and 250 mg/kg bw per day. Total systemic exposure at 50, 85, 125 and 250 mg/kg bw per day was lower on day 7 than on day 1, which was reflected in accumulation ratios of less than or equal to one, indicating no potential for accumulation.

None of the plasma samples analysed from control animals was found to contain the metabolite SYN547305.

Oral gavage administration of spiropidion to non-pregnant New Zealand White rabbits at dose levels of 250 and 125 mg/kg bw per day for seven days was not well tolerated, resulting in negligible food intake and marked body weight losses, showing that these doses exceeded the maximum tolerated dose. Slight body weight losses were observed at 50 mg/kg bw per day and 85 mg/kg bw per day but were only accompanied by a reduction in food intake at 85 mg/kg bw per day (Hackford, 2016c).

Study 2

In a non-GLP and guideline dose range-finding study, spiropidion (purity 98.4%) was administered from GD 6 to GD 27 by gavage to four groups of ten time-mated New Zealand White rabbits at dose levels of 0, 15, 50 or 75 mg/kg bw per day, dose volume of 5 ml/kg bw in 0.5% w/v aqueous CMC, acidified to pH 5. Animals were examined twice daily for mortality and morbidity and were clinically examined daily. Body weights were recorded daily from the day of arrival at the laboratory to GD 28, inclusive. Food consumption was recorded over GD 5 to GD 6 and every two days thereafter. On GD 28 the females were killed, and the number of corpora lutea and the number and distribution of implantations in each uterine horn recorded. Implantations were classified as early resorptions, late resorptions, dead fetuses or live fetuses. A blood sample was obtained the day of termination for proof of absorption. The live fetuses were removed from the uterus, weighed, their sex determined and they were examined for external and visceral abnormalities. Placenta and gravid uterus weights were also recorded.

At 75 mg/kg bw per day one female was found dead on GD 28. Fluid was present in the thoracic cavity which may have resulted from dosing trauma; the female was pregnant, showed overall body weight loss and low food intake, which was generally very low from GD 8. Another female at 75 mg/kg bw per day aborted on GD 26 and lost weight over GD 6–15, although body weight was generally gained thereafter. Red-stained bedding had been noted for both animals prior to their being found dead or terminated. There were no findings at necropsy that were considered to be treatment-related.

No clinical observations were considered to be treatment-related.

There was a dose-related reduction (not statistically significant) in mean body weight gain/loss over the first days of dosing (GDs 6 to 9) at 50 and 75 mg/kg bw per day. At 75 mg/kg bw per day, mean body weight gain remained lower than for controls for the rest of the study, while body weight gain at 50 mg/kg bw per day returned to being similar to controls by the end of the exposure period. Females given 15 mg/kg bw per day had mean body weight gains which were similar to, or greater than, those of the controls. Mean terminal body weight, adjusted for the weight of the gravid uterus, was unaffected by treatment. There was a dose-related reduction in group mean food intake over the treatment period that was statistically significant; lower than the control in the groups given 50 or 75 mg/kg bw per day (–25% and –35% below controls, respectively). There was no effect on food intake at 15 mg/kg bw per day, with values similar to the controls.

There were no macroscopic abnormalities that were considered related to treatment.

Caesarean section evaluation showed that the mean number of live fetuses per dam was higher at 15 mg/kg bw per day when compared to controls with statistical significance. Litter weight was reported to be greater at 15 and 50 mg/kg bw per day when compared to controls (statistically significant), but with no dose–effect relationship. In the absence of relevant changes at higher dose levels these changes were considered not to be related to treatment. Mean fetal weight was lower (with statistical significance) at 75 mg/kg bw per day when compared to controls. The value was outside HCD data from the laboratory, therefore, this change was considered to be treatment-related. A statistically significant decrease in the percentage of pre-implantation loss was reported at all dose levels when compared to controls.

There were no external or visceral abnormalities that were considered to be treatment-related.

Major abnormalities were noted in one fetus in the group given 15 mg/kg bw per day (acephaly) and one fetus in the group given 50 mg/kg bw per day (severely enlarged heart and aortic arch). Since these abnormalities were not seen at the subsequent higher doses they were considered not to be test item-related but spontaneous in origin.

Table 26. Key findings of the preliminary developmental toxicity study in rabbits

	Dose level (mg/kg bw per day)			
Finding	**0**	**15**	**50**	**75**
Body weight (kg) ± SD				
GD 0	3.498 ± 0.25	3.466 ± 0.29	3.499 ± 0.27	3.374 ± 0.25
GD 6	3.411 ± 0.24	3.388 ± 0.30	3.394 ± 0.24	3.340 ± 0.20
GD 9	3.430 ± 0.26	3.398 ± 0.29	3.382 ± 0.23	3.313 ± 0.22
GD 12	3.459 ± 0.25	3.433 ± 0.28	3.432 ± 0.22	3.346 ± 0.20
GD 19	3.519 ± 0.22	3.505 ± 0.29	3.495 ± 0.23	3.419 ± 0.18
GD 28	3.690 ± 0.22	3.734 ± 0.28	3.688 ± 0.21	3.557 ± 0.21
Body weight gain (kg) ± SD				
Gestation day intervals				
GDs 0–6	−0.086 ± 0.04	−0.079 ± 0.09	−0.105 ± 0.06	−0.034 ± 0.07
GDs 6–9	0.019 ± 0.03	0.010 ± 0.04	−0.012 ± 0.06	−0.027 ± 0.05
GDs 6–12	0.048 ± 0.05	0.045 ± 0.06	0.038 ± 0.09	0.006 ± 0.08
GDs 6–19	0.108 ± 0.09	0.118 ± 0.06	0.101 ± 0.11	0.079 ± 0.14
GDs 6–28	0.279 ± 0.2	0.346 ± 0.1	0.294 ± 0.1	0.217 ± 0.2

	Dose level (mg/kg bw per day)			
Finding	**0**	**15**	**50**	**75**
Food consumption (g/animal per day) ± SD				
GDs 5–6	96.8 ± 32.9	95.0 ± 35.4	71.5 ± 43.1	91.3 ± 45.4
GDs 6–8	113.3 ± 25.5	102.1 ± 30.4	49.8 ± 30.8**	55.6 ± 29.8**
GDs 10–12	124.9 ± 14.5	98.1 ± 26.0	85.2 ± 30.5*	58.8 ± 37.0**
GDs 18–20	115.3 ± 33.5	108.1 ± 25.0	101.1 ± 12.7	97.5 ± 46.6
GDs 26–28	121.2 ± 30.5	96.8 ± 24.2	108.2 ± 19.9	95.6 ± 16.1
GDs 6–28	118.59 ± 23.5	99.99 ± 14.6	89.36 ± 19.0*	77.30 ± 25.4**
Caesarian observations				
Animals mated	10	10	10	10
Animals pregnant	8	8	10	8
Nonpregnant	2	2	0	1
Died pregnant	0	0	0	1
Terminated nonpregnant	0	0	0	1
Totally resorbed	0	0	0	0
Dams with live fetuses	8	8	10	7
Fetal weights (g)				
Mean fetal weight	40.46	36.33	37.95	35.26*
(HCD Mean fetal weight, Range: 38.5–42.5g)				
Males	40.26	37.76	37.50	35.66
Females	39.71	34.60	38.36	35.09
Pre-implantation loss (%)	47.3	6.8**	11.5*	10.4*

GD: Gestation day; Source: adapted from Hackford, 2016d

* $p < 0.05$; ** $p < 0.01$ statistically significant difference from control group mean (Dunnett's test)

HCD: Historical control data from 12 studies conducted at the same test facility between Sept. 2008 and Oct. 2012; Examined 260 does

The NOAEL for maternal toxicity was 50 mg/kg bw per day, based on, decreased body weight gain and decreased food consumption at 75 mg/kg bw per day.

The NOAEL for developmental toxicity was 50 mg/kg bw per day, based on reduced fetal weight at 75 mg/kg bw per day (Hackford, 2016d).

Study 3

In the main developmental toxicity study spiropidion (purity 98.4%) was administered once daily by gavage to time-mated female New Zealand White rabbits (22 per dose group) at dose levels of 0, 10, 30 or 60 mg/kg bw per day, at a dose volume of 10 mL/kg bw in 0.5 % w/v CMC, acidified to pH ≤5), from GD 6 to GD 27 inclusive. Animals were examined twice daily for mortality and morbidity and were given a detailed clinical examination daily. Body weights were recorded daily from GD 5 to GD 28. The amount of food consumed by each animal was recorded over GD 4 to GD 6 and every two days thereafter during gestation. On GD 28 the females were terminated and uterine examination carried out. The live fetuses were removed from the uterus, weighed, their sex determined, and they were examined for external and visceral abnormalities. Placenta and gravid uterus weights were also recorded.

There were six early decedents during the study; none was considered to be test item-related and all prematurely killed females were pregnant. One control female (F203) died shortly after dosing on GD 16, this animal showed no clinical signs prior to death; an oesophageal rupture was found at necropsy and therefore this death was considered to be due to accidental dosing trauma. Another control female (F206) was killed on GD 23 due to poor clinical condition (loose faeces and nasal discharge from GD 22, decreased activity and cold body surface on GD 23), inappetence and body weight loss (–10%).

At necropsy, the animal was found to have: abnormal gelatinous material in the stomach, multiple raised red areas on the cardiac mucosa at the entrance of the oesophagus, a thin bile duct, distended gall bladder with pale areas and dark material, and a pale liver. This death was considered to be incidental.

One female (F288) at 60 mg/kg bw per day was killed on GD 9 due to a rapid deterioration in clinical condition. Clinical signs included pale extremities, laboured breathing and prostration, however there were no findings at necropsy which would account for the clinical condition of this animal. The death was considered to be incidental and not related to the test item.

One female (F223) at 10 mg/kg bw per day and one (F281) at 60 mg/kg bw per day showed signs of abortion during the study (red stained bedding) and were killed on GD 24 and GD 21, respectively. One female (F242) at 10 mg/kg bw per day aborted on GD 21 and was subsequently euthanised. One female (F287) at 60 mg/kg bw per day aborted three fetuses on GD 28 and had nine live fetuses at necropsy.

There were no clinical observations considered to be related to treatment.

At 60 mg/kg bw per day, there was a group mean body weight loss over the first six days of dosing (GDs 6–11). From GD 12 onwards, animals in this group started to gain weight; however, mean body weight gain for these animals was 33 % lower than the control group over the entire dosing period (GDs 6–28). Consequently, mean body weight on GD 28 was 5% lower than the controls, although statistical significance was not attained.

At 30 mg/kg bw per day, mean body weight gains were lower over the first eight days of treatment, up to 100% lower than control. From GD 14 onwards, animals in this group started to gain weight; however, mean body weight gain for these animals was 28% lower than the control group over the entire dosing period (GD 6–28).

At 10 mg/kg bw per day mean body weight gains were slightly reduced over GDs 6–9 only and were similar to, or greater than the controls thereafter.

Mean terminal body weight, adjusted for the weight of the gravid uterus, was slightly lower in the females given 60 mg/kg bw per day compared with controls (–3 %) and weight was similar to controls in the groups given 10 or 30 mg/kg bw per day.

Females at 60 mg/kg bw per day ate statistically significantly less than the controls from GD 6 to GD 18 so that their overall mean food intake for the entire dosing period was 19% lower than the controls. At 10 or 30 mg/kg bw per day, mean food intake was generally similar to control values.

There were no treatment-related maternal necropsy findings.

Caesarean section data showed that one female at 10 mg/kg bw per day, and two females in each of the groups given 30 and 60 mg/kg bw per day were not pregnant, and one female at 60 mg/kg bw per day suffered total resorption of the litter. This resulted in 20, 19, 20 and 17 females respectively with live fetuses on GD 28, in the groups given 0, 10, 30 or 60 mg/kg bw per day. At 60 mg/kg bw per day the mean incidence of post-implantation loss was slightly higher than that for the controls, however, this was mainly due to one female with total resorption; this female had only one implantation that was an early intrauterine death. The mean number of live fetuses per female was similar in all groups. Uterine/implantation parameters at 10 or 30 mg/kg bw per day were comparable with controls.

At 60 mg/kg bw per day, group mean fetal and placental weights were lower than control values (–7 % and –6 %, respectively), but not with statistical significance. These values were at the lower level of the historical control ranges. There was no effect on these parameters at 10 or 30 mg/kg bw per day. Mean sex ratio was unaffected by test item administration.

Fetal examination did not show treatment-related major abnormalities. There were no statistically significant differences from controls in the combined or various fetal examination types' overall incidences of minor and variant fetal abnormalities in the groups given spiropidion.

At 60 mg/kg bw per day, there was a slight but statistically significant increase (by trend analysis) in the number of litters with fetuses showing the minor skeletal defects described as increased anterior and posterior fontanelle and non-ossification of the pubis. In addition, at 60 mg/kg bw per day only, an

increase in the number of fetuses showing an extra thirteenth ribs with costal cartilage was also seen. The majority of these findings were outside the historical background range.

A statistically significant increase (trend analysis) in the number of litters with fetuses showing the variant cartilage defects described as incomplete second cartilaginous dorsal plate of the cervical vertebrae and incomplete xiphoid cartilage of the sternum, were noted at 30 and 60 mg/kg bw per day. The incidences of these skeletal variations were outside the historical data range.

A statistically significant increase in litters with fetuses showing interrupted costal cartilage of the ribs was noted at 30 and 60 mg/kg bw per day, compared with controls. The incidences of litters with this skeletal variation at 30 mg/kg bw per day were within the background data.

Increases in the minor or variant fetal abnormalities associated with skeletal ossification noted at 60 mg/kg bw per day were considered to be related to the lower fetal weight in this group and as such, represent a slight developmental delay. The slightly higher numbers of litters with fetuses showing two cartilage variants in the group given 30 mg/kg bw per day are considered to be incidental findings and of no toxicological significance.

Table 27. Key findings of main developmental toxicity study in rabbits

	Dose level (mg/kg bw per day)				HCD	
Finding	**0**	**10**	**30**	**60**	**Mean**	**min–max**
Body weight gain (g) ± SD						
Gestation day intervals						
GDs 6–9	0.023 ± 0.04	0.019 ± 0.04	0.011 ± 0.07	−0.005 ± 0.06		
(difference from control)		(↓17.4%)	(↓52.2%)	(↓78.3%)		
GDs 6–12	0.018 ± 0.08	0.029 ± 0.06	0.000 ± 0.08	−0.027 ± 0.07		
(difference from control)			(↓100%)	(↓250%)		
GDs 6–15	0.097 ± 0.08	0.108 ± 0.08	0.076 ± 0.09	0.054 ± 0.09		
(difference from control)			(↓21.7)	(↓44.3%)		
GDs 6–18	0.097 ± 0.07	0.106 ± 0.07	0.075 ± 0.08	0.062 ± 0.10		
(difference from control)			(↓ 22.7)	(↓36.1%)		
GDs 6–28	0.358 ± 0.20	0.279 ± 0.28	0.256 ± 0.14	0.241 ± 0.17		
(difference from control)		(↓22.1%)	(↓28.5%)	(↓32.7%)		
GDs 6–28 adjusted for gravid uterus weight	−0.19 ± 0.1	−0.16 ± 0.1	−0.19 ± 0.2	−0.21 ± 0.1		
(difference from control)		(↑15.8%)	(0.0)	(↓10.5%)		
Food consumption (g/animal/day) ± SD						
GDs 6–8	129.2 ± 29.7	113.7 ± 32.7	113.7 ± 41.4	92.9 ± 35.0**		
(difference from control)				(↓28.1%)		
GDs 8–10	117.7 ± 31.7	111.7 ± 27.9	111.2 ± 33.6	81.8 ± 38.3**		
(difference from control)				(↓30.5%)		
GDs 10–12	104.5 ± 30.9	109.7 ± 22.9	101.2 ± 35.9	75.3 ± 35.2*		
(difference from control)				(↓28.0%)		

	Dose level (mg/kg bw per day)				HCD	
Finding	**0**	**10**	**30**	**60**	**Mean**	**min–max**
GDs 12–14	108.9 ± 30.4	100.4 ± 32.7	90.1 ± 38.3	64.5 ± 37.8**		
(difference from control)			(↓17.3%)	(↓40.8%)		
GDs 14–16	105.7 ± 28.7	87.1 ± 23.9	97.2 ± 38.1	70.7 ± 28.7**		
(difference from control)		(↓17.6%)	(↓8.0%)	(↓33.1%)		
GDs 16–18	106.8 ± 31.6	102.2 ± 22.3	101.5 ± 28.0	81.7 ± 31.0*		
(difference from control)				(↓23.5%)		
GDs 18–20	108.0 ± 26.1	111.2 ± 22.2	103.6 ± 28.2	100.9 ± 26.0		
GDs 20–22	99.6 ± 21.3	105.9 ± 34.2	103.0 ± 29.3	106.2 ± 25.7		
GDs 22–24	100.7 ± 20.0	98.6 ± 30.5	101.9 ± 28.1	103.2 ± 29.2		
GDs 24–26	99.1 ± 21.2	103.4 ± 21.8	95.9 ± 24.4	97.3 ± 27.0		
GDs 26–28	96.2 ± 22.7	91.6 ± 33.9	79.5 ± 21.2	85.6 ± 31.9		
GDs 6–28	107.57 ± 20.7	102.90 ± 18.7	99.35 ± 22.9	87.28 ± 19.5*		
(difference from control)		(↓4.3%)	(↓7.6%)	(↓18.9%)		
Caesarian observations						
Animals mated	22	22	22	22		
Animals pregnant	22	21	20	20		
Animals not pregnant	0	1	2	2		
Died/killed/aborted pregnant	2	2	0	2		
Terminated non-pregnant	0	0	0	0		
Totally resorbed	0	0	0	1		
Dams with live fetuses	20	19	20	17		
Mean number of live fetuses per dam ± SD	8.1 ± 2.3	7.1 ± 1.7	7.4 ± 2.7	7.9 ± 2.2		
Intra-uterine deaths/per dam						
Number of early deaths (litters affected)	11 (7)	12 (8)	4 (2)	9 (8)		
Number of late deaths (litters affected)	3 (2)	6 (4)	8 (5)	6 (5)		
Fetal weights (g)						
Litter weight	290.88	265.87	272.86	275.89		
Mean fetal weight	36.61	37.78	38.27	33.90		32.6–43.6
Males	36.80	36.88	38.66	33.96		
Females	36.66	37.42	37.38	32.99		

	Dose level (mg/kg bw per day)				HCD	
Finding	**0**	**10**	**30**	**60**	**Mean**	**min–max**
Pre-implantation loss (%)	19.1	16.8	15.5	10.1		
Post-implantation loss (%)	7.6	10.2	6.6	16.1		
Mean placental weight (g)	4.07	4.28	4.34	3.82		3.5–4.6
Fetal abnormalities – skeletal					**Fetal incidence (*n*)**	**Litters affected (*n*)**
Number of fetuses examined	83	65	73	72		
Number of litters examined	20	19	19	17		
Fontanelle – anterior, increased in size						
Number of fetuses [%]	0 [0.0]	0 [0.0]	0 [0.0]	2 [2.6]^{J}	0.8–1.3%	1–2
Number of litters [%]	0 [0.0]	0 [0.0]	0 [0.0]	2 [11.8]$^{\#}$		
Fontanelle – posterior: increased in size						
Number of fetuses [%]	0 [0.0]	0 [0.0]	0 [0.0]	2 [2.6]^{J}	0.9–1.3%	1–1
Number of litters [%]	0 [0.0]	0 [0.0]	0 [0.0]	2 [11.8]$^{\#}$		
Pelvic girdle – pubis- uni- or bilateral: not ossified						
Number of fetuses [%]	0 [0.0]	0 [0.0]	0 [0.0]	6 [4.0]^{JJ}	0.4–2.3%	1–2
Number of litters [%]	0 [0.0]	0 [0.0]	0 [0.0]	3 [17.6]$^{\#\#}$		
Cervical vertebra – 2nd cartilaginous dorsal plate: incomplete						
Number of fetuses [%]	39 [22.2]	27 [19.8]	45 [36.6]^{J}	55 [38.2]^{JJ}	14.4–14.4%	7–7
Number of litters [%]	10 [50.0]	13 [68.4]	17 [85.0]$^{\#}$	15 [88.2]$^{\#\#}$		
Rib – 13th extra- uni- or bilateral: with costal cartilage						
Number of fetuses [%]	19 [13.1]	25 [20.7]	12 [7.9]	44 [31.5]^{J}	15.3–33.6%	9–18
Number of litters [%]	10 [50.0]	12 [63.2]	8 [40.0]	14 [82.4]		
Rib – one or more: costal cartilage interrupted						
Number of fetuses [%]	4 [2.6]	10 [8.3]	8 [4.5]	18 [13.3]^{J}	0.8–7.8%	1–8
Number of litters [%]	4 [20.0]	9 [47.4]	6 [30.0]$^{\#}$	12 [70.6]$^{\#\#}$		
Sternum – xiphoid cartilage: incomplete						
Number of fetuses [%]	4 [2.6]	1 [0.5]	14 [8.4]^{J}	15 [8.6]^{JJ}	0.8–7.0%	1–4
Number of litters [%]	2 [10.0]	1 [5.3]	7 [35.0]$^{\#}$	6 [35.3]$^{\#\#}$		

GD: Gestation day; SD: standard deviation; Source: adapted from King, 2017

HCD: Historical control data for caesarean section from 29 studies conducted at the same test facility between Dec. 2004 and June 2016; examined 601 does; HCD for skeletal abnormalities from 4305 fetuses and 547 litters.

* $p < 0.05$; ** $p < 0.01$ statistically significant difference from control group mean (Dunnett's test)

^{J} $p < 0.05$, ^{JJ} $p < 0.01$ Jonckheere test, one-sided

$^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ Cochran–Armitage test, one-sided

The NOAEL for maternal toxicity was 10 mg/kg bw per day based on body weight stasis in the first six days of treatment and decreased body weight gain thereafter at 30 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity was 30 mg/kg bw per day, based on increased incidence of skeletal effects (unossified pelvic girdle and incomplete xyphoid cartilage) at 60 mg/kg bw per day (King, 2017).

2.6 Special studies

(a) Neurotoxicity

Acute neurotoxicity

In a preliminary acute neurotoxicity study aimed at identifying the maximum tolerable dose and the time to peak effect, spiropidion (purity 98.4%) was administered by gavage to four groups of Han Wistar rats (three animals/sex per group) at dose levels of 0, 50, 500 or 1000 mg/kg bw and a dose volume of 10 mL/kg bw in 0.5% w/v CMC acidified to pH ≤5. The animals were then maintained for a seven-day post dose observation period. During the study, clinical condition, body weight, food consumption and neurobehavioural screening observations were undertaken. Neurobehavioural assessments were performed once prior to dosing, and then at 1, 2, 4, 6, and 8 hours post dose.

One 1000 mg/kg bw female was observed with a convulsion at approximately 7.5 hours. This female subsequently experienced two further convulsions, necessitating her unscheduled termination for welfare reasons. On the day after dose administration (day 2) another 1000 mg/kg bw female exhibited irritable behaviour, vocalisation, piloerection, uncoordinated gait and a generally poor clinical condition, leading to her early (unscheduled) termination for welfare reasons. Two 1000 mg/kg bw males each displayed decreased activity, piloerection, swaying and uncoordinated gait on day 2 and/or day 3 after dose administration, but had recovered by day 4.

Body weight losses occurred over the first day after dosing (day 1 to 2) at 500 mg/kg bw and 1000 mg/kg bw for both sexes, with the magnitude of body weight losses being dose-related for males but not females. Over days 2 to 4 of the observation period there was further body weight loss for one 1000 mg/kg bw male and only minimal body weight gains for the remaining two males at that same dose. Minimal body weight gains over this period were also noted for the single surviving 1000 mg/kg bw female, and for one 500 mg/kg bw male.

Based on the findings in this study, 500 mg/kg bw was recommended as the highest dose level for the definitive acute neurotoxicity study since the weight loss that occurred following dose administration showed clear recovery during the eight-day observation period. A 500 mg/kg bw dose was anticipated not to compromise the animals over a two-week, post-dose assessment period.

The incidence of clinical signs at eight hours post dose (specifically the repeated convulsions in one female and the absence of rearing counts in two of three females at 1000 mg/kg bw) indicated that eight hours post-dose had been the time of peak effect for spiropidion (Christine, 2017).

In the main acute neurotoxicity study, spiropidion (purity 98.4%) was administered as a single oral (gavage) dose to four groups of 10 Han Wistar rats/sex per group at dose levels of 0, 50, 150 or 500 mg/kg bw, with a vehicle dose volume of 10 mL/kg of aqueous 0.5% w/v CMC acidified to pH ≤5, followed by a 14-day observation period. Animals were dosed sequentially and in random order over four days. During the study, clinical condition, body weight, food consumption, neurobehavioural screening observations (in cage, in hand and arena observations, reactivity investigation, and motor activity), brain weight, macropathology and histopathology investigations were undertaken involving an adequate number of target tissues.

At approximately eight hours after dosing, four females at 500 mg/kg bw had convulsions that lasted approximately 1–2 minutes. Consequently these animals, together with another female at 500 mg/kg bw which did not show these signs but displayed a generally poor clinical condition, were killed for welfare reasons. As a result of these severe adverse clinical observations, the remaining five females allocated to the group were not dosed and were removed from the study. There were no treatment-related signs recorded at 500 mg/kg bw in the males, or either in sex at 150 mg/kg bw or 50 mg/kg bw.

An initial body weight loss in the majority of animals over the first day following treatment was observed in males at 500 mg/kg bw and in females at 150 mg/kg bw. Mean body weight stasis was reported in males at 150 mg/kg bw during the first day after treatment. Between days 2 and 15 body weight gain for groups dosed at 150 mg/kg bw per day and above was higher or comparable to controls. Since the lower mean body weight gains noted for the 150 mg/kg bw males and females over the first day following dosing were slight and transient with no effect on absolute body weights, these effects on

body weight gain at 150 mg/kg bw were considered not to be adverse. There were no treatment-related effects on body weights in animals at 50 mg/kg bw.

No relevant treatment-related effects were reported on food consumption.

There were no treatment-related effects on the in-cage or in-hand clinical observations performed on day 1 (eight hours post dose), day 8 or 15.

There were no treatment-related effects on the arena observations performed on days 1 (at eight hours post dose). Increased activity and rearing counts (statistically significant) were reported in males at 500 mg/kg bw on observation days 8 and 15 compared to controls, however activity and rearing counts for these animals were similar to the pretreatment observations and there was no similar finding at eight hours after dose administration (day 1). The increase in activity and rearing counts was not observed during the motor activity assessments on these days and consequently the findings reported in the standard arena were considered spurious and were not attributed to treatment.

A small but statistically significant increase in landing foot splay was reported in females at 150 mg/kg bw on observation day 15. Given the small size of the change and that the values were within the historical control data ranges (69–80 mm) this isolated finding was considered to be of no toxicological significance.

On day 1 (at the time of peak effect) there were slight reductions in the mean high- (rearing) and low-beam (cage-floor activity) counts for the majority of the 60-minute recording period in males at 500 mg/kg bw; these reductions were not different from control values in a statistically significant sense, and were not attributed to treatment. Similarly, slight reductions in the high- and low-beam scores were also noted for 50 or 150 mg/kg bw females on day 1, achieving statistical significance at 50 mg/kg bw but not at 150 mg/kg bw. Due to the absence of a clear dose–response relationship the reduced motor activity scores for the 50 mg/kg bw females on day 1 were considered not to be treatment-related. In any case, in the absence of any other neurobehavioural findings these slight reductions in motor activity noted on day 1 were considered not to be toxicologically relevant.

On days 8 and 15 there were no treatment-related motor activity findings since the mean high-beam and low-beam scores for the treated animals were similar to those seen during pre-treatment investigations.

There were no treatment-related effects on brain weight, macroscopic lesions or histopathology findings in the nervous system tissues.

Table 28. Key findings of main acute neurotoxicity study in rats

	Dose level (mg/kg bw)						
	Males				Females		
Finding	**0**	**50**	**150**	**500**	**0**	**50**	**150**
Body weight (g) ± SD							
Predose (−7 days)	117 ± 10.8	113 ± 11.2	117 ± 10.4	117 ± 10.0	117 ± 9.7	116 ± 9.5	113 ± 10.2
Day 1	159 ± 12.2	154 ± 12.3	158 ± 12.5	156 ± 10.5	139 ± 13.1	140 ± 8.4	136 ± 11.1
Day 2	163 ± 11.7	158 ± 12.4	158 ± 11.8	144 ± 14.5	141 ± 11.1	141 ± 8.8	135 ± 10.2
Day 4	174 ± 12.1	166 ± 12.5	169 ± 12.0	156 ± 13.7	148 ± 12.6	147 ± 8.9	141 ± 11.2
Day 8	195 ± 10.8	188 ± 14.0	191 ± 13.4	180 ± 13.0	160 ± 12.3	160 ± 10.6	155 ± 11.8
Day 15	232 ± 10.8	230 ± 17.5	235 ± 14.2	225 ± 12.9	174 ± 11.5	177 ± 10.3	173 ± 13.8
Body weight gain (g) ± SD							
Days 1–2	4 ± 1.8	4 ± 2.3	1 ± 1.6**↓	−13 ± 7.7**↓	2 ± 4.3	1 ± 3.4	−1 ± 2.5
Days 2–4	10 ± 2.1	9 ± 1.9	11 ± 2.4	12 ± 3.1	7 ± 3.1	6 ± 2.6	6 ± 2.3
Days 4–8	21 ± 3.9	22 ± 3.4	22 ± 3.4	24 ± 3.2*↑	12 ± 2.8	13 ± 2.0	14 ± 1.6
Days 8–15	38 ± 5.5	42 ± 4.7	44 ± 4.3*	45 ± 6.5**↑	14 ± 5.1	17 ± 4.4	18 ± 6.9
Days 1–15	73 ± 7.2	76 ± 6.9	77 ± 3.9	69 ± 5.1	35 ± 8.9	37 ± 7.0	37 ± 7.2

	Dose level (mg/kg bw)						
	Males				Females		
Finding	**0**	**50**	**150**	**500**	**0**	**50**	**150**
Activity count ± SD							
Predose	11.5 ± 5.8	18.0 ± 4.5	16.5 ± 6.2	16.8 ± 5.3	20.9 ± 7.8	17.0 ± 3.1	18.4 ± 5.5
Day 1	6.0 ± 4.7	9.1 ± 4.2	8.9 ± 7.7	8.1 ± 4.8	19.4 ± 10.6	24.1 ± 2.9	20.8 ± 5.7
Day 8	5.8 ± 6.4	9.9 ± 5.1	11.1 ± 8.3	17.2 ± 6.8**↑	25.7 ± 8.7	26.4 ± 3.8	24.6 ± 4.7
Day 15	6.5 ± 6.3	8.3 ± 4.9	6.7 ± 4.7	14.7 ± 7.8*↑	27.7 ± 11.5	29.4 ± 4.0	26.9 ± 4.3
Rearing count ± SD							
Predose	6.7 ± 2.8	9.3 ± 2.5	7.3 ± 2.7	7.3 ± 2.4	13.3 ± 6.5	8.3 ± 4.0	10.5 ± 3.9
Day 1	3.9 ± 2.8	5.5 ± 3.7	2.8 ± 2.2	4.3 ± 3.6	13.1 ± 8.7	12.4 ± 4.5	12.4 ± 5.3
Day 8	3.5 ± 3.5	5.3 ± 3.4	5.5 ± 4.3	8.9 ± 3.5**↑	17.3 ± 7.5	16.5 ± 3.9	16.0 ± 5.8
Day 15	3.0 ± 4.1	4.0 ± 3.3	3.2 ± 2.7	7.0 ± 4.1*↑	16.9 ± 8.5	21.0 ± 5.7	18.2 ± 5.0
Landing foot splay (mm) ± SD							
Pre-dose	77 ± 11	66 ± 9	74 ± 10	79 ± 18	64 ± 8	70 ± 11	66 ± 11
Day 1	75 ± 13	73 ± 10	83 ± 10	72 ± 14	73 ± 7	75 ± 9	77 ± 12
Day 8	77 ± 12	75 ± 15	80 ± 10	80 ± 17	73 ± 6	72 ± 14	79 ± 15
Day 15	80 ± 17	79 ± 15	83 ± 12	81 ± 15	71 ± 6	76 ± 6	80 ± 9*↑
High-beam motor activity (total in 60 min) ± SD							
Predose	196.9 ± 66.3	172.2 ± 80.1	192.4 ± 79.8	238.0 ± 89.2	273.6 ± 64.6	213.6 ± 116.1	302.2 ± 135.7
Day 1	190.8 ± 124.0	149.8 ± 46.2	157.0 ± 68.8	116.8 ± 87.3	298.6 ± 103.9	173.8 ± 103.4**↓	205.1 ± 55.4
Day 8	289.9 ± 102.9	229.9 ± 75.8	287.0 ± 101.1	329.0 ± 135.4	425.4 ± 189.1	341.7 ± 146.6	434.2 ± 115.8
Day 15	310.5 ± 178.6	260.0 ± 76.4	289.7 ± 140.7	357.8 ± 132.0	360.1 ± 100.7	324.6 ± 137.1	394.0 ± 199.9
Low-beam motor activity (total in 60 min) ± SD							
Predose	696.6 ± 159.8	663.4 ± 209.9	787.77 ± 284.1	848.7 ± 224.5	871.7 ± 216.2	808.2 ± 219.0	938.9 ± 259.0
Day 1	701.3 ± 269.4	601.3 ± 131.2	671.2 ± 188.8	546.4 ± 165.4	957.3 ± 351.6	662.4 ± 201.2*↓	779.2 ± 260.7
Day 8	660.1 ± 173.3	589.4 ± 152.2	629.6 ± 142.4	767.7 ± 208.8	941.5 ± 340.3	752.9 ± 240.9	952.0 ± 241.1
Day 15	804.0 ± 156.4	760.6 ± 201.0	692.1 ± 232.9	836.5 ± 211.5	899.4 ± 231.9	741.2 ± 192.8	953.6 ± 374.6

SD: standard deviation; Source: adapted from Leggett, 2018

* $p < 0.05$; ** $p < 0.01$ statistically significant difference from control group mean (Dunnett's test)

The NOAEL for systemic toxicity was 150 mg/kg bw based on body weight loss in males at 500 mg/kg bw.

The NOAEL for neurotoxicity was 150 mg/g bw based on clinical sign (convulsions) in females at 500 mg/kg bw (Leggett, 2018).

(b) Mechanistic studies

An ex vivo and one in vitro study were conducted (as summarized in Table 29) to assess treatment-related thyroid effects observed in the short-term and reproductive toxicity studies in rats.

Table 29. Summary of mechanistic studies

Type of test organism Tested doses	Critical effects	Purity (%) Batch Number	Reference
Spiropidion only UDP-glucuronosyltransferase induction, rat liver samples, in diet 0, 2500 and 1500 ppm M and F equal to 159/110 mg/kg bw/day M and F	Rise in rate of T4 glucuronidation, (2.5- to 2-fold increase, M and F) Spiropidion is an inducer of hepatic UDP-GT activity	Not specified Not specified	Madden, 2014
Spiropidion and metabolite SYN547305 Rat thyroid peroxidase activity, microsomal preparation 0, 0.05, 0.5, 2, 5 and 10 µM	No significant effects on rat thyroid peroxidase activity; Spiropidion and metabolite SYN547305 are not inhibitors of rat thyroid peroxidase activity in vitro.	98.4 CSH001-002-001	Lake, 2014

M: Male; F: Female UDP-GT: Uridine diphosphate glucuronyltransferase

In an ex vivo study the potential spiropidion induction of uridine diphosphate glucuronyltransferase (UDP-GT) enzyme was assessed in liver samples from 10 Han Wistar rats/sex per dose at 0, 2500 or 1500 ppm (equal to 0, 159 and 110 mg/kg bw per day). Microsomes were prepared from each liver sample and the rate of [^{125}I]thyroxine phenolic glucuronidation (T4 glucuronidation) was determined for each microsomal preparation. For comparison the rate of reaction in microsomes prepared from animals pretreated with known inducers of xenobiotic metabolism (phenobarbital, 3-methylcholanthrene or β-napthoflavone and clofibric acid) was also assessed.

Dietary exposure of male and female animals to spiropidion for 90 days at dietary dose levels of 2500 and 1500 ppm for males and females respectively, resulted in statistically significant increases in the rate of T4 glucuronidation. In males, a 2.5-fold increase was observed whilst in females a 2-fold increase was seen.

It was concluded that spiropidion is an inducer of hepatic UDP-GT activity in both male and female Han Wistar rats (Madden, 2014).

In an in vitro study spiropidion (purity 98.4%) and metabolite SYN547305 (purity 95-99%) were assessed for their ability to inhibit rat thyroid peroxidase activity. A pooled thyroid gland microsomal preparation from 10 Han Wistar rats was exposed to 0, 0.05, 0.5, 2, 5 and 10 µM spiropidion or metabolite SYN547305. Thyroid peroxidase activity was assayed by determining the monoiodination of *L*-tyrosine. Incubations were conducted in triplicate, except for the control incubations (DMSO only) where four replicates were used. As a positive control the effect of 6-propyl-2-thiouracil (PTU; 10 µM) on rat thyroid peroxidase activity was also determined.

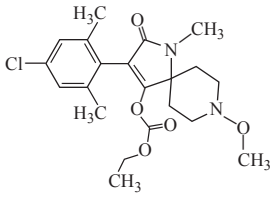
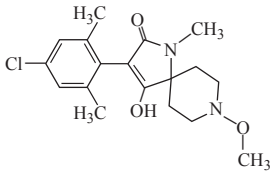
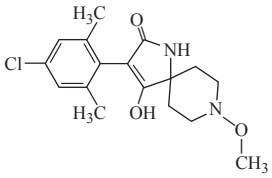
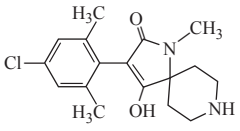
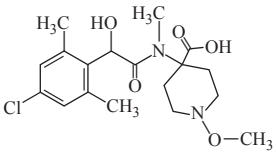
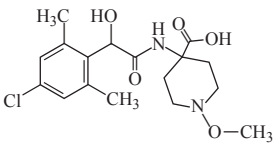
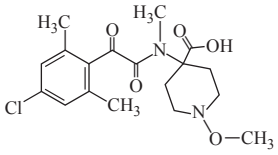
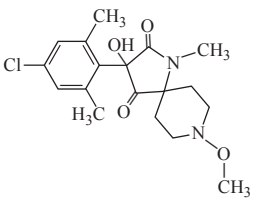
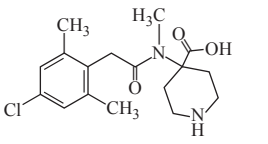
Treatment with spiropidion or metabolite SYN547305 had no significant effect on rat thyroid peroxidase activity at any concentration tested. Treatment with the positive control, PTU, resulted in a greater than 99.9% inhibition of thyroid peroxidase activity in both experiments.

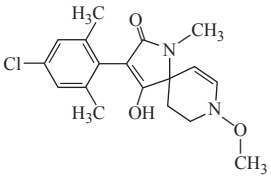
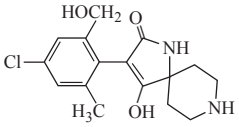
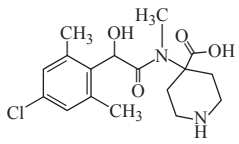
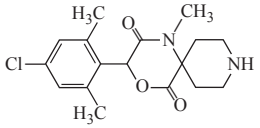
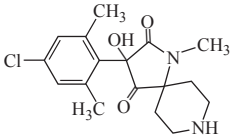
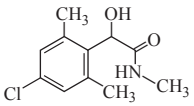
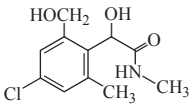
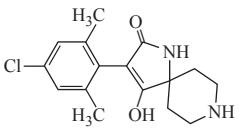
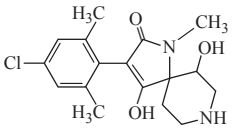
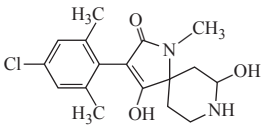
It was concluded that spiropidion and metabolite SYN547305 are not inhibitors of rat thyroid peroxidase activity in vitro.

(c) Toxicity studies on metabolites and impurities

Table 30 summarises the occurrence of spiropidion (SYN546330) and dietary metabolites in various crops and animals.

Table 30. Dietary metabolites of spiropidion

Compound code (other names)	Description	Chemical structure	Matrix
SYN546330 Spiropidion	[3-(4-chloro-2,6-dimethyl-phenyl)-8-methoxy-1-methyl-2-oxo-1,8-diazaspiro[4.5] dec-3-en-4-yl] ethyl carbonate		**Plants:** Tomato, potato, cotton **Animals:** Rat
SYN547305 (CSCD710959)	3-(4-chloro-2,6-dimethyl-phenyl)-4-hydroxy-8-methoxy-1-methyl-1,8-diazaspiro[4.5] dec-3-en-2-one		**Plants:** Tomato, potato, cotton **Animals:** Goat, hen, rat
SYN547435	3-(4-chloro-2,6-dimethyl-phenyl)-4-hydroxy-8-methoxy-1,8-diazaspiro[4.5] dec-3-en-2-one		**Plants:** Tomato, potato, cotton **Animals:** Rat
SYN548430	3-(4-chloro-2,6-dimethyl-phenyl)-4-hydroxy-1-methyl-1,8-diazaspiro[4.5] dec-3-en-2-one		**Plants:** Tomato, potato, cotton **Animals:** Goat, hen, rat
SYN550839	4-[[2-(4-chloro-2,6-dimethyl-phenyl)-2-hydroxy-acetyl]-methyl-amino]-1-methoxy-piperidine-4-carboxylic acid		**Plants:** Cotton
SYN550820	4-[[2-(4-chloro-2,6-dimethyl-phenyl)-2-hydroxy-acetyl] amino]-1-methoxy-piperidine-4-carboxylic acid		**Plants:** Cotton
SYN548388	4-[[2-(4-chloro-2,6-dimethyl-phenyl)-2-oxo-acetyl]-methyl-amino]-1-methoxy-piperidine-4-carboxylic acid		**Plants:** Tomato leaves
SYN548033	3-(4-chloro-2,6-dimethyl-phenyl)-3-hydroxy-8-methoxy-1-methyl-1,8-diazaspiro[4.5] decane-2,4-dione		**Plants:** Tomato, potato foliage and rotational crops
SYN551003	4-[[2-(4-chloro-2,6-dimethyl-phenyl)acetyl]-methyl-amino]piperidine-4-carboxylic acid		**Plants:** Cotton

Compound code (other names)	Description	Chemical structure	Matrix
Dehydrated/Reduced SYN547305	3-(4-chloro-2,6-dimethyl-phenyl)-4-hydroxy-8-methoxy-1-methyl-1,8-diazaspiro[4.5] deca-3,6-dien-2-one		**Plants:** Tomato **Animals:** Rat
SYN548939-OH U5 (Proposed structure)	3-[4-chloro-2-(hydroxymethyl)-6-methyl-phenyl]-4-hydroxy-1,8-diazaspiro[4.5] dec-3-en-2-one		**Plants:** Rotated crops
SYN550838	4-[[2-(4-chloro-2,6-dimethyl-phenyl)-2-hydroxy-acetyl]-methyl-amino] piperidine-4-carboxylic acid		**Plants:** Rotated crops
SYN550821	3-(4-chloro-2,6-dimethyl-phenyl)-1-methyl-4-oxa-1,9-diazaspiro[5.5] undecane-2,5-dione		**Plants:** Rotated crops
SYN549937	3-(4-chloro-2,6-dimethyl-phenyl)-3-hydroxy-1-methyl-1,8-diazaspiro[4.5] decane-2,4-dione		**Plants:** Rotated crops
SYN549098	2-(4-chloro-2,6-dimethyl-phenyl)-2-hydroxy-N-methyl-acetamide		**Plants:** Rotated crops
SYN549098-OH (Proposed structure)	2-[4-chloro-2-(hydroxymethyl)-6-methyl-phenyl]-2-hydroxy-N-methyl-acetamide		**Plants:** Rotated crops
SYN548939	3-(4-chloro-2,6-dimethyl-phenyl)-4-hydroxy-1,8-diazaspiro[4.5]dec-3-en-2-one		**Animals:** Goat, hen
OH-SYN548430_I1 (Proposed structure)	3-(4-chloro-2,6-dimethyl-phenyl)-4,6-dihydroxy-1-methyl-1,8-diazaspiro[4.5] dec-3-en-2-one		**Animals:** Goat
OH-SYN548430_I2 (Proposed structure)	3-(4-chloro-2,6-dimethyl-phenyl)-4,7-dihydroxy-1-methyl-1,8-diazaspiro[4.5] dec-3-en-2-one		**Animals:** Goat

An assessment of genotoxic potential has been made for all 18 dietary metabolites of spiropidion (SYN546330) identified in primary crop, rotational crop and/or livestock metabolism studies. Each metabolite of spiropidion was analysed using three quantitative structure–activity relationship (QSAR) programs:

- DEREK Nexus (v. 6.0.1) – an expert knowledge-based system,
- VEGA platform (CAESAR mutagenicity models, v. 2.1.13) – a statistically-based system and additionally
- QSAR Toolbox (v. 4.3) – a hybrid system; mechanistic, end-point specific and empiric (functional group) profilers were used.

Full details of the predictions and adequate references to the models used were provided.

Following QSAR analysis, a chemical read-across approach was proposed using knowledge of chemical reactivity with respect to genotoxic end-points. The metabolites were divided into five categories (see below) based on structural similarity (derived from expert knowledge and physical chemistry parameters), and their genotoxicity potential assessed using comparative QSAR analysis with reference to parent or other metabolites for which genotoxicity data was already available.

Spiropidion was provided with a full genotoxicity package and was not considered to be of genotoxic concern. Metabolite SYN547305 is formed under aqueous conditions by hydrolysis of the carbonate group. Metabolite SYN548430 is the major metabolite present in the rat and consequently neither of these are considered to be of genotoxic concern. At the current time, there are no in vitro or in vivo data available for any other metabolite.

Several spiropidion metabolites exist as tautomers (keto and enol forms). For this QSAR assessment both tautomeric forms of the relevant metabolites were considered, however in biological systems (in vitro or in vivo) both may exist in a dynamic equilibrium. Therefore the metabolite is considered as a single molecule which can exist as either or both tautomers.

Based on this analysis it is proposed that the metabolites be included in the proposed category 1 given their structural similarity to parent spiropidion and the absence of additional structural alerts for genotoxicity.

The read across and QSAR assessment is summarised below:

Category 1

Metabolites SYN547305, dehydrated-SYN547305, OH-SYN548430-I1, SYN547435, SYN548430, SYN548939-OH U5, OH-SYN548430-I2 and SYN548939 exist as two tautomers (T1 and T2).
The QSAR assessment showed that these metabolites reported some alerts and were structurally similar to parent (spiropidion) – read-across to parent is proposed. No genotoxic concern for these metabolites.

Category 2 (locked ketones)

Metabolites SYN548033 and SYN549937 were considered to be structurally different to parent and of no genotoxic concern based on their QSAR assessment.

Category 3 (breaking of the dione ring)

Metabolites SYN548388, SYN550839, SYN550820, SYN550838 and SYN551003 were considered to be structurally different to parent and of no genotoxic concern based on their QSAR assessment.

Category 4 (lactones)

Metabolite SYN550821 was considered to be structurally different to parent and of no genotoxic concern based on the QSAR assessment.

Category 5 (acetamides)

Metabolites: OH-SYN549098-I2 and SYN549098 were considered to be structurally different to parent and of no genotoxic concern based on their QSAR assessment.

QSAR genotoxicity assessment

Based on the read across analysis, metabolites SYN548033, SYN548388, SYN550821 and SYN549098 were selected as representative metabolites for categories 2, 3, 4 and 5.

Like all the other compounds including the parent, metabolites in categories 2, 3, 4 and 5 were predicted to be nonmutagenic by both the Derek and Caesar models.

No DNA alerts for AMES, CA and MNT by OASIS, DNA binding by OASIS, protein-binding alerts for chromosomal aberration by OASIS, in vitro mutagenicity (Ames test) alerts by ISS profilers were found for their structures, like all the other compounds including the parent.

For metabolites the SYN548033, SYN548388, SYN550821 and SYN549098 the same "H-acceptor-path3-H-acceptor" alert was found for in vivo mutagenicity (micronucleus) alerts by the ISS profiler, as with the parent and all the other compounds.

For SYN548033 (category 2), SYN548388 (category 3) and SYN550821 (category 4), DNA-binding by OECD profiler produced the same alert as for the parent ("SN1 >> Iminium Ion Formation; SN1 >> Iminium Ion Formation >> Aliphatic tertiary amines") while for SYN549098 there were no alerts as was the case for the other metabolites.

SYN548033 (category 2) and SYN548388 (category 3) triggered an alert by OASIS for protein-binding, as did all the other keto tautomer forms, but by OECD triggered the same alert for protein-binding as with the parent. Metabolite SYN549084 (category 5) produced no alerts from these two profilers.

Only for SYN550821 (category 4) were different alerts produced, with protein-binding by OASIS and protein binding by the OECD profiler (not specific for genotoxicity) giving: "Activated alkyl esters and thioesters" and "Allyl acetates and related chemicals", respectively. (See Appendix 1 for the detailed description of those alerts as reported by QSAR Toolbox.)

General toxicity assessment of dietary metabolites

A general toxicity assessment f has been conducted for the dietary metabolites SYN547305, SYN548430 and SYN547435, for which residue levels in primary crop are > 10% of total readioactive residue (TRR) and > 0.01 mg/kg bw. Spiropidion is considered to have been adequately assessed by the toxicology data package outlined in this evaluation. Metabolite spiropidion-enol (SYN547305) is formed by rapid hydrolysis of the parent compound and is the precursor of all other metabolites identified in rats. It is very likely that this metabolite is responsible for spiropidion's toxicity, as substantial exposure to spiropidion-enol was demonstrated in several toxicity studies. Therefore the results from toxicology studies on the parent are considered to adequately cover the toxicity testing of spiropidion-enol (SYN547305).

Metabolite SYN548430 is a major mammalian metabolite with 42.8% and 35.1% of the AD recovered in the urine of intact rats at 5 and 250 mg/kg bw, respectively, seen in the rat biotransformation study of Strathdee & Pulner (2017). The toxicological database and reference values for parent spiropidion (SYN546330) were considered applicable to SYN548430.

Metabolite SYN547435 was not recovered at high enough systemic exposure levels (less than 1% in urine) to be considered adequately tested in toxicity studies with the parent. SYN547435 was therefore assessed for repeat-dose toxicity using a chemical read-across approach which examined the structural similarity of SYN547435, spiropidion (SYN546330), SYN547305 and SYN548430 via the appropriate chemistry parameters. A QSAR analysis for all toxicity end-points of SYN547435 and the read-across candidates was performed to provide supporting information for the read-across assessment. The toxicological database and reference values for parent spiropidion (SYN546330) are considered applicable to SYN547435.

(d) Microbiological effects

The pro-insecticide, spiropidion was evaluated at JMPR 2021 to determine its impact on the microbiota of the gastrointestinal tract. Since no data was submitted by the sponsors, a literature search was performed using a number of search engines. These included Google Scholar (http://scholar.google.com/)/ Google search engine (https://www.google.com/), PubMed (http://www.ncbi.nlm.nih.gov/pubmed), WEB OF SCIENCE (https://apps.webofknowledge.com), BioOne (http://www.bioone.org/) and ScienceDirect (http://www.sciencedirect.com/).

The searching strategy used included the input keywords of the insecticide chemical name (spiropidion), chemical structure, antimicrobial mode of action, antimicrobial spectrum of activity, antimicrobial resistance, resistance mechanisms and genetics, microbiome, microbiota, gut microbiota, gut microbiome, gastrointestinal microbiota, gastrointestinal microbiome, etc.) and the Boolean operators (AND, OR, and NOT). The extensive search and review of the scientific literature did not find any reports of the effects of the spiropidion on the intestinal microbiome which we could include in the toxicological risk assessment.

3. Observations in humans

In reports on manufacturing plant personnel, no adverse health effects were noted. No information on accidental or intentional poisoning in humans is available.

Comments

Biochemical aspects

In disposition studies rats were administered [^{14}C]spiropidion labelled at either the phenyl or spirodecanone ring as a single oral dose of 5 or 250 mg/kg bw body weight (bw), or as a single intravenous dose of 1 mg/kg bw of [^{14}C]spiropidion labelled at the phenyl ring. Excretion was >95% (urine, faeces and cage wash) of the administered dose (AD), of which more than 90% was excreted within 48 hours of oral dosing, or 24 hours when dosing was intravenous. There were no significant sex-, dose- or radiolabel-related differences in either the rates or routes of excretion. The major route of excretion was through the urine, accounting for approximately 60% and 50% of the AD in animals given an oral dose of 5 mg/kg bw or 250 mg/kg bw respectively, or 60% of the AD when an intravenous dose of 1 mg/kg bw was administered. In animals given an oral dose of 5 mg/kg bw, an oral dose of 250 mg/kg bw or an intravenous dose of 1 mg/kg bw, faecal excretion accounted for approximately 30%, 40% and 30% of the AD respectively.

In bile duct-cannulated rats, excreted radioactivity in urine, faeces and cage wash was >96% of the AD following either a single dose of 5 or 250 mg/kg bw. Elimination via urine was comparable to that of intact animals. Following a single low dose of 5 mg/kg bw the majority of the dose was excreted in the urine (65–66%) in the first 48 hours, in faeces (14–15%), and via biliary excretion (6–11%). Following a single oral administration of 250 mg/kg bw the majority of the dose was excreted in the urine (45–64%) in the first 48 hours. Elimination of spiropidion via the faeces accounted for 19–30% of the dose and biliary excretion for 13–14% of the dose (Punler, Tomlinson & Hutton, 2016a). The oral absorption was 72–77% in male and female rats, based on urinary, biliary excretion, cage wash and carcass.

Following intravenous administration of 1 mg/kg bw, blood concentrations of radioactivity steadily declined to 72 hours post dose. The systemic exposure (represented by the area under the concentration–time curve, $AUC_{(0-t)}$) was comparable irrespective of sex, and the estimated half-life ($t_{½}$) of total radioactivity was longer in males (42 hours) than females (22 hours). In animals given a single oral dose of 5 or 250 mg/kg bw, peak whole blood and plasma concentrations (C_{max}) were observed at 1–2 h or 1–4 h, respectively. Overall total systemic exposure was comparable between whole blood and plasma with the same dose levels and radiolabel position. Systemic exposure to total radioactivity increased in a broadly proportional manner with dose. The estimated $t_{½}$ of total radioactivity in whole blood was longer in males (65 hours) than females (44 hours) administered 5 mg/kg bw, while no sex differences were observed in animals given 250 mg/kg bw (Punler, Tomlinson & Hutton, 2016b).

The pharmacokinetics of non-radiolabelled spiropidion were investigated in the rat following repeated oral exposure at dose levels of 3, 30 and 300 mg/kg bw per day over seven days, or after a single intravenous administration at a dose level of 1 mg/kg bw. Spiropidion blood concentrations were generally very low (< 10 ng/mL or below the limit of quantification). The low and variable blood concentrations of spiropidion are due to its rapid hydrolysis to metabolite SYN547305 after both oral and intravenous administration. This is supported by the concentrations of metabolite SYN547305 observed after intravenous and oral administration of spiropidion. There was no evidence supporting accumulation (Lewsley & Hewitt, 2013).

Radioactivity was widely distributed, with the highest concentrations observed in the gastrointestinal tract and its contents, followed by liver and kidney, consistent with the excretion profile of spiropidion. Radioactivity in all other tissues was lower or equal to 0.1% of the AD. The tissue depletion half-life of radioactivity in the majority of tissues was either similar, or shorter, than in plasma. However, longer estimates were obtained in whole blood. At termination (96 h post dose), total tissue and carcass residues accounted for equal or less than 2.5% of the administered dose.

Metabolism occurred rapidly in both intact and bile duct-cannulated rats. Unchanged spiropidion was found in faeces at 2.7% and 7% respectively for intact or cannulated animals given 250 mg/kg bw. The observed metabolites were qualitatively and quantitatively similar in urine, faeces, bile and plasma irrespective of dose. Spiropidion was extensively metabolized in rat via ester hydrolysis and subsequent loss of the methoxy moiety in the piperidine ring. No cleavage of the cyclic moieties (phenyl, spirodecanone or piperidine) was observed. In rat excreta, only SYN548430 and an oxidised product of SYN548430, accounted for more than 10% of the AD. The major components (> 10% of total radioactivity AUC) identified in plasma were SYN547305 and SYN548430. In an in vitro study, metabolism of spiropidion in human liver microsomes was extensive and qualitatively comparable to that in rat microsomes, irrespective of the radiolabel position (Strathdee & Punler, 2017).

Toxicological data

In rats, the acute oral median lethal dose (LD_{50}) was greater than 2000 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 1.12 mg/L. Spiropidion was not irritating to the skin of rabbits but was mildly irritating to the eyes of rabbits. Spiropidion was sensitizing to the skin of Guinea pigs and not phototoxic in vitro (Gherke, 2018; Pooles, 2015; Tarcai, 2016a b; Török-Bathó, 2015; Tóth, 2017; Váliczkó, 2016).

The short-term toxicity of spiropidion was orally tested in mice, rats and dogs, and its long-term toxicity and carcinogenicity tested in mice and rats. The most common effects in rodent studies were on body weights along with reductions in cholesterol and triglycerides. In dogs, critical effects consisted of severe clinical signs indicative of systemic neurotoxicity, which warranted unscheduled termination. No necropsy or microscopic treatment-related effects were reported that were associated with those observations.

In a 90-day toxicity study in mice in which spiropidion was administered at a dietary concentration of 0, 250, 700 or 1500 ppm (equal to 0, 35.2, 105 and 236 mg/kg bw per day for males, 0, 44.1, 115 and 252 mg/kg bw per day for females) the NOAEL was 700 ppm (equal to 105 mg/kg bw per day) based on reduced body weight in females and reduced body weight gain, reduced food utilization, and changes in clinical biochemistry parameters in both sexes at 1500 ppm (equal to 236 mg/kg bw per day) (Shearer, 2018b).

In a 90-day rat toxicity study in which spiropidion was administered at dietary concentrations of 0, 100, 500 or 2500 ppm for males, the high dose being 1500 ppm in the case of females (equal to 0, 6.2, 31.5 and 159 mg/kg bw per day in males, 0, 7.0, 36.1 and 110 mg/kg bw per day in females) the NOAEL was 100 ppm (equal to 6.2 mg/kg bw per day) based on reduced cholesterol in both sexes and triglycerides in females at 500 ppm (equal to 31.5 mg/kg bw per day) (Shearer, 2018c).

In a 28-day dog study in which spiropidion was administered via gelatine capsules at dose levels of 0, 10, 30 or 65↑100 mg/kg bw per day, the NOAEL was 30 mg/kg bw per day, based on clinical signs and mortality at 65↑100 mg/kg bw per day. Severe clinical signs were observed after one or two doses of 100 mg/kg bw per day, and after 2–4 doses of 65 mg/kg bw per day (Shearer, 2018d).

In a 90-day study in dogs in which spiropidion was administered via gelatine capsules at dose levels of 0, 5, 15 or 30 mg/kg bw per day, the NOAEL was 15 mg/kg bw per day, based on mortality and clinical signs in females at 30 mg/kg bw per day (Shearer, 2018e).

In a one-year dog study in which spiropidion was administered via gelatine capsules at dose levels of 0, 1, 3, 10 or 30 mg/kg bw per day, the NOAEL was 10 mg/kg bw per day, based on mortality and clinical signs in two animals at 30 mg/kg bw per day (Shearer, 2018f).

Combining the 90-day and one-year studies, the Meeting identified an overall NOAEL of 15 mg/kg bw per day.

In a carcinogenicity study in mice, spiropidion was administered at dietary concentrations of 0, 50, 250 or 500 ppm (equal to 0, 6.4, 31.8 and 65.4 mg/kg bw per day for males, 0, 7.0, 36.8 and 72.8 mg/kg bw per day for females). The NOAEL was 50 ppm (equal to 6.4 mg/kg bw per day) based on lower body weight and reduced body weight gain at 250 ppm (equal to 31.8 mg/kg bw per day). No treatment-related increases in tumour incidence were observed in this study (Shearer, 2018g).

In a two-year toxicity and carcinogenicity study in rats, spiropidion was administered at dietary concentrations of 0, 50, 100 or 500 ppm in males, the high dose being 300 ppm for females (equal to 0, 2.4, 4.7 and 24.0 mg/kg bw per day for males, 0, 3.1, 6.1 and 18.7 mg/kg bw per day for females). The NOAEL for non-neoplastic lesions was 100 ppm (equal to 4.7 mg/kg bw per day), based on increased incidence of luminal dilatation and inflammation in the bile duct observed in males at 500 ppm (equal to 24.0 mg/kg bw per day). The NOAEL for carcinogenicity was 50 ppm (equal to 2.4 mg/kg bw per day) based on an equivocal increase in testicular interstitial cell adenomas at 100 ppm (equal to 4.7 mg/kg bw per day). Equivocal increases in malignant ependymomas in males were seen at the highest dose level (Shearer, 2018h).

The Meeting concluded that spiropidion is not carcinogenic in mice and female rats, and produced equivocal carcinogenic effects in male rats.

Spiropidion was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found.

The Meeting concluded that spiropidion is unlikely to be genotoxic.

In view of the lack of genotoxicity, the absence of carcinogenicity in mice and the fact that only an equivocal increase in testicular interstitial cell adenomas and ependymomas were seen in male rats, the Meeting concluded that spiropidion is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation reproductive toxicity study in rats spiropidion was administered at dietary concentrations of 50, 100 or 500 ppm for males, the high dose for females being 300 ppm (equal to 0, 3, 6 and 31 mg/kg bw per day for males, 0, 4, 8 and 24 mg/kg bw per day, for females, pre-pairing). The NOAEL for parental toxicity was 100 ppm (equal to 7.8 mg/kg bw per day), based on increased incidence of thyroid follicular hypertrophy in females of both generations at 300 ppm (equal to 23 mg/kg bw per day). The NOAEL for offspring toxicity was 300 ppm (equal to 23 mg/kg bw per day), the highest dose tested. The NOAEL for reproductive toxicity was 300 ppm (equal to 23 mg/kg bw per day) the highest dose tested (King, 2018).

In a developmental toxicity study in rats given spiropidion by gavage at dose levels of 0, 10, 30 or 100 mg/kg bw per day from gestation day (GD) 6 to GD 19, the NOAEL for maternal toxicity was 30 mg/kg bw per day, based on body weight loss, reduced body weight gain and food consumption at 100 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 100 mg/kg bw per day, the highest dose tested (Pottle, 2017).

In a developmental toxicity study in rabbits given spiropidion once daily by gavage at dose levels of 0, 10, 30 or 60 mg/kg bw per day from GD 6 to GD 27, the NOAEL for maternal toxicity was 10 mg/kg bw per day based on body weight stasis in the first six days of treatment and decreased body weight gain thereafter at 30 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 30 mg/kg bw per day, based on increased incidence of skeletal effects (unossified pelvic girdle and incomplete xyphoid cartilage) at 60 mg/kg bw per day (King, 2017).

The Meeting concluded that spiropidion is not teratogenic.

In an acute neurotoxicity study, spiropidion was given to rats by gavage at a dose of 0, 50, 150 or 500 mg/kg bw. There were no histopathological changes in the nervous system. The NOAEL for neurotoxicity was 150 mg/kg bw based on clinical signs (convulsions) in females at 500 mg/kg bw. The NOAEL for systemic toxicity was 150 mg/kg bw based on body weight loss in males at 500 mg/kg bw (Leggett, 2018).

Although there were no indications of neuropathological effects due to spiropidion, the Meeting concluded that spiropidion may cause acute neurobehavioural effects at high doses.

No evidence of immunotoxicity was reported in routine toxicological studies with spiropidion.

The Meeting concluded that spiropidion is unlikely to be immunotoxic.

An ex vivo and an in vitro study were conducted to assess treatment-related thyroid effects observed in the short term and reproductive toxicity studies in rats. Results indicated that spiropidion is an inducer of hepatic uridine diphosphate glucuronosyltransferase (UDP-GT) activity in both male and female rats, and that it does not inhibit rat thyroid peroxidase activity in vitro.

Microbiological data

No information on the potential effects of spiropidion on the microbiome of the human gastrointestinal tract was available.

Toxicological data on metabolites and/or degradates

The Meeting noted that the ethyl carbonate group of spiropidion (SYN546330) is rapidly removed by hydrolysis to leave the metabolite spiropidion-enol (SYN547305). It is very likely this metabolite that is responsible for spiropidion′s toxicity, as substantial exposure to spiropidion-enol was demonstrated in several toxicity studies.

Spiropidion-enol (SYN547305) free and conjugate

Spiropidion-enol is a metabolite found in the rat (< 1% in urine), in plants (tomato, potato, cotton), and other animals (goat, hen, eggs).

No specific toxicological data are available. However, toxicity studies with the parent include exposure to SYN547305. The parent molecule, spiropidion, is a pro-insecticide which is hydrolysed to SYN547305; it is very likely that SYN547305 is responsible for the insecticidal effect. SYN547305 is the precursor of all the other metabolites identified in rats. It was found in plasma in the rat biotransformation study (Strathdee & Punler, 2017) and exposure was demonstrated in several toxicological studies. The Meeting concluded that the toxicity of spiropidion-enol (SYN547305) is covered by that of the parent compound.

Spiropidion-enol (SYN547305) dehydrogenated

SYN547305 is a metabolite found in plants (tomato).

No specific toxicological data are available. Based on structural considerations the Meeting concluded that is unlikely to be genotoxic. For chronic toxicity, the TTC approach can be applied using Cramer class III, 1.5 µg/kg bw per day.

Metabolite SYN548430

SYN548430 is a major metabolite in the rat (> 30% in urine), in plants (tomato, potato, cotton) and animals (goat, hen, milk, eggs).

As SYN548430 is a major rat metabolite the Meeting concluded that it would be covered by toxicological studies on the parent.

Metabolite SYN547435

SYN547435 is a metabolite in the rat (< 1% in urine) and in plants (tomato, potato).

No specific toxicological data are available. A chemical read-across approach examined the structural similarities of SYN547435, spiropidion (SYN546330), SYN547305 and SYN548430 via the appropriate chemistry parameters, and an OECD QSAR Toolbox analysis (v. 4.3) for all toxicity end-points of SYN547435. On the basis of this analysis, the Meeting concluded that metabolite SYN547435 would be covered by toxicological studies on the parent.

Metabolites SYN550820, SYN550839, SYN548033 and SYN549098 (free and conjugate)

SYN550820, SYN550839, SYN548033 and SYN549098 (free and conjugate) are metabolites of plants (cotton, gin trash or rotational crops).

No specific toxicological data are available. The Meeting noted that these metabolites are structurally different from the parent compound. According to the results of evaluations made using Derek Nexus (v. 6.0.1), VEGA platform (CAESAR mutagenicity models, v. 2.1.13) and the OECD QSAR Toolbox (v. 4.3), no alert for genotoxicity was identified. For chronic toxicity the TTC approach can be applied using Cramer class III, 1.5 µg/kg bw per day.

Metabolite SYN548939

SYN548939 is a metabolite in animals (goat, poultry).

No specific toxicological data are available. The Meeting noted that the metabolite is structurally similar to the parent compound and is of no genotoxicological concern based on the results of Derek Nexus (v. 6.0.1), VEGA platform (CAESAR mutagenicity models, v. 2.1.13) and the OECD QSAR Toolbox (v. 4.3) evaluations.

The Meeting concluded that metabolite SYN548939 is not of greater toxicity than spiropidion and would be covered by toxicological studies on the parent.

Human data

In reports on manufacturing plant personnel, no adverse health effects were noted. No information on accidental or intentional poisoning in humans is available.

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

Toxicological evaluation

The Meeting established an ADI of 0–0.02 mg/kg bw, based on a NOAEL of 2.4 mg/kg bw per day for an equivocal increase in testicular interstitial cell adenomas at 100 ppm (equal to 4.7 mg/kg bw per day) in the two-year rat study, and using a safety factor of 100.

The Meeting established an ARfD of 0.3 mg/kg bw based on a NOAEL of 30 mg/kg bw for mortality and clinical signs in a 28-day dog study, using a safety factor of 100. This NOAEL is supported by the NOAEL of 30 mg/kg bw for an initial body weight loss (GDs 6–9) in dams in the rat developmental study.

Levels relevant to risk assessment of spiropidion

Species	Study	Effect	NOAEL	LOAEL
Mouse	80-week carcinogenicity [a]	Toxicity	50 ppm, equal to 6.4 mg/kg bw per day	250 ppm, equal to 31.8 mg/kg bw per day
		Carcinogenicity	500 ppm, equal to 65.4 mg/kg bw per day [b]	-
Rat	Two-year toxicity and carcinogenicity [a]	Toxicity	100 ppm, equal to 4.7 mg/kg bw per day	500 ppm, equal to 24.0 mg/kg bw per day
		Carcinogenicity	50 ppm, equal to 2.4 mg/kg bw per day	100 ppm, equal to 4.7 mg/kg bw per day
	Two-generation reproductive toxicity [a]	Reproduction/ fertility	300 ppm, equal to 23 mg/kg bw per day [b]	-
		Parental toxicity	100 ppm, equal to 7.8 mg/kg bw per day	300 ppm, equal to 23 mg/kg bw per day
		Offspring toxicity	300 ppm, equal to 23 mg/kg bw per day [b]	-
	Developmental toxicity study [c]	Maternal toxicity	30 mg/kg bw per day	100 mg/kg bw per day
		Fetal toxicity	100 mg/kg bw per day [b]	-
	Acute oral neurotoxicity study [c]	Neurotoxicity	150 mg/kg bw	500 mg/kg bw
Rabbit	Developmental toxicity study [c]	Maternal toxicity	10 mg/kg bw per day	30 mg/kg bw per day
		Fetal toxicity	30 mg/kg bw per day	60 mg/kg bw per day
Dog	28 day toxicity [d]	Toxicity	30 mg/kg bw per day	65/100 mg/kg bw per day
	13-week and one-year studies of toxicity [d, e]	Toxicity	15 mg/kg bw per day	30 mg/kg bw per day

a Dietary administration

b Highest dose tested

c Gavage administration

d Gelatine capsule administration

e Two studies combined

Acceptable daily intake (ADI) for spiropidion, spiropidion-enol (SYN547305 free and conjugate), SYN548430, SYN547435 and SYN548939, expressed as spiropidion:

0–0.02 mg/kg bw

Acute reference dose (ARfD) for spiropidion, spiropidion-enol (SYN547305 free and conjugate), SYN548430, SYN547435 and SYN548939, expressed as spiropidion:

0.3 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 spiropidion

Absorption, distribution, excretion, and metabolism in mammals	
Rate and extent of oral absorption	Oral absorption of 72–77% in male and female rats, based on urinary, biliary excretion, cage wash and carcass (excluding gastrointestinal content) within 72 hours The majority of a 5 mg/kg bw oral dose was systemically available, based on the urinary excretion ratio following oral and intravenous administration
Distribution	Widely distributed, with highest concentrations of radioactivity observed in the gastrointestinal tract, liver and kidney
Rate and extent of excretion	> 94 % of the administered dose was excreted within 48 h with excretion complete by 168 h; the major route of excretion was via urine (65%), followed by faeces (~ 15%) and bile (~ 10%) and < 0.7% of dose recovered in the carcass
Potential for accumulation	No evidence of accumulation
Metabolism in mammals	SYN546330 was extensively metabolized in rat via ester hydrolysis to form SYN547305 and subsequent loss of the methoxy moiety in the piperidine ring to form SYN548430. SYN548430 and an oxidised moiety of SYN548430, individually accounted for > 10% of the administered dose in excreta. The major components (> 10% of total radioactivity AUC) identified in plasma were SYN547305 and SYN548430
Acute toxicity	
Rat LD_{50} oral	> 2000 mg/kg bw
Rat LD_{50} dermal	> 5000 mg/kg bw
Rat LC_{50} inhalation (nose-only exposure)	> 1.12 mg/L
Rabbit, skin irritation	Non-irritating
Rabbit, eye irritation	Mildly irritating
Mouse LLNA, skin sensitization	Sensitizing (EC3 value: 0.13%)
In vitro 3T3 NRU phototoxicity test	Non-phototoxic
Short-term studies of toxicity	
Target/critical effect	Reduced cholesterol and triglycerides
Lowest relevant oral NOAEL	6.2 mg/kg bw per day (rat)
Lowest relevant dermal NOAEL	300 mg/kg bw per day (rat)
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Body weight (mouse), equivocal increase in Leydig cell adenomas and in malignant ependymomas (rat)
Lowest relevant oral NOAEL	2.4 mg/kg bw per day (male rats)
Carcinogenicity	Not carcinogenic in mice; equivocal increase in Leydig cell adenomas and in malignant ependymomas (rat) [a]
Genotoxicity	Unlikely to be genotoxic [a]

Reproductive toxicity	
Target/critical effect	Parental: thyroid follicular hypertrophy
Lowest relevant parental NOAEL	7.8 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	23 mg/kg bw per day, highest dose tested (rat)
Lowest relevant reproductive NOAEL	23 mg/kg bw per day, highest dose tested (rat)
Developmental toxicity	
Target/critical effect	Parental: decreased body weight gain (rabbit); body weight loss and food consumption (rat)
Lowest relevant maternal NOAEL	10 mg/kg bw per day (rabbit)
Lowest relevant developmental NOAEL	30 mg/kg bw per day (rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	150 mg/kg bw (rat)
Subchronic neurotoxicity NOAEL	Not available
Developmental neurotoxicity NOAEL	Not available
Immunotoxicity	Not available
Mechanism studies	UDP glucuronosyltransferase induction, rat liver samples, diet: SYN546330 is an inducer of hepatic UDP-GT activity Rat thyroid peroxidase activity, microsomal preparation: SYN546330 and SYN547305 are not inhibitors of rat thyroid peroxidase activity in vitro
Studies on toxicologically relevant metabolites or impurites	
SYN547305	See rat thyroid peroxidase activity above
SYN550820, SYN550839, SYN548033, SYN548939, SYN549098 (free and conjugate)	Unlikely to be genotoxic (QSAR)
SYN547435	Structurally similar to spiropidion and rat metabolites for all toxicity end-points (read-across and QSAR)
Human data	Limited information as this is a new substance

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

Summary

	Value	Study	Safety factor
ADI [a]	0–0.02 mg/kg bw	Two-year study (rat)	100
ARfD [a]	0.3 mg/kg bw	28-day study (dog)	100

[a] Applies to spiropidion, spiropidion-enol (SYN547305 free and conjugate), SYN548430, SYN547435 and SYN548939, expressed as spiropidion

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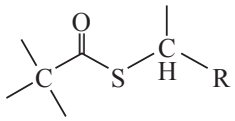
Appendix 1

Mechanistic Domain: S_N2

Mechanistic Alert: S_N2 Reaction at a sp^3 carbon atom

Structural Alert: Activated alkyl esters and thioesters

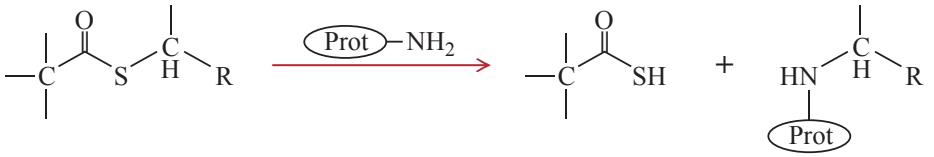
The chemical is a strong sensitizer as a result of nucleophilic substitution on an activated carbon atom:



R = −COOH, −NO_2, −CN, −SO_3H, C{ar}, C{sp^2}, C{sp}

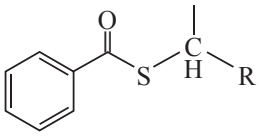
Mechanism

An S_N2 mechanism occurring at the activated carbon has been suggested as being responsible for the protein reactivity of these chemicals,
(Roberts, Patlewicz, Kern et al., 2007; Roberts, Patlewicz, Dimitrov et al., 2007).



R = −COOH, −NO_2, −CN, −SO_3H, C{ar}, C{sp^2}, C{sp} Prot = protein

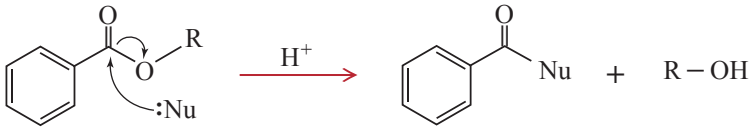
The chemicals are weak sensitizers as a result of nucleophilic substitution on an activated carbon atom:



R = C{ar}, C{sp^2}, C{sp}

Mechanism

An S_N2 mechanism occurring at the activated carbon has been suggested as being responsible for the protein reactivity of these chemicals (Roberts et al., 2007a, b).

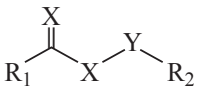


:Nu = a biological nucleophile, for example cysteine or lysine

Figure A1. S_N2 mechanism for alkyl esters

Note: The necessary conditions for eliciting direct or indirect protein interaction described in this general mechanistic profile are met. However, the specific structural boundaries providing sufficient interaction with proteins may not be identified. These specific structural boundaries are examined in the corresponding end-point-specific profile.

Structural alert: allyl acetates and related chemicals

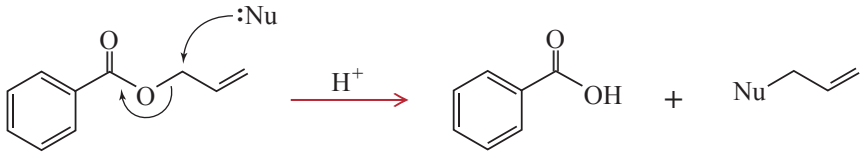


X = oxygen or sulfur R_1 = any carbon atom Y = CH_2 or CH

R_2 = carbon atom part of an alkene, alkyne, aromatic or heteroaromatic or heterocyclic ring

Mechanism

An S_N2 mechanism occurring at the activated carbon (atom Y in the alert) has been suggested as being responsible for the protein reactivity of these chemicals (Roberts et al., 2007a, b).



:Nu = a biological nucleophile, for example cysteine or lysine

Figure A2. S_N2 mechanism for allyl acetate and derivatives

TETRANILIPROLE

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)-2*H*-tetrazol-2-yl]methyl}-1*H*-pyrazole-5-carboxamide (IUPAC), with the Chemical Abstract Service 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 insect ryanodine receptors.

Tetraniliprole 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).

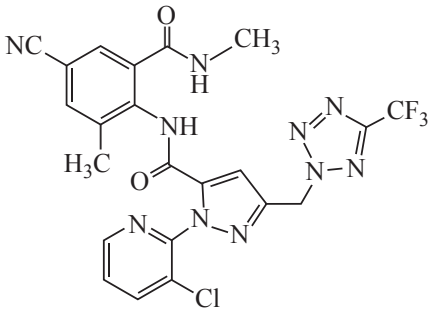
All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with relevant national or international test guidelines, unless otherwise specified. No additional information from a literature search was identified that complemented the toxicological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

Evaluation for acceptable intake

1. Biochemical aspects

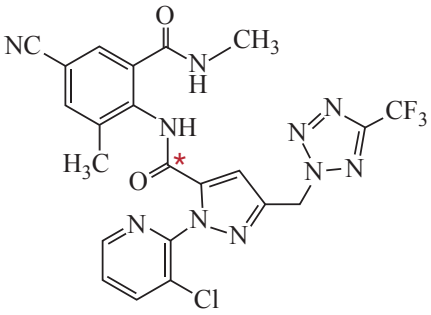
The absorption, distribution, metabolism and excretion (ADME) of tetraniliprole (Fig. 1), as well as its toxicokinetics, have been investigated in Wistar rats. Summaries of the relevant data are presented below.

Figure 1. Chemical structure of tetraniliprole

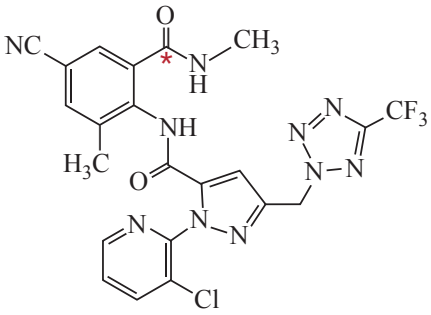


The ADME of tetraniliprole were investigated using tetraniliprole ^{14}C labelled in four positions: typyrazole-carboxamide, pyridinyl-2, phenyl-carbamoyl and tetrazolyl (see Fig. 2 for structures). The test item was a mixture of labelled and unlabelled tetraniliprole. The study design is summarized in Table 1.

Figure 2. Four ^{14}C labelling positions used in ADME studies with tetraniliprole

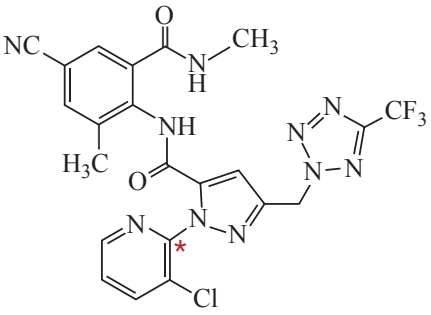


[*Pyrazole-carboxamide*-^{14}C]tetraniliprole

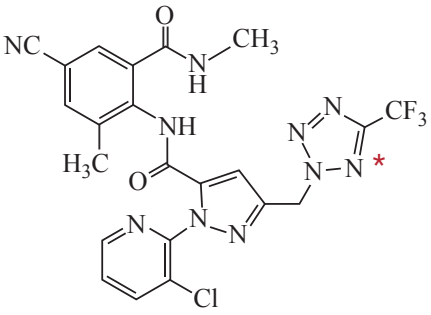


[*Phenyl-carbamoyl*-^{14}C]tetraniliprole

* indicates position of ^{14}C label



[*Pyridinyl-2*-^{14}C]tetraniliprole



[*Tetrazolyl*-^{14}C]tetraniliprole

Table 1. Dosing groups for ADME experiments with ^{14}C-labelled tetraniliprole

Test group	Target dose (mg/kg bw) [route]	Animals of each sex	Remarks	Reference
ADME study Wistar rats – low dose	2 [oral]	4M, 4F	Single-dose mixture of [*pyrazole-carboxamide*-^{14}C] tetraniliprole (purity 97.9%) and unlabelled tetraniliprole with target radioactivity of 3.29 MBq/mg in 0.5% aqueous tragacanth. Collection of urine, faeces, plasma, organs, and carcass at 72 hours	Bongartz & Miebach, 2016a
ADME study Wistar rats – high dose	200 [oral]	4M, 4F	Single-dose mixture of [*pyrazole-carboxamide*-^{14}C] tetraniliprole (purity 97.9%) and unlabelled tetraniliprole with target radioactivity of 3.29 MBq/mg in 0.5% aqueous tragacanth. Collection of urine, faeces, plasma, organs, and carcass at 72 hours	Bongartz & Miebach, 2016a
ADME study Wistar rats – repeat dose	2 [oral]	4M	Multiple doses of unlabelled tetranipiprole for 14 days and a single-dose mixture of [*pyrazole-carboxamide*-^{14}C] tetraniliprole (purity 97.9%) and unlabelled tetraniliprole with target radioactivity of 3.29 MBq/mg in 0.5% aqueous tragacanth. Collection of urine, faeces, plasma, organs, and carcass at 72 hours	Bongartz & Miebach, 2016a
ADME study Wistar rats – low-dose, with bile duct cannulation	2 [oral]	6M , 6F	Single-dose mixture of [*pyrazole-carboxamide*-^{14}C] tetraniliprole (purity 97.9%) and unlabelled tetraniliprole with target radioactivity of 3.29 MBq/mg in 0.5% aqueous tragacanth. Collection of urine, faeces, organs, and carcass at 48 hours	Bongartz & Miebach, 2016a
ADME study Wistar rats – mid-dose	20 [oral]	4M, 4F	Single-dose mixture of [*pyrazole-carboxamide*-^{14}C] tetraniliprole (purity 97.9%) and unlabelled tetraniliprole with target radioactivity of 3.29 MBq/mg in 0.5% aqueous tragacanth. Collection of urine, faeces, plasma, organs, and carcass at 72 hours	Bongartz & Miebach, 2016a
ADME study Wistar rats – low dose	2 [oral]	4M, 4F	Single-dose mixture of [*phenyl-carbamoyl*-^{14}C] tetraniliprole (purity > 99%) and unlabelled tetraniliprole with target radioactivity of 3.29 MBq/mg in 0.5% aqueous tragacanth. Collection of urine, faeces, plasma, organs, and carcass at 72 hours	Bongartz & Miebach, 2016b
ADME study Wistar rats – low dose	2 [oral]	4M, 4F	Single-dose mixture of [*pyridinyl-2*-^{14}C] tetraniliprole (purity >99%) and unlabelled tetraniliprole with target radioactivity of 3.29 MBq/mg in 0.5% aqueous tragacanth. Collection of urine, faeces, plasma, organs, and carcass at 72 hours	Bongartz & Miebach, 2016c
ADME study Wistar rats – low dose	2 [oral]	4M, 4F	Single-dose mixture of [tetrazolyl-14C]-tetraniliprole (purity >99%) and unlabelled tetraniliprole with target radioactivity of 3.29 MBq/mg in 0.5% aqueous tragacanth. Collection of urine, faeces, plasma, organs, and carcass at 72 hours	Bongartz & Miebach, 2016d

Whole body radioagraphy – pilot metabolism	5 [oral]	4M	Single-dose mixture of [*pyrazole-carboxamide*-^{14}C] tetraniliprole (purity > 98%) and unlabelled tetraniliprole with target radioactivity of 3.29 MBq/mg in 0.5% aqueous tragacanth. One animal each killed at 1, 4, 8 and 24 hours with collection of urine and blood at terminal kill in all groups, faeces in 24 h group and liver and kidney at terminal kill from all animals	Koester, 2015
Whole body radioagraphy – quantitative whole body radioactivity	5 [oral]	8M 8F	Single-dose mixture of [*pyrazole-carboxamide*-^{14}C] tetraniliprole (purity > 98%) and unlabelled tetraniliprole with target radioactivity of 3.29 MBq/mg in 0.5% aqueous tragacanth. One animal/sex killed at 1, 4, 8, 24, 48, 72, 120 and 168 hours with collection of urine and blood at 1 h (in 1 h group only) 4, 8, 24, 48, 72, 96, 120, 144 and 168 h; faeces at 24, 48, 72, 96, 120 ,144 and 168 h; expired air at 24 and 28 h; whole body sections at terminal kill	Koester, 2015
Seven day blood kinetics	3000 ppm equal to 145 in males, 168 in females	5M 5F	Seven-day administration of unlabelled tetraniliprole (purity 92.6%) in diet. Collection of blood at 8 am, 2 pm and 5 pm on day following last adminstration	Odin-Feurtet, 2013
Toxicity and biokinetic screening study – biokinetic	150 [oral)	2M 2F	Single-dose unlabelled tetraniliprole (purity 98.6%) in 0.5% aqueous methylcellulose. Collection of blood at 0, 3, 5, 7 and 24 hrs and fat at 24 hrs	Odin-Feurtet, 2010
Toxicity and biokinetic screening study – bio-accumulation	0 [oral)	5M 5F	Fourteen doses of 0.5% aqueous methylcellulose. Collection of blood, fat, liver, adrenals, kidneys and thyroid at 24 hours after last administration	Odin-Feurtet, 2010
Toxicity and biokinetic screening study – bio-accumulation	150 [oral)	5M 5F	Fourteen doses of unlabelled tetraniliprole (purity 98.6%) in 0.5% aqueous methylcellulose. Collection of blood, fat, liver, adrenals, kidneys and thyroid at 24 hours after last administration	Odin-Feurtet, 2010

1.1 Absorption, distribution and excretion

(a) Oral route

Rat

In an absorption, distribution and excretion assay on Wistar rats, animals were allocated to one of the following treatments:

- a single gavage dose of 2, 20 or 200 mg/kg bw [*pyrazole-carboxamide*-^{14}C] tetraniliprole (four rats/sex per dose; Fig. 2)
- fourteen doses of unlabelled tetraniliprole at 2 mg/kg bw per day, followed by a single 2 mg/kg bw dose of [*pyrazole-carboxamide*-^{14}C] tetraniliprole (four male rats)
- a single dose of 2 mg/kg bw of [*pyrazole-carboxamide*-^{14}C] tetraniliprole (three rats/sex per dose sampled from six rats/sex per dose originally planned) in a bile excretion assay.

In the excretion, retention and recovery assays, the predominant route of excretion was the faeces, (Table 2) accounting for 94–110% of the administered dose (AD). Excretion in the urine was negligible for rats receiving more than 2 mg/kg bw; it was just 5–7% of the AD at the low dose in single or repeat dose groups and in bile duct-cannulated animals. Excretion in the bile was 39% in males and 25% in females. Excretion was complete, with less than 0.5% AD recovered in the carcass in all but one group provided the (GI) tract is excluded. The exception were the male, bile duct-cannulated rats, where 1% of AD was retained in the carcass. The overall absorption, calculated as a sum of urine, bile and carcass, was 46% in males and 30% in females.

Table 2. Summary of mean cumulative excretion balance and recovery data (% of AD) up to 72 hours after administration of single or multiple oral gavage doses of [pyrazole-carboxamide-^{14}C]tetraniliprole to rats

Time after dosing (hours)	Dose regime								
	Single dose 2 mg/kg bw		Single dose 200 mg/kg bw		Multiple dose 2 mg/kg bw	Bile duct cannulation: Single dose 2 mg/kg bw		Single dose 20 mg/kg bw	
	Male	Female	Male	Female	Male	Male	Female	Male	Female
					Urine				
4	0.30	0.62	NC	0.12	1.30	0.38	1.17	0.07	0.06
8	2.15	2.91	0.05	0.28	3.28	1.28	3.06	0.22	0.22
12	3.49	NS	NS	NS	NS	NS	NS	NS	NS
24	4.43	5.91	0.08	0.39	4.75	4.46	4.29	0.31	0.40
48	4.63	6.59	0.09	0.50	4.92	5.66	4.45	0.33	0.41
72	4.66	6.69	0.09	0.53	4.95	NS	NS	0.34	0.41
					Bile				
4	NS	NS	NS	NS	NS	10.88	11.23	NS	NS
8	NS	NS	NS	NS	NS	22.33	18.25	NS	NS
24	NS	NS	NS	NS	NS	37.09	24.04	NS	NS
48	NS	NS	NS	NS	NS	38.92	24.71	NS	NS
					Faeces				
24	93.69	74.99	105.63	30.15	93.03	55.40	70.04	104.39	107.00
48	98.32	93.42	108.78	73.94	102.33	59.64	71.55	106.97	109.53
72	98.58	94.47	108.86	93.69	103.09	NS	NS	107.02	109.75
Total excreted	103.24	101.16	108.95	94.22	108.05	104.22	100.71	107.35	110.16
Carcass (w/o GIT)	0.189	0.396	NC	0.011	0.204	1.009	0.459	0.012	0.013
GIT	0.029	0.151	NC	0.178	0.073	4.870	0.066	0.002	0.003
Total in body	0.218	0.547	NC	0.189	0.278	5.879	0.525	0.014	0.017
Total recovery	103.46	101.71	108.95	94.40	108.32	110.10	101.24	107.37	110.18

NS: No sample; NC: Not calculated; Source: Tables 4-5, pp.69–70, Bongartz & Miebach, 2016a

GIT: Gastrointestinal tract

Of the radioactivity still retained in the carcass 72 hours after dosing the highest concentrations were found in the liver (Table 3a). There was no evidence of bioaccumulation following repeated dosing. Based on equivalent concentrations, the amount of radioactivity from [*Pyrazole-carboxamide*-^{14}C]-labelled tetraniliprole retained in the tissue plateaued at 20 mg/kg bw.

Table 3. Mean tissue distribution data (% of AD) at 72 hours after administration of single or multiple oral gavage doses of [pyrazole-carboxamide-^{14}C]tetraniliprole to rats

	Dose regime						
	Single dose 2 mg/kg bw		Single dose 200 mg/kg bw		Multiple dose 2 mg/kg bw	Single dose 20 mg/kg bw	
Matrix	**Male**	**Female**	**Male**	**Female**	**Male**	**Male**	**Female**
Red blood cells[a]	0.0015	0.0021	NC	NC	0.0012	NC	NC
Plasma[a]	0.0027	0.0032	NC	NC	0.0035	0.0001	0.0002
Carcass	0.0023	0.1066	NC	NC	NC	NC	NC
Heart	0.0002	0.0005	NC	NC	0.0002[b]	NC	NC
Brain	NC	NC	NC	NC	NC	NC	NC
Kidneys	0.0024	0.0039	NC	NC	0.0019	0.0002	0.0002
Liver	0.1396	0.2210	NC	0.0106	0.1558	0.0118	0.0129
Testes	0.0005	NA	NC	NA	0.0008	NC	NA
Ovaries	NA	0.0002	NA	NC	NA	NA	NC
Uterus	NA	0.0006	NA	NC	NA	NA	NC
Adrenal gland	NC	0.0001	NC	NC	NC	NC	NC
Thyroid gland	NC	NC	NC	NC	NC	NC	NC
Spleen	NC	0.0003	NC	NC	NC	NC	NC
Lung	0.0007	0.0017	NC	NC	0.0011	NC	NC
Skin	0.0184	0.0494	NC	NC	0.0231	NC	NC
Femur[a]	NC	NC	NC	NC	NC	NC	NC
Perirenal fat[a]	NC	0.0058	NC	NC	0.0012	NC	0.0001[b]
Muscle[b]	NC	NC	NC	NC	0.0035	NC	NC

NA: Not applicable; NC: Not calculated; Source: Table 9, p.74, Bongartz & Miebach, 2016a

[a] Reported result pertains only to a portion of the tissue sampled; remainder of radioactivity in tissue is included in the carcass

[b] Mean value calculated with half of lowest level of quantitation (LLOQ)

In the pharmacokinetics assays, the calculated time to reach maximum concentration (T_{max}) was roughly 1.5 hours in low-dose males and females and in mid-dose males (Table 4.). After repeated dosing the T_{max} was one hour. Mid-dose females exhibited a T_{max} of approximately four hours. The calculated maximum concentration (C_{max}) and area under the concentration–time curve (AUC) were higher in females than males; C_{max} was also higher at the low dose than the mid dose. The absolute half-lives were under an hour, indicating rapid elimination. The difference in kinetics elicited by the 2 and 20 mg/kg bw doses was considered to be a result of lower absorption with increasing dose in rats.

Table 4. Pharmacokinetic parameter estimates after administration of single or multiple oral gavage dose of [pyrazole-carboxamide-^{14}C]tetraniliprole to rats [a]

	Dose regime				
	Single dose 2 mg/kg bw		Multiple dose 2 mg/kg bw	Single dose 20 mg/kg bw	
Parameter	Male	Female	Male	Male	Female
T_{max} ; observed (h)	1	1	1	1	7
T_{max} ; calculated (h)	1.59	1.60	1.02	1.35	3.97
C_{max} ; measured (µg/mL)[a]	0.138	0.199	0.131	0.00665	0.00812
C_{max} ; calculated (µg/mL)[a]	0.151	0.214	0.131	0.00695	0.00940
$t_{½\ abs.}$ (h)	0.72	0.40	0.20	0.49	0.18
$t_{½\ elim.}$ (h)	27.9	18.0	30.1	14.3	4.1
$AUC_{0-\infty}$ (g × h/g)	1.21	2.36	1.27	0.06	0.12

[a] Dose-normalized value Source: Table 11, p.76 of Bongartz & Miebach, 2016a

T_{max}: Time to maximum concentration; T_{max}: Maximum concentration; $t_{½}$: Half-life

$AUC_{0-\infty}$: Total area under the time–concentration curve

In separate absorption, distribution and excretion assays performed on Wistar rats, four/sex per radiolabel were given a single gavage dose of 2 mg/kg bw [*phenyl-carbamoyl*-^{14}C]tetraniliprole, [*pyridinyl-2*-^{14}C]tetraniliprole or [*tetrazolyl*-^{14}C]tetraniliprole (Fig. 2) in 0.5% aqueous tragacanth. Blood samples were collected at 0.25, 0.5, 1, 7, 24, 48 and 72 hours post dose. Urine samples were collected individually at 4, 8, 12 (males only), 24, 48, and 72 hours post dose. Faeces samples were collected individually at 24, 48, and 72 hours post dose.

In the [*phenyl-carbamoyl*-^{14}C]tetraniliprole excretion, retention and recovery assay, more than 99% of the AD was recovered within 72 hours of dosing. The majority of the radioactivity was recovered between 24 and 48 hours with 95.7% and 96.6% of the administered radioactivity recovered in the faeces in males and females respectively (Table 5).

In the [*pyridinyl-2*-^{14}C]tetraniliprole assay, greater than 100% of the AD was recovered within 72 hours of dosing. The majority of radioactivity was recovered within 24 hours with the vast majority in the faeces (102.0% in males, 101.8% in females). Urinary excretion accounted for 3.0% and 2.5% of the AD in males and females respectively.

In the [*tetrazolyl*-^{14}C]tetraniliprole assay, greater than 100% of the AD was recovered within 72 hours of dosing. As with the other radiolabels, the vast majority was excreted in the faeces (98% in males, 96% in females), however slightly higher amounts of radioactivity were excreted in the urine than with the other radiolabels

Table 5. Summary mean cumulative excretion balance and recovery data (% of AD) up to 72 hours after administration to rats of single oral gavage doses of 2 mg/kg of radiolabelled tetraniliprole

	Dose regime					
Time after dosing (hours)	[*phenyl-carbamoyl*-^{14}C] tetraniliprole[a]		[*pyridinyl-2*-^{14}C] tetraniliprole[b]		[*tetrazolyl*-^{14}C] tetraniliprole[c]	
	Male	Female	Male	Female	Male	Female
			Urine			
4	0.82	0.74	0.81	0.91	2.76	0.93
8	2.46	2.18	1.94	1.45	4.17	3.15
12	3.18	NS	2.55	NS	4.80	NS
24	3.90	4.18	2.94	2.38	5.20	5.63
48	4.02	4.53	3.01	2.47	5.33	5.86
72	4.05	4.60	3.04	2.51	5.38	5.94

Time after dosing (hours)	Dose regime					
	[*phenyl-carbamoyl*-^{14}C] tetraniliprole[a]		[*pyridinyl-2*-^{14}C] tetraniliprole[b]		[*tetrazolyl*-^{14}C] tetraniliprole[c]	
	Male	Female	Male	Female	Male	Female
			Faeces			
24	93.03	84.50	100.18	100.58	96.53	93.27
48	95.50	95.80	101.86	101.61	97.73	95.73
72	95.70	96.58	102.01	101.77	97.88	96.02
Total excreted	99.75	101.18	105.06	104.28	103.26	101.96
Carcass (without GIT)	0.245	0.380	0.293	0.162	0.246	0.257
GIT	0.033	0.106	0.037	0.029	0.043	0.046
Total in body	0.278	0.486	0.330	0.191	0.289	0.304
Total recovery	100.03	101.66	105.39	104.47	103.55	102.26

NS: No sample; GIT: Gastrointestinal tract

[a] Data obtained from Tables 4 and 5, pp.56–57 of Bongartz & Miebach, 2016b

[b] Data obtained from Tables 3 and 4, pp.54–55 of Bongartz & Miebach, 2016c

[c] Data obtained from Appendices 9 and 13, pp.118 and 123 of Bongartz & Miebach, 2016d
2 mg/kg bw, $n = 4$ per sex (applies to all above studies)

In the case of all three radiolabels the highest levels of radioactivity remaining 72 hours after administration were in the liver, followed by the general carcass and skin (Table 6a). There were slight differences between the radiolabels as to the places where trace amounts of radioactivity were detected, such as eyes, perirenal fat and muscle.

Table 6. Mean tissue distribution data (% of dose) at 72 h after administration of single oral gavage doses of radiolabelled-tetraniliprole to rats.

Matrix	Dose regime					
	[*phenyl-carbamoyl*-^{14}C] tetraniliprole[a]		[*pyridinyl-2*-^{14}C] tetraniliprole[b]		[*tetrazolyl*-^{14}C] tetraniliprole[c]	
	Male	Female	Male	Female	Male	Female
Red blood cells[d]	0.0016	0.0018	0.0026	0.0020	0.0020	0.0020
Plasma[d]	0.0028	0.0033	0.0026	0.0019	0.0027	0.0026
Carcass	0.0313[e]	0.1099	0.0599	NC	0.0539	0.0629
Heart	0.0004	0.0005	0.0006	NC	0.0007	0.0005
Brain	NC	NC	NC	NC	0.0005[e]	0.0004[e]
Kidneys	0.0028	0.0034	0.0045	0.0027[e]	0.0024	0.0025
Liver	0.1772	0.2070	0.1757	0.1426	0.1475	0.1505
Testes	0.0007	NA	0.0011	NA	0.0013	NA
Ovaries	NA	0.0002	NA	0.0001	NA	0.0001
Uterus	NA	0.0007	NA	0.0003[e]	NA	0.0006
Adrenal gland	NC	0.0001[e]	0.0001	0.0001	NC	0.0001
Thyroid gland	NC	NC	0.0001	NC	NC	NC
Spleen	NC	0.0002	0.0003	0.0002[e]	0.0002[e]	0.0003
Lung	0.0011	0.0015	0.0017	0.0009	0.0014	0.0012
Eye	NC	NC	NC	0.0001	0.0002	0.0002
Skin	0.0310	0.0449	0.0437	NC	0.0326	0.0312

	Dose regime					
	[*phenyl-carbamoyl*-^{14}C] tetraniliprole[a]		[*pyridinyl-2*-^{14}C] tetraniliprole[b]		[*tetrazolyl*-^{14}C] tetraniliprole[c]	
Matrix	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Femur[d]	NC	NC	NC	NC	NC	NC
Perirenal fat[d]	NC	0.0057	NC	NC	NC	0.0016
Muscle[d]	NC	0.0007[e]	NC	NC	0.0008	0.0008

[a] Data obtained from Table 9, p.61 of Bongartz & Miebach, 2016b

[b] Data obtained from Table 8, p.59 of Bongartz & Miebach, 2016c

[c] Data obtained from Table 8, p.62 of Bongartz & Miebach, 2016d
2 mg/kg bw, $n = 4$ per sex (applies to all above studies)

[d] Result pertains only to portion of the tissue sampled; remainder of radioactivity in tissue is included with the carcass

[e] Mean value calculated with half of lowest level of quantitation for values lower than the lowest level of quantitation

NC Not calculated

NA Not applicable

In the plasma distribution and pharmacokinetic assays, in the case of all three assays, measurable concentrations of the radiolabel were found in the plasma 0.25 h after dose administration. The T_{max} values (calculated) ranged from 0.80 h to 1.79 h, with times for males generally shorter than for females. Values of C_{max} and AUC were lower in males. The $t_{½}$ for the absorption phase was shorter in females in the [*phenyl-carbamoyl*-^{14}C] tetraniliprole assay, and the $t_{½}$ for the elimination phase was shorter in females in the assays with [*pyridinyl-2*-^{14}C] tetraniliprole and [*tetrazolyl*-^{14}C] tetraniliprole. However, with the exception of the elimination phase in the [*pyridinyl-2*-^{14}C] tetraniliprole assay, the differences between sexes and radiolabels were relatively small in size (Table 7; Bongartz & Miebach, 2016b, c, d).

Table 7. Pharmacokinetic parameter estimates after administration of single oral gavage doses of radiolabelled-tetraniliprole to rats.

	Dose regime					
	[*phenyl-carbamoyl*-^{14}C] tetraniliprole[a]		[*pyridinyl-2*-^{14}C] tetraniliprole[b]		[*tetrazolyl*-^{14}C] tetraniliprole[c]	
Matrix	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
T_{max}: observed (h)	1	1	1	1	1.00	1.00
T_{max}: calculated (h)	1.69	1.79	0.80	1.42	1.03	1.55
C_{max}: measured (µg/mL)[d]	0.144	0.205	0.096	0.108	0.161	0.208
C_{max}: calculated (µg/mL)[d]	0.161	0.235	0.096	0.111	0.163	0.232
$t_{½abs}$ (h)	0.70	0.46	0.14	0.36	0.22	0.45
$t_{½elim}$ (h)	22.9	25.0	36.0	11.3	31.6	25.0
$AUC_{0-\infty}$ (g × hour/g)[d]	1.29	2.32	1.04	1.21	1.27	2.00

[a] Data from Table 11, p.63 of Bongartz & Miebach, 2016b

[b] Data from Table 10, p.61 of Bongartz & Miebach, 2016c

[c] Data from Table 10, p.64 of Bongartz & Miebach, 2016d; 2 mg/kg bw, $n = 4$/sex (applies to all above studies)

[d] Dose-normalized value

T_{max}: Time to maximum concentration; T_{max}: Maximum concentration; $t_{½}$: Half-life

$AUC_{0-\infty}$: Total area under the time–concentration curve

In a whole body autoradiography assay, Wistar Hsd/Cpb: WU rats were given a single dose of 5 mg/kg bw of [*pyrazole-carboxamide*-^{14}C] tetraniliprole; 12 males and eight females were thus dosed. The absorption of radioactivity from the GI tract, and its distribution to and elimination from blood, organs and tissues, were analysed qualitatively and quantitatively by whole-body autoradioluminography (WBAL) using eight rats per sex. One rat was assigned for cryosectioning at each of the time interval, 1, 4, 8, 24, 48, 72, 120 and 168 hours after dosing. The amount of radioactivity in excreta (urine and faeces) and exhaled

carbon dioxide were additionally determined for selected time periods. For evaluation of metabolism, one male rat was sacrificed at each time point, 1, 4, 8, and 24 hours after dosing. Samples of urine, faeces, plasma, liver and kidney were collected and prepared for chromatographic evaluation by high-performance liquid chromatography (HPLC) with radiometric detection of the metabolic profiles.

Radioactivity was quickly absorbed from the GI tract, however absorption levels were low, resulting in comparatively low levels of radioactivity in the blood, organs, tissues and urine. Peak concentrations in most organs and tissues in males were reached one hour after dosing, with the exception of the testes and nasal mucosa, which reached peak radioactivity concentrations four hours after dosing. In females, peak organ and tissue concentrations were reached slightly later and peak concentrations in the blood, perirenal fat and ovaries were reached after four hours, with peak concentrations in the uterus, brain, spinal cord, nasal mucosa and vitreal body reached at eight hours after dosing (Table 8). Otherwise there were no significant sex-related differences in blood or tissue concentrations. Highest concentrations were found in the liver and kidney, but radioactivity was also found preferentially in glandular organs and fatty tissues. Radioactivity was below the LOQ in most tissues by 72 hours after dosing, with the exception of the liver in males and females and the kidneys in males.

Table 8. Individual equivalent concentrations of radioactivity in blood, organs and tissues of male rats after administration of [pyrazole-carboxamide-^{14}C]tetraniliprole (Maximum CEQ values shown in bold)

Time of sacrifice after dosing (hours)	Equivalent concentration CEQ (µg tetraniliprole equiv./g)							
	1	4	8	24	48	72	120	168
				Males				
Blood	**0.251**	0.105	0.043	0.009	<LOQ	<LOQ	<LOQ	-
Liver	**2.566**	1.494	0.574	0.125	0.070	0.057	0.026	0.015
Renal cortex	**0.529**	0.215	0.075	0.015	0.008	0.008	<LOQ	0.005
Renal medulla	**0.479**	0.223	0.079	0.016	0.008	0.006	<LOQ	<LOQ
Kidney total	**0.504**	0.219	0.077	0.015	0.008	0.007	<LOQ	<LOQ
Brown fat	**0.602**	0.261	0.071	0.016	-	-	-	-
Perirenal fat	**0.192**	0.112	0.080	0.022	<LOQ	<LOQ	-	-
Skeleton muscle	**0.169**	0.081	0.025	<LOQ	<LOQ	<LOQ	-	-
Myocardium	**0.426**	0.172	0.054	0.008	<LOQ	-	-	-
Lung	**0.258**	0.087	0.031	0.006	<LOQ	<LOQ	<LOQ	-
Spleen	**0.299**	0.116	0.036	0.005	<LOQ	<LOQ	<LOQ	-
Pancreas	**0.511**	0.199	0.053	0.008	<LOQ	<LOQ	-	-
Bone marrow	**0.205**	0.095	0.029	<LOQ	-	-	-	-
Testis	0.044	**0.082**	0.037	<LOQ	<LOQ	<LOQ	-	-
Brain	< LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	-	-
Spinal cord	**0.008**	0.006	<LOQ	<LOQ	-	-	-	-
Pituitary gland	**0.297**	0.125	0.034	0.007	-	-	-	-
Pineal body	**0.356**	0.131	0.045	0.006	-	-	-	-
Adrenal gland	**1.121**	0.402	0.144	0.018	<LOQ	<LOQ	-	-
Thymus	**0.185**	0.094	0.027	<LOQ	<LOQ	-	-	-
Thyroid gland	**0.452**	0.178	0.056	0.009	-	-	-	-
Salivary gland	**0.431**	0.181	0.051	0.009	<LOQ	<LOQ	-	-
Nasal mucosa	0.061	**0.088**	0.059	0.016	0.006	<LOQ	-	-
Vitreal body	**0.026**	0.017	<LOQ	<LOQ	<LOQ	-	-	-
Harderian gland	**0.339**	0.211	0.060	0.010	<LOQ	-	-	-

Time of sacrifice after dosing (hours)	Equivalent concentration CEQ (µg tetraniliprole equiv./g)							
	1	4	8	24	48	72	120	168
				Females				
Blood	0.156	**0.173**	0.158	0.017	<LOQ	-	-	-
Liver	**1.746**	1.470	1.303	0.201	0.091	0.045	0.026	0.015
Renal cortex	**0.376**	0.268	0.302	0.037	0.011	0.007	-	-
Renal medulla	**0.305**	0.254	0.263	0.028	0.008	0.005	-	-
Kidney total	**0.341**	0.261	0.282	0.033	0.009	0.006	-	-
Brown fat	**0.517**	0.425	0.439	0.057	0.011	-	-	-
Perirenal fat	0.135	**0.245**	0.220	0.065	0.017	<LOQ	-	-
Skeleton muscle	**0.147**	0.116	0.104	0.009	<LOQ	<LOQ	-	-
Myocardium	**0.358**	0.283	0.279	0.027	<LOQ	-	-	-
Lung	**0.178**	0.174	0.172	0.018	<LOQ	-	-	-
Spleen	**0.255**	0.183	0.195	0.019	<LOQ	<LOQ	-	-
Pancreas	**0.471**	0.294	0.343	0.030	<LOQ	<LOQ	-	-
Bone marrow	**0.187**	0.135	0.163	0.014	-	-	-	-
Ovary	0.161	**0.163**	-	0.025	<LOQ	-	-	-
Uterus	0.151	0.143	**0.229**	0.025	<LOQ	-	-	-
Brain	< LOQ	0.006	**0.006**	<LOQ	<LOQ	-	-	-
Spinal cord	< LOQ	0.007	**0.009**	<LOQ	-	-	-	-
Pituitary gland	**0.280**	0.220	0.230	0.020	-	-	-	-
Pineal body	**0.324**	0.244	0.222	0.026	-	-	-	-
Adrenal gland	**0.827**	0.565	0.646	0.064	0.008	-	-	-
Thymus	**0.196**	0.173	0.153	0.015	<LOQ	-	-	-
Thyroid gland	**0.367**	0.258	0.285	0.026	-	-	-	-
Salivary gland	**0.392**	0.298	0.282	0.027	<LOQ	-	-	-
Nasal mucosa	0.041	0.173	**0.223**	0.033	0.011	<LOQ	-	-
Vitreal body	0.020	0.024	**0.035**	<LOQ	<LOQ	-	-	-
Harderian gland	**0.324**	0.261	0.253	0.022	-	-	-	-

LOQ: Limit of quantitation; CEQ: Equivalent concentration Source: pp.14 and 45 of Koester, 2015

- : Organ or tissue was visible in the rat sections but not discernible in the radioluminograms

Excretion was primarily via the faecal route in the assay relating to Table 8, however, biliary excretion was not assessed. With both males and females, race amounts of radioactivity (<0.01%) were excreted in the expired air after 24 hours. In urine, faeces, plasma, liver and kidney, unchanged tetraniliprole was the most prominent component. Metabolites formed included BCS-CL73507-*N*-methyl-quinazolinone.

A small increase in metabolite BCS-CL73507-*N*-methyl-quinzolinone occurred during storage of plasma in all the samples investigated. Unchanged tetraniliprole remained the most prominent component at all time points (Koester, 2015).

In a seven-day blood kinetics assay, Wistar554 Rj:WI (IOPS HAN) rats were given dietary concentrations of 3000 ppm of unlabelled tetraniliprole (five rats of each sex; equal to 145 mg/kg bw per day in males, 163 mg/kg bw per day in females) for seven days in order to determine the optimal time for sampling. Animals were kept on cycle of a 4 am to 4 pm dark, 4 pm to 4 am light. Blood samples were taken at three time points on day 8 of the study (8 am, 2 pm and 5 pm) to determine plasma concentrations.

Values of T_{max} and C_{max} could not be established as there were too few blood sampling time points and too high an interanimal variability. However, study authors concluded that under environmental conditions of the long-term studies (light period: 7 am to 7 pm and dark period: 7 pm to 7 am), taking blood sampls at approximately 8 am should provide a good indication of the maximum plasma concentration of the test item (Odin-Feurtet, 2013).

1.2 Biotransformation

The metabolite profiles for urine, bile and faeces were determined for pyrazole-caboxamide-labelled tetraniliprole by Bongartz & Miebach (2016a). In this study the metabolites, along with the parent compound, accounted for 90–108% of the AD and the vast majority of metabolites were identified.

Unchanged parent was the major component in all test groups regardless of dose or sex. In low-dose groups, unchanged parent was 51.3–66.4% of the AD, while unchanged parent made up 88.8–108% of AD in the 20 and 200 mg/kg bw dose groups. Unchanged parent, however, was not seen in the bile of either males or females. The most common metabolites accounted for 1–9% of the AD and included the tetraniliprole-deshydrochloro-dihydrate (faeces and bile in males and females, urine in males), tetraniliprole-dihydroxy (faeces and urine in males and females) and tetraniliprole-hydroxy-*N*-methyl (faeces, urine and bile in males and females). Other prominent tetraniliprole metabolites (ranging from approximately 1–4% of AD) included:

-benzylalcohol-glucuronide (faeces and bile in males and females and urine in males),

-hydroxypyridyl-glucuronide (bile in males and females),

-deschloro-desmethyl-amide-dihydroxy (faeces and bile in males and females),

-despyridyl (faeces, urine and bile in males and females),

-benzylalcohol, (faeces and urine in males and females),

-pyridinyl-pyrazole-5-carboxylic acid (faeces, urine and bile in males and females) and

-hydroxypyridine (faeces and bile in males and females) metabolites.

The other identified metabolites accounted for most of remaining products and were present in quantities of equal or less than 2.5% of AD (Bongartz & Miebach, 2016a).

With the phenyl-carbamoyl-labelled tetraniliprole, parent compound was the major component in both sexes and comprised 53% of the AD in males and 60% in females. Quantification of individual metabolites did not indicate any clear sex-related patterns, although hydroxylated metabolites appeared to be more abundant in males than females. Major metabolites were:

-tetraniliprole-deshydrochloro-dihydrate,

-tetraniliprole-dihydroxy (for males), tetraniliprole-despyridyl (for females),

-tetraniliprole-benzylalcohol,

-tetraniliprolehydroxyN-methyl and

-tetraniliprole-hydroxypyridine

Presence of the above metabolites ranged from 3.2% to 7.3% of AD.

Other prominent metabolites were:

tetraniliprole-dihydroxy (for females),

tetraniliprole-deschlorodesmethyl-amide-dihydroxy and

tetraniliproledespyridyl (for males)

cocentrations of which ranged from 2.2–2.7% of AD. The remainder of the identified metabolites amounted to less than 2.1% of AD (Bongartz & Miebach, 2016b).

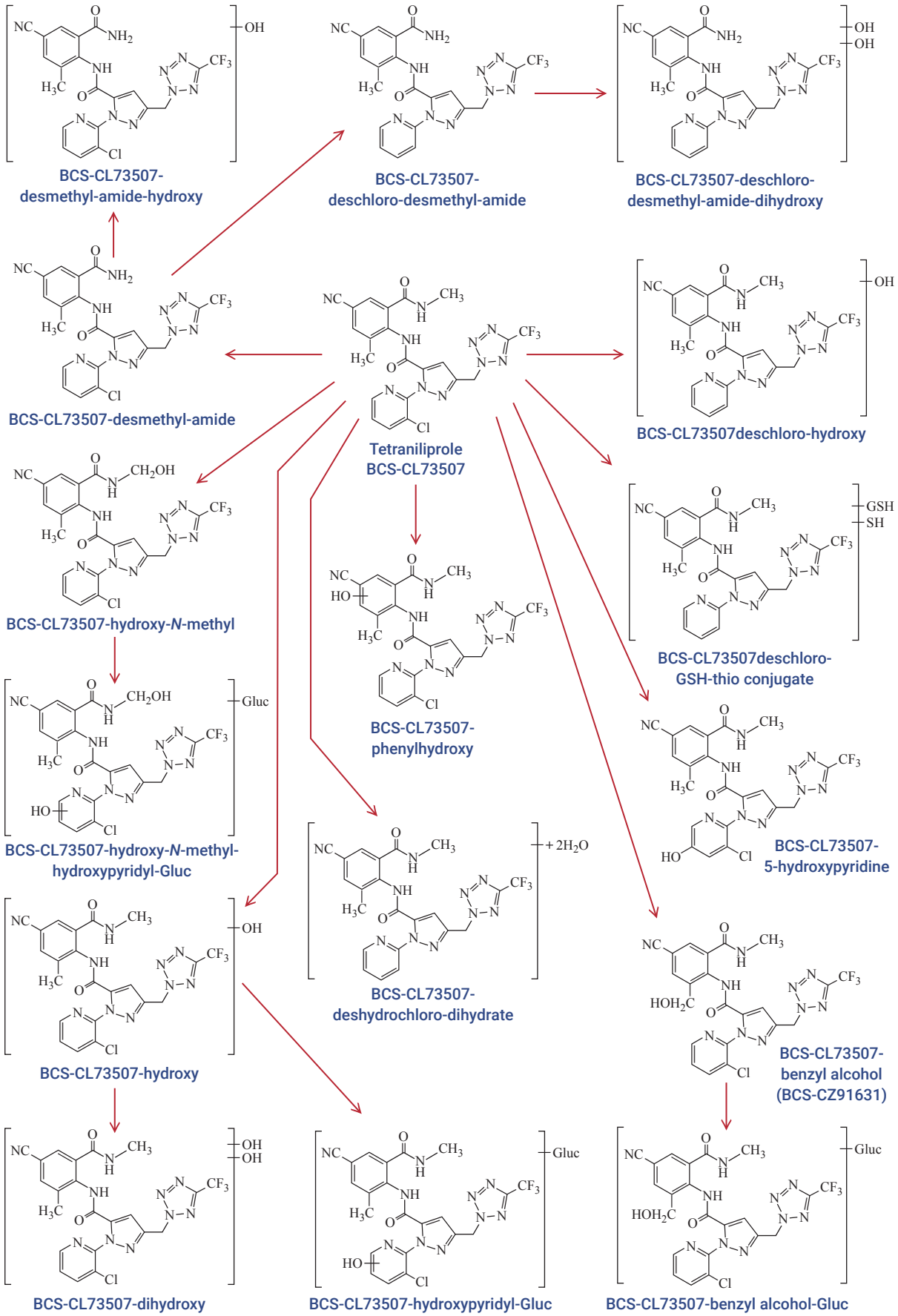
Primary metabolic reactions with tetraniliprole (Fig. 3a) consisted of hydroxylation in the pyridinyl moiety, the *N*-methyl moiety and the methyl group of the phenyl moiety, resulting in mono- and/or dihydoxy compounds, with more hydroxylation occurring in males than in females. Although hydroxylation in other positions was detected, these locations were not determined by structural elucidation. Conjugation with glucuronic acid was observed as a secondary reaction after hydroxylation.

A second major reaction was the intramolecular condensation (cyclization) of the parent resulting in quinzalinone compounds, one of which was a primary plasma metabolite that was identified as BCS-CQ63359 or tetraniliprole-*N*-methyl-quinazolinone. In this case the phenyl moiety

is cleaved, resulting in an amide, with subsequent oxidation to a carboxylic acid or methylation to an *N*-methyl amide. There were additional cleavage reactions involving the pyridine and tetrazole rings. Cleavage of the tetrazole ring was followed by oxidation, resulting in a carboxylic acid.

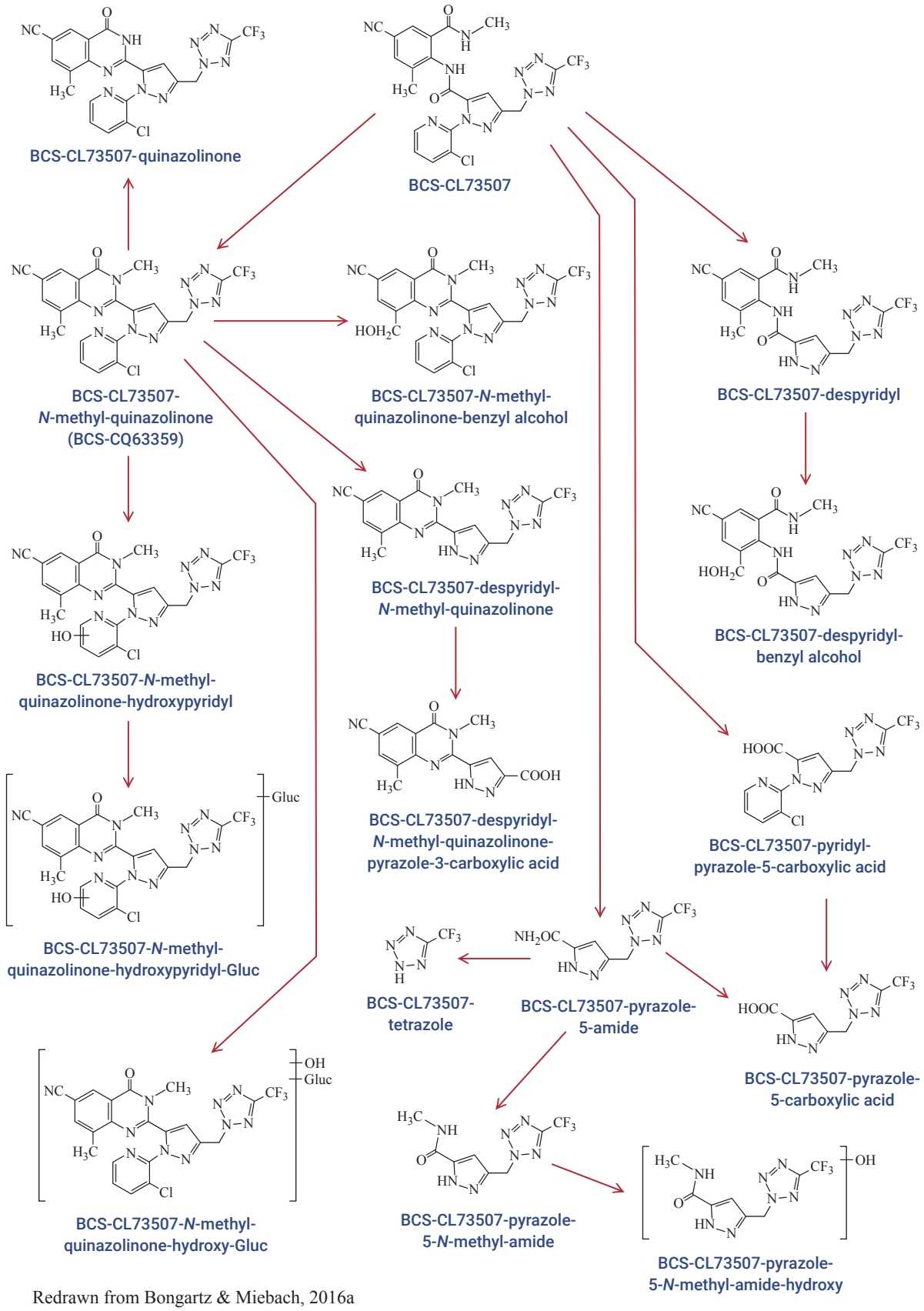
In addition to the two major pathways, minor metabolic reactions included the demethylation of the *N*-methyl group and deschlorodination in the pyridine ring. There were also two separate conjugations of the parent compound (after deschlorodination) with glutathione, followed by degradation of one of the glutathione groups to a mercapto alcohol.

Figure 3a. Proposed metabolic pathway of tetraniliprole in the rat (Part A)



Redrawn from Bongartz & Miebach, 2016a

Figure 3b. Proposed metabolic pathway of tetraniliprole in the rat (Part B)



Redrawn from Bongartz & Miebach, 2016a

Results from assays on other radiolabels suggest similar metabolic reactions and proposed pathways (Bongartz & Miebach, 2016b, c, d).

1.3 Biokinetics

In a toxicity and biokinetics screening study, Wistar Rj:WI (IOPS HAN) rats were given gavage doses of unlabelled tetraniliprole or a related insecticide, BCS-CO79240, in 0.5% methylcellulose as a single dose of 150 mg/kg bw (two rats per sex) or daily doses of 0 or 150 mg/kg bw per day for 14 consecutive days (five rats/sex per dose). In the single-dose group, blood samples were taken at 0, 3, 5, 7 and 24 hours for a biokinetic assay and fat samples were taken at terminal kill 24 hours after dosing for bioaccumulation determinations on blood and fat. In the repeat-dose group, blood, fat, liver, adrenal glands, kidneys and the thyroid glands were sampled at necropsy 24 hours after the administration of the last dose to determine bioaccumulation in the blood and fat, and for histopathology.

In the single dose assay, T_{max} was 7 h in males and 3 h in females. The C_{max} values in females were 1.3 times those in males, and 24 hours after dosing, plasma concentrations were still elevated in females compared to males (Table 9). Likewise, fat concentrations were 2.8 times higher in females than in males.

Table 9. Pharmacokinetic parameter estimates after administration of single and multiple oral gavage doses of tetraniliprole to rats

Parameter	Single dose		Repeat dose	
	Male	Female	Male	Female
Tetraniliprole				
T_{max}: observed (hours)	7	3	NC	NC
Plasma concentration: calculated (µg/L)[a]	1591.0 ± 550.1	2096.0 ± 817.4	264.6 ± 135.3	544.2 ± 383.6
Fat concentrations (µg/g)[b]	0.45 ± 0.02	1.24 ± 025	0.91 ± 0.64	2.09 ± 1.08
BCS-CO79240				
T_{max}: observed (hours)	3	5	NC	NC
Plasma concentration: calculated (µg/L)[a]	558.0 ± 5.7	579.5 ± 33.2	ND	ND
Fat concentrations (µg/g)[b]	1.19 ± 1.16	4.12 ± 4.15	ND	2.06 ± 1.17

NC: Single time point measured,not calculated; ND: Not detected; Data from p.43 of Odin-Feuretet (2010).

[a] At T_{max} in single dose study or 24 hours after administration in multiple dose study

[b] Twenty-four hours after administration
2 mg/kg bw, $n = 4$/sex

In the repeat-dose assay there were no effects on mortality, treatment-related clinical signs of toxicity, body weight or food consumption or any histopathological findings. Twenty-four hours after the last repeat dose, plasma and fat concentrations were higher in females than in males (2.0-fold in plasma and 2.3-fold in fat). While plasma concentrations were similar in the single- and repeat-dose studies 24 hours after the end of dosing, fat concentrations were 2.0-fold and 1.7-fold higher in males and females, respectively, in repeat-dose experiments (Odin-Feurtet, 2010).

In a series of in vitro interspecies liver microsome metabolic comparison assays, 10 µM [*pyrazole-carboxamide*-^{14}C]tetraniliprole, [*pyridinyl-2*-^{14}C]tetraniliprole or [*tetrazolyl-4*-^{14}C]tetraniliprole were incubated with pooled liver microsomes from 1220 male or 800 female mice, 200 male or 100 female rats, eight male rabbits, nine male dogs or a combined pool of 50 male and female humans. The liver microsomes were exposed to the test substance and sampled at 0, 30 and 60 minutes. Metabolites were identified by HPLC but the structures were not elucidated. Tetraniliprole was incubated in the absence of microsomes for 60 minutes to determine the stability in the test sample.

Five metabolites were identified and labelled BCS-1 through BCS-5. Metabolite BCS-5 was present in the negative control assay just below the lower LOQ, indicating that it is a hydrolysis compound.

Mice and human microsomes had the highest activity with 73–82% and 74–78% unchanged tetraniliprole, respectively, remaining after 60 minutes. Female rats had the least activity microsomes with 98–100% unchanged tetraniliprole remaining after 60 minutes. Metabolite BCS-3 was the major

metabolite in all groups and accounted for 3.8–25.1% of the recovered radioactivity in all but the female rat tests.

In dogs, BCS-1, BCS-3 and BCS-5 were found at low levels. The major metabolite was BCS-3, but still found at no more than 5.1% of AD. Between 96.6% and 97.2% of AD remained unchanged in dogs after 30 minutes, and this decreased to between 91.3% and 96.2% of AD after 60 minutes.

There were no metabolites unique to the human assay. Qualitatively, the findings in humans were most similar to male rabbits, however quantitatively they were most similar to mice.

The majority of the metabolism occurred within the first 30 minutes of the assay and there were no major differences due to labelling position (Mura, 2015a, b; Sola, 2015).

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

In an acute oral toxicity study, female Wistar rats were given doses of tetraniliprole (purity 89.6%) in propylene glycol 400 at 2000 mg/kg bw. There were no mortalities and no clinical signs of toxicity. All animals gained weight throughout the observation period and there were no gross changes at necropsy (Matting, 2013a).

In an acute oral toxicity study, male Wistar rats were given doses of tetraniliprole (purity 89.6%) in propylene glycol 400 at 2000 mg/kg bw. There were no mortalities and no clinical signs of toxicity. All animals gained weight throughout the observation period and there were no gross changes at necropsy (Matting, 2014).

In an acute dermal toxicity study, Wistar rats were given a limit dose of tetraniliprole (purity 89.6%) dampened with water at 2000 mg/kg bw. There were no mortalities and no signs of systemic toxicity or irritation. All animals gained weight throughout the study and there were no changes at gross necropsy (Matting, 2013b).

In an acute inhalation toxicity study, Wistar rats were given a maximum achievable concentration of tetraniliprole (purity 89.6%) at 5.01 mg/L as a dust for four hours. One male in the main study was found dead four hours after dosing. Treated animals exhibited clinical signs including slightly laboured respiration, sneezing, noisy respiration, decreased activity and hunched posture within two days of exposure. No clinical signs were observed three days after exposure with the exception of one female rat which displayed fur loss around the eyes until 11 days after exposure. All surviving animals gained weight over the 14-day observation period. Gross necropsy of the deceased male revealed dark/red discoloration of the non-collapsed lungs. No necropsy findings were noted (Nagy, 2013).

Tetraniliprole was therefore of low acute oral, dermal and inhalation toxicity in rats (Table 10).

Table 10. Summary of acute toxicity studies in rats with tetraniliprole

Species	Strain	Sex	Route	Purity	Result	Reference
Rat	Wistar	Female	Oral	89.6%	LD_{50} > 2000 mg/kg bw	Matting, 2013a
Rat	Wistar	Male	Oral	89.6%	LD_{50} > 2000 mg/kg bw	Matting, 2014
Rat	Wistar	Male & Female	Dermal	89.6%	LD_{50} > 2000 mg/kg bw	Matting, 2013b
Rat	Wistar	Male & Female	Inhalation	89.6%	LC_{50} > 5.01 mg/L	Nagy, 2013

bw: Body weight; LC_{50}: Median lethal concentration; LD_{50}: Median lethal dose

(b) Dermal irritation

In a dermal irritation study, 0.5 g of tetraniliprole (purity 89.6%) was moistened with water and applied under a 10 × 10 cm gauze patch to the skin of three male New Zealand White rabbits for four hours. Slight erythema was noted in the first 24 hours following treatment. Tetraniliprole was considered to be minimally irritating to the skin of rabbits (Matting, 2013c).

(c) Ocular irritation

In an eye irritation study, 0.1 mg of tetraniliprole (purity 89.6%) was instilled into the conjunctival sac of the right eyes of six male New Zealand white rabbits. Minor to moderate conjunctival redness and chemosis were noted in the first 48 hours following treatment; however, there were no signs of irritation by 72 hours post-instillation. Tetraniliprole was considered to be transiently-irritating to the eyes of rabbits (Matting, 2013d).

(d) Dermal sensitization

In a dermal sensitization study using a local lymph node assay (LLNA), four female CBA/JRj mice per group were exposed to tetraniliprole (purity 89.6%). Each group was tested with either vehicle only, or the test substance at concentrations of 10%, 25% or 50% weight for volume (w/v) in dimethyl sulfoxide (DMSO). A 25% solution of α-hexylcinnamaldehyde dissolved in DMSO was run concurrently as a reliability check. Alopecia was noted on days 2–6 and 3–6 for test sites treated with 50% and 25% doses respectively. Rigid ears were also noted in mice treated with a 50% dose of tetraniliprole. The number of responses increased with increasing concentration. The stimulation index (SI) value at a concentration of 25% was above 3 and the EC3 (the amount of test substance needed to induce a three-fold increase in cell proliferation in a LLNA) was calculated to be 21.7%. There were no signs of dermal irritation observed for any vehicle control animal. The positive control elicited the expected response. Tetraniliprole was a dermal sensitizer in this assay (Hargaitai, 2013).

In a dermal sensitization study using a local lymph node assay (LLNA), five female CBA/Ca mice per group were exposed to tetraniliprole (purity 89.0%). Each group was tested with either vehicle only, or with the test substance at concentrations of 10%, 25% or 50% (w/v) in DMSO. A 25% solution of α-hexylcinnamaldehyde dissolved in DMSO was run concurrently as a reliability check. Alopecia of the ears was observed in some animals treated with 50% and 25% doses. Tetraniliprole precipitation was observed in all dose groups and an increase in mean ear thickness (> 25%) was noted in animals treated with 50% and 25% doses. The number of responses increased with increasing concentration. While all the SI values in treated groups were above 3 without a consistent dose–response relationship, the EC3 was estimated to be below 10% (w/v). No signs of dermal irritation were observed for any vehicle control animal during the study. The positive control elicited the expected response. Body weight loss and no weight gain were noted in all dose groups. Tetraniliprole was a dermal sensitizer in this assay (Varga-Kanizsai, 2016).

2.2 Short-term studies of toxicity

(a) Oral administration

Mouse

In a 28-day toxicity study, groups of five C57BL/6J mice received tetraniliprole (purity 96.0%) in the diet at a concentration of 0, 600, 3000 or 6000 ppm (equal to 0, 100, 523 and 1010 mg/kg bw per day for males, 0, 113, 576 and 1159 mg/kg bw per day for females). Animals were inspected daily for mortality, moribundity and clinical signs of toxicity. Detailed clinical observations were performed weekly. Body weight, body weight gain and feed consumption were measured weekly. Clinical chemistry parameters were analysed from samples taken prior to terminal kill. Gross examinations were performed on all animals, including those found dead, killed moribund or killed at the end of the study. Select organs were weighed. Histopathological examinations were performed on the liver, kidneys, thyroid glands and on macroscopic findings for all animals, and also on the liver, spleen, brain, pituitary, adrenal glands and reproductive tissues of control and high-dose animals.

There were no effects on mortality, clinical signs of toxicity, body weight, body weight gain, feed consumption, clinical chemistry parameters or post-mortem findings.

The NOAEL for oral toxicity of tetraniliprole in a 28-day study in mice was 6000 ppm (equal to 1010 mg/kg bw per day), the highest dose tested (Blanck, 2011).

In a 90-day toxicity study in C57BL/6J mice, groups of 10 animals were fed tetraniliprole (purity 92.6%) in the diet at a concentration of 0, 900, 2700 or 6000 ppm (equal to 0, 145, 426 or 973 mg/kg bw per day for males, 0, 180, 544 or 1224 mg/kg bw per day for females). Animals were inspected daily for mortality, moribundity and clinical signs of toxicity. Detailed clinical observations were performed weekly. Body weight, body weight gain and feed consumption were measured weekly. Clinical chemistry parameters were analysed from samples taken prior to terminal kill. Gross examinations were performed on all animals, including those found dead, killed moribund or killed at the end of the study. Select organs were weighed. Histopathological examinations were performed on the liver, kidneys, thyroid glands and on macroscopic findings for all animals, and also on the liver, spleen, brain, pituitary, adrenal glands and reproductive tissues of control and high-dose animals.

There were no treatment-related effects on mortality, body weight, body weight gain, feed consumption, clinical signs of toxicity, clinical chemistry parameters or post-mortem findings. There was a linear, but less than proportional increase in unchanged tetraniliprole in plasma samples. Plasma levels were higher in females given the low dose than in males given the high dose (Table 11).

Table 11. Plasma concentration of tetraniliprole in treated mice

	Dose level (ppm)							
	Male				Female			
	0	**900**	**2700**	**6000**	**0**	**900**	**2700**	**6000**
Week 12 concentration (mg/L ± SD)	<LOQ	0.364 ±0.066	0.412 ±0.064	0.570 ±0.109	<LOQ	0.697 ±0.114	0.774 ±0.094	1.009 ±0.183

<LOQ: Below the lower limit of quantitation of 0.2 mg/L; SD: Standard deviation Source: p.28, Odin, 2016a

The NOAEL for oral toxicity of tetraniliprole in a 90-day study in mice was 6000 ppm (equal to 973 mg/kg bw per day), the highest dose tested (Odin, 2016a).

Rat

In a 28-day toxicity study, groups of five Wistar rats received tetraniliprole (purity 96.0%) in the diet at a concentration of 0, 500, 2000 or 8000 ppm (equal to 0, 38, 148 and 599 mg/kg bw per day for males, 0, 43, 171 and 700 mg/kg bw per day for females). Animals were inspected daily for mortality, moribundity and clinical signs of toxicity. Detailed clinical observations were performed weekly. Body weight, body weight gain and food consumption were measured weekly. Haematological and clinical chemistry parameters were analysed from samples taken prior to terminal kill. Gross examinations were performed on all animals, including those found dead, killed moribund or killed at the end of the study. Select organs were weighed. Histopathological examinations were performed on the liver, kidneys, thyroid glands and on macroscopic findings for all animals, and also on the liver, spleen, brain, pituitary, adrenal glands and reproductive tissues of control and high-dose animals. Portions of the liver were homogenized for microsomal preparations to determine cytochrome P-450 isoenzyme profile and uridine diphosphate glucuronosyltransferase (UDP-GT) activity.

There were no effects on mortality, clinical signs of toxicity, body weight, body weight gain, feed consumption, haematology or postmortem findings. Ethoxyresorufin-O-deethylase (EROD) activity was increased in males in all treated groups and in females at the highest dose tested.

The NOAEL for oral toxicity of tetraniliprole in a 28-day study in rats was 8000 ppm (equal to 599 mg/kg bw per day), the highest dose tested (Lasserre, 2011).

In a 90-day toxicity study, groups of 10 Wistar rats received tetraniliprole (purity 92.6%) in the diet at a concentration of 0, 900, 3000 or 10 000 ppm (equal to 0, 55.5, 178 or 608 mg/kg bw per day for males, 0, 65.7, 213 or 723 mg/kg bw per day for females). In addition, 10 animals per sex were added to the control (0 ppm) and high-dose groups (10 000 ppm) to assess the reversibility of any effects observed at the high dose. After the treatment period these animals were maintained on the control diet for an additional month. Animals were inspected daily for mortality, moribundity and clinical signs of toxicity. Detailed clinical observations were performed weekly and all surviving animals were subjected to a neurotoxicity assessment using functional observation battery (FOB)

parameters during weeks 11–12 of the study. Ophthalmoscopic evaluations were performed during acclimatization and during week 12 in control and high-dose animals. Body weight, body weight gain and feed consumption were measured weekly. Haematological, clinical chemistry and urinalysis parameters were determined from samples taken prior to terminal kill and blood samples were additionally analysed for plasma concentrations of the test compound. All animals were necropsied, selected organs weighed and a range of tissues were taken, fixed, and examined microscopically. Based on the absence of findings in the neurological and ophthalmological examinations during the treatment phase, these parameters were not evaluated during the recovery phase. Tissues were sampled at the end of the recovery period but in the absence of findings over the 90-day treatment period, they were not examined microscopically.

There were no effects on mortality, clinical signs of toxicity, FOB parameters, body weight, body weight gain, food consumption, ophthalmology, haematology, clinical chemistry, urinalysis or postmortem findings. Plasma concentrations in males were linear, but lower than proportional. In females, plasma concentrations were higher than those in males in all treated groups, but did not exhibit a dose–response relationship (Table 12).

Table 12. Plasma concentrations of tetraniliprole (BCS-SL73507)

	Dose level (ppm)					
	Male			**Female**		
	900	**3000**	**10 000**	**900**	**2700**	**6000**
Plasma concentration (mg/L ± SD)	0.266 ±0.075	0.315 ±0.079	0.406 ±0.036	0.875 ±0.211	0.778 ±0.362	0.915 ±0.221

SD: Standard deviation

Source: p.28, Odin, 2016b

The NOAEL for oral toxicity of tetraniliprole in a 90-day study in rats was 10 000 ppm (equal to 608 mg/kg bw per day), the highest dose tested (Odin, 2016b).

Dog

Study 1

In a 28-day range-finding study, groups of two beagle dogs per sex received tetraniliprole (purity 92.6%) in the diet at concentrations of 0, 2000, 6000 or 17 000 ppm (equal to 0, 62.2, 190 and 555 mg/kg bw per day for males, 0, 60.5, 222 and 597 mg/kg bw per day for females). Animals were inspected daily for mortality, moribundity and clinical signs of toxicity. Detailed clinical observations were performed weekly. Ophthalmoscopic evaluations were performed during acclimatization and at the end of treatment. Body weight and body weight gain were measured weekly and food consumption measured daily. Haematological, clinical chemistry and urinalysis parameters were determined from samples taken prior to treatment and at the end of the study, and blood samples were analysed for plasma concentrations of the test compound. All animals were necropsied, selected organs weighed and a range of tissues taken, fixed and examined microscopically.

There were no effects on body weight, food consumption, ophthalmoscopy, haematology, urinalysis, nor were there any postmortem findings. Salivation was increased in females at 6000 ppm and above and blood cholesterol levels increased in males and females at 6000 ppm and above. Plasma concentrations of unchanged tetraniliprole in males were linear, but lower than proportional to dosing. In females, plasma concentrations were neither linear nor proportional to dosing (Table 12; Kennel, 2012).

Table 12. Plasma concentration of tetraniliprole (BCS-CL73507) in treated dogsat the end of the study

Dose (ppm)	Animal number	Individual measured concentration (mg/L)					
			Time after treatment (hours)				
		Prestudy	0	1	2	4	6
	Male						
0[a]	T1M2226	<VL	<VL	-	-	-	-
	T1M2227	<VL	<VL	-	-	-	-
2000	T2M2230	<VL	3.07	2.95	3.87	3.91	3.97
	T2M2231	<VL	3.27	3.42	3.96	4.23	4.14
6000	T3M2234	<VL	3.75	3.73	4.37	4.60	4.86
	T3M2235	<VL	3.36	4.37	4.97	5.08	5.20
17 000	T4M2238	<VL	4.72	5.66	6.78	7.20	6.95
	T4M2239	<VL	4.82	5.67	6.67	7.03	6.82
	Female						
0[a]	T1F2228	<VL	<VL	-	-	-	-
	T1F2229	<VL	<VL	-	-	-	-
2000	T2F2232	<VL	4.03	4.43	4.74	5.22	5.33
	T2F2233	<VL	3.57	3.30	4.22	3.52	4.91
6000	T3F2236	<VL	2.81	3.49	4.04	4.53	4.33
	T3F2237	<VL	6.09	6.45	8.04	8.37	8.10
17 000	T4F2240	<VL	2.41	3.32	4.01	3.94	3.81
	T4F2241	<VL	2.51	2.78	3.82	3.93	3.92

VL: Validation limit time; [a] Prior to feeding; Source: p.24 of Kennel, 2012

Study 2

In a 90-day toxicity study, groups of four beagle dogs per sex received tetraniliprole (purity 89.6%) in the diet at concentrations of 0, 800, 3200 or 12 800 ppm (equal to 0, 25.6, 126 or 440 mg/kg bw per day for males, 0, 29.9, 138 or 485 mg/kg bw per day for females). Animals were inspected daily for mortality, moribundity and clinical signs of toxicity. Detailed clinical observations were performed weekly and a detailed physical examination was performed monthly. Ophthalmoscopic evaluations were performed during acclimatization and at the end of treatment. Body weight and body weight gain were measured weekly, and feed consumption was measured daily. Haematological, clinical chemistry and urinalysis parameters were determined from samples taken prior to treatment, at week 7 and at the end of the study. Blood samples from the end of the study were analysed for plasma concentrations of the test compound and metabolite BCS-CQ63359. All animals were necropsied, selected organs weighed, and a range of tissues were taken, fixed, and examined microscopically.

There were no effects on food consumption, ophthalmoscopy, haematology or urinalysis parameters and no histopathological changes. There were increases in salivation and blood cholesterol at the mid dose in males and females. Due to the lack of a dose–response relationship for all but cholesterol level at week 13 in males, the changes were not considered adverse. At week 13 cholesterol was increased at the mid and high dose in males; however, both of these values were similar to the prestudy low-dose values and therefore not considered treatment-related or adverse. At the high dose, body weight and body weight gain were decreased in males and females, however based on the lack of treatment-related body weight changes in the one-year study, the changes were not considered adverse. Liver weights were increased in high-dose males and alkaline phosphatase (ALP) was increased in high-dose females. As in the case of increased liver weights and hepatocellular hypertrophy, recent publications (Yokoyama et al., 2019, 2021) have indicated that ALP is not a sensitive end-point in dogs. As such, in the absence of other markers of hepatotoxicity, the changes seen here in ALP values would not be considered adverse. Both males and females exhibited linear (but lower than proportional) trends in unchanged compound and major metabolite levels in the blood plasma (Table 13).

Table 13. Discussion table: select findings in the 90-day dog study (± SD)

	Sex and dose level (ppm)							
	Male				Female			
Parameter	**0**	**800**	**3200**	**12 800**	**0**	**800**	**3200**	**12 800**
Body weight (kg)								
Day 1	7.95 ± 0.420	7.98 ± 0.695	7.73 ± 0.655	7.75 ± 0.896	6.55 ± 0.465	6.40 ± 0.716	6.40 ± 0.678	6.55 ± 0.332
Day 92	9.45 ± 0.794	8.95 ± 0.451	8.73 ± 1.201	8.33 ± 1.926 (↓12)	8.10 ± 0.648	7.68 ± 0.903	7.83 ± 0.754	7.18 ± 0.369 (↓11)
Body weight gain (%)								
Day 92	1.50 ± 0.779	0.98 ± 0.519	1.0 ± 0.804	0.58 ± 1.118 (↓61)	1.55 ± 0.238	1.28 ± 0.512	1.43 ± 0.189	0.63 ± 0.411** (↓59)
Salivation	0	0	3	2	0	0	15	6
Alkaline phosphatase (IU/L)								
Prestudy	144.0 ± 62.18	154.8 ± 55.55 (↑8)	136.8 ± 23.17 (↓5)	144.0 ± 47.92 (0)	111.5 ± 14.27	126.5 ± 30.86 (↑13)	129.5 ± 20.44 (↑16)	134.0 ± 31.61 (↑20)
Week 7	141.8 ± 38.99	152.8 ± 32.07 (↑8)	112.3 ±2 9.24 (↓2)	181.0 ± 98.88 (↑28)	142.8 ± 37.88	115.5 ± 17.37 (↓19)	121.0 ± 17.05 (↓15)	218.0 ± 16.99** (↑53)
Week 13	103.5 ± 27.33	112.3 ± 29.24 (↑9)	116.0 ± 39.34 (↑12)	163.3 ± 88.46 (↑58)	89.3 ± 27.11	80.5 ± 17.54 (↓10)	102.8 ± 18.63** (↑15)	191.5 ± 16.54** (↑114)
Blood cholesterol (mmol/L)								
Prestudy	3.233 ± 0.3764	3.530 ± 0.1787	3.245 ± 0.2468	3.053 ± 0.3428	3.190 ± 0.1701	2.995 ± 0.2205	3.090 ± 0.2550	2.945 ± 0.2266
Week 7	2.838 ± 0.3439	3.115 ± 0.6251	3.533 ± 1.2887 (↑24)	3.440 ± 0.4392 (↑21)	3.645 ± 0.9719	3.128 ± 0.2666	3.163 ± 0.6653	3.628 ± 1.2279
Week 13	2.795 ± 0.3426	3.238 ± 0.5178	3.425 ± 1.0300 (↑23)	3.548 ± 0.3137 (↑27)	3.428 ± 0.3694	3.338 ± 0.5527	3.948 ± 1.3109	3.580 ± 0.9185
Liver weight								
Absolute (g)	261.28 ± 40.331	280.50 ± 25.947	293.49 ± 30.327 (↑12)	299.27 ± 52.964 (↑15)	253.21 ± 30.660	252.18 ± 19.125	268.72 ± 17.756 (↑6)	255.45 ± 28.042 (↑1)
Relative (%)	2.76 ± 0.370	3.13 ± 0.145 (↑13)	3.41 ± 0.580 (↑24)	3.69 ± 0.774 (↑34)	3.13 ± 0.326	3.32 ± 0.474 (↑6)	3.47 ± 0.501 (↑11)	3.56 ± 0.281 (↑14)

* $p = 0.05$, ** $p = 0.01$, Dunnett's LSD test

Source: pp. 36 & 38, 110–127 of Kennel, 2015a

Table 14. Plasma concentrations of parent tetraniliprole and metabolite BCS-CQ63359 in dogs at week 13

	Sex and dose level (ppm)							
	Male				Female			
Parameter	0	800	3200	12 800	0	800	3200	12 800
Tetraniliprole, BCS-CL73507 concentration (mg/L)	0.012	2.010	2.920	4.645	0.031	2.680	3.890	4.940
BCS-CQ63359 concentration (mg/L)	< 0.010[a]	0.405	0.770	1.700	< 0.025[a]	0.400	1.140	1.625

Source: p. 38 of Kennel, 2015a

[a] For series with part of individual values found below the limit of quantification, the value of LOQ was used for mean calculation. The result was expressed as "< of the mean value"

The NOAEL for oral toxicity of tetraniliprole in a 90-day study in dogs was 12 800 ppm (equal to 440 mg/kg bw per day), the highest dose tested (Kennel, 2015a).

Study 3

In a one-year toxicity study, groups of four beagle dogs per sex received tetraniliprole (purity 89.6%) in the diet at concentrations of 0, 650, 2900 or 12 800 ppm (equal to 0, 19.8, 91.2 or 440 mg/kg bw per day for males, 0, 18.3, 88.8 or 408 mg/kg bw per day for females). Animals were inspected daily for mortality, moribundity and clinical signs of toxicity. Detailed clinical observations were performed. Ophthalmoscopic evaluations were performed during acclimatization and at the end of treatment. Body weight, body weight gain were measured weekly and food consumption was measured daily. Haematological, clinical chemistry and urinalysis parameters were determined from samples taken prior to treatment, at months 3–4, 6–7 and at the end of the study. Blood samples from month 4 and the end of the study were analysed for plasma concentrations of the test compound and metabolite BCS-CQ63359. All animals were necropsied, selected organs weighed, and a range of tissues taken, fixed, and examined microscopically.

There were no effects on feed consumption, ophthalmology, haematology or urinalysis parameters. Body weight and body weight gains were depressed compared to controls in the high-dose group (Table 15). In females at the low dose and mid dose, changes in the last months of the study were due to one control female with a high body weight. Changes in clinical signs of toxicity were limited to an increase in salivation in males and females at mid dose and above. Salivation was considered to be related to the irritation profile of the test compound, and while treatment-related, it was not adverse. Liver changes consisted of an increase in cholesterol in males at the mid dose and above, increased ALP activity in males and females at the high dose and increased relative liver weights in males only in the high-dose group. While there was a dose-responsive increase in cholesterol in males, as evidenced by the values in females and the large standard deviations, a wide range of cholesterol values were apparent and the change was not considered treatment-related. In the absence of other liver findings, the change in ALP activity was not considered adverse. There was an increase in diffuse vacuolation of the adrenal zona glomerulosa in males and females, however as there was no functional deficit observed that corresponded with this histopathological change and no equivalent changes were seen in the 90-day study, the change was not considered treatment-related and adverse. Likewise, an apparent increase in hypospermatogenesis and segmental tubular atrophy/hypoplasia of the testes at the mid dose and above were considered incidental to treatment. Hypospermatogenesis is a relatively common change in the dog and both hypospermatogenesis and atrophy were seen in the control dogs in the 90-day oral toxicity study. Additionally, in the absence of cell debris, reduced epididymis, testicular and prostate weights and lack of macroscopic or other microscopic changes, the findings were considered unlikely to be treatment-related.

Table 15. Select findings in the one-year beagle dog study with tetraniliprole; (percentages shown indicate % of control)

	Sex and dose level (ppm)							
	Male				Female			
Parameter	**0**	**800**	**3200**	**12 800**	**0**	**800**	**3200**	**12 800**
Body weight (bw) and body weight gain								
Day 1, initial bw (% ± SD)[a]	7.88 ± 0.70	7.95 ± 0.85	7.98 ± 0.66	7.90 ± 0.41	6.88 ± 0.58	6.88 ± 0.50	6.83 ± 0.38	6.95 ± 0.30
Day 100 (% ± SD)[a]	9.08 ± 1.01	9.23 ± 1.18	8.83 ± 1.15	8.15 ± 0.19 (↓10%)	8.38 ± 1.04	7.78 ± 0.67	8.08 ± 1.10	7.70 ± 0.70 (↓5%)
Day 204 (% ± SD)[a]	9.28 ± 1.03	9.40 ± 1.24	9.35 ± 1.09	8.25 ± 0.53 (↓11%)	8.75 ±.1.11	8.43 ± 0.70	8.38 ± 0.96	7.93 ± 0.49 (↓9%)
Day 302 (% ± SD)[a]	9.80 ± 1.20	9.93 ± 1.36	9.78 ± 1.28	8.50 ± 0.73 (↓13%)	9.15 ± 1.16	8.43 ± 1.04 (↓8%)	8.40 ± 1.04 (↓8%)	8.03 ± 0.60 (↓12%)
Day 365, final bw (% ± SD)[a]	9.75 ± 1.31	9.55 ± 1.36	9.70 ± 1.24	8.40 ± 0.66 (↓14%)	9.08 ± 1.09	8.15 ± 0.47 (↓10%)	8.20 ± 0.91 (↓10%)	7.90 ± 0.39 (↓13%)
Individual animal weights; day 365 (kg)	8.1 9.9 9.7 11.3	7.6 10.7 9.7 10.2	10.9 9.2 10.5 8.2	8.5 9.2 8.3 7.6	9.4 10.2 9.1 7.6	7.6 8.7 8.3 8.0	7.4 7.6 9.4 8.4	7.4 7.8 8.3 8.1
Body weight gain; weeks 1–52, (= days 1–365) (kg ± SD)	1.88 ± 0.61	1.60 ± 0.67 (↓15%)	1.73 ± 0.85 (↓8%)	0.50 ± 0.74 (↓73%)	2.20 ± 0.53	1.28 ± 0.33 (↓42%)	1.38 ± 0.78 (↓33%)	0.95 ± 0.27* (↓57%)
Salivation[a]	0 (0)	0 (0)	1 (6)	1 (15)	0 (0)	0 (0)	2 (33)	3 (41)
Clinical chemistry								
Alkaline phosphatase activity ± SD (IU/L)								
Prestudy	103.5 ± 21.61	117.8 ± 55.00 (+14%)	97.8 ± 19.47 (–6%)	102.5 ± 33.13 (–1%)	123.5 ± 23.80	144.0 ± 70.70 (+17%)	124.3 ± 73.09 (+1%)	138.0 ± 37.61 (+12%)
Month 4	67.0 ± 12.68	66.3 ± 31.34 (–1%)	75.0 ± 12.73 (+12%)	120.8 ± 48.86 (+80%)	96.8 ± 27.93	99.3 ± 41.50 (+3%)	102.8 ± 31.08 (+6%)	136.5 ± 59.36 (+41%)
Month 6	56.0 ± 10.49	50.3 ± 22.77 (–10%)	65.5 ± 16.46 (+17%)	130.0* ± 52.25 (+132%)	97.0 ± 33.74	111.3 ± 34.43 (+15%)	74.5 ± 27.63 (–23%)	116.5 ± 28.20 (+20%)
Month 12	46.0 ± 7.35	47.8 ± 24.14 (+4%)	78.5 ± 5.80 (+71%)	138.0* ± 34.46 (+200%)	72.3 ± 18.89	149.0 ± 81.21 (+106%)	104.3 ± 59.08 (+44%)	124.8 ± 34.59 (+73%)

	Sex and dose level (ppm)							
	Male				Female			
Parameter	**0**	**800**	**3200**	**12 800**	**0**	**800**	**3200**	**12 800**
Cholesterol ± SD (mmol/L)								
Prestudy	3.310 ± 0.5570	3.068 ± 0.1362	3.235 ± 0.4223	3.023 ± 0.2223	2.913 ± 0.3699	3.273 ± 0.7031	3.030 ± 0.0931	3.190 ± 0.5529
Month 3	2.850 ± 0.2941	2.998 ± 0.2506	3.160 ± 0.3451	3.695 ± 0.7155 (+29%)	3.658 ± 1.0737	3.798 ± 0.8880	4.118 ± 1.1450	3.845 ± 0.6598
Month 6	2.753 ± 0.2650	3.015 ± 0.1790	3.170 ± 0.3201 (+15%)	3.510 ± 0.5832 (+27.5%)	3.853 ± 0.5447	4.655 ± 1.8112 (+22%)	3.610 ± 0.1997 (–6%)	3.573 ± 0.3494 (–7%)
Month 12	2.668 ± 0.2354	3.070 ± 0.2459 (+15%)	3.278 ± 0.4891 (+23%)	3.308 ± 0.6381 (+24%)	3.438 ± 0.2924	5.565[#] ± 1.5207 (+62%)	4.073 ± 0.2559 (+18%)	4.323 ± 0.9708 (+26%)
Organ weights ± SD								
Liver weight: absolute (g)	274.28 ± 26.944	252.97 ± 10.868	266.63 ± 24.164	283.31 ± 29.084 (↑3)	226.27 ± 40.219	261.16 ± 62.675 (↑15)	221.16 ± 46.503	259.40 ± 22.643 (↑15)
Liver weight: relative (%)	2.85 ± 0.148	2.69 ± 0.369	2.81 ± 0.158	3.44 ± 0.411* (↑21)	2.57 ± 0.377	3.24 ± 0.854 (↑26)	2.72 ± 0.459	3.33 ± 0.287 (↑30)
Histopathology – adrenal glands								
Zona glomerulosa vacuolation: diffuse								
minimal	0	1	2	1	0	0	0	0
slight	0	1	1	3	0	1	2	3
Total	**0**	**2**	**3**	**4**	**0**	**1**	**2**	**3**
Zona glomerulosa vacuolation: multifocal								
minimal	1	0	0	0	1	0	0	0
Histopathology – testes								
Hypospermatogenesis: bilateral: multifocal/diffuse								
minimal	0	2	3	2				
slight	0	1	1	2	-	-	-	-
Total	**0**	**3**	**4**	**4**				
Segmental tubular atrophy/hypoplasia: bilateral								
minimal	0	0	0	1				
slight	0	0	1	0				
moderate	0	0	0	1	-	-	-	-
Total	**0**	**0**	**1**	**2**				
Segmental tubular atrophy/hypoplasia: unilateral								
minimal	0	0	1	0				
slight	0	0	1	0	-	-	-	-
Total	**0**	**0**	**2**	**0**				

	Sex and dose level (ppm)							
	Male				Female			
Parameter	**0**	**800**	**3200**	**12 800**	**0**	**800**	**3200**	**12 800**
Histopathology – prostate								
Secretory depletion: diffuse								
minimal	0	2	1	2				
slight	0	0	0	1	-	-	-	-
Total	**0**	**2**	**1**	**3**				
Mean plasma concentrations (mg/L)[b]								
Month 4								
Tetraniliprole	0.025	1.975	2.748	4.745	<0.036[c]	1.700	2.558	5.858
BCS-CQ63359	0.013[c]	0.322	0.793	2.433	<0.012[c]	0.342	0.708	1.993
	<0.012[c]							
Month 12								
Tetraniliprole	0.049	1.808	3.590	6.350	0.038	2.848	2.370	7.335
BCS-CQ63359	<0.013	0.516	1.133	2.823	<0.011[c]	0.685	1.371	3.035
	0.014							

Source: pp.42, 44, 97–110, 199–225, 286–289 and 870 of Kennel, 2016a

[a] Animals with finding (number of incidents of finding)

[b] Mean values calculated from raw data

[c] For series individual value(s) found below the limit of quantification (LOQ), the value of LOQ (0.01 mg/L) was used for calculation of the mean; the result is expressed as "< of the mean value"

* $p \leq 0.05$, ** $p \leq 0.01$, Dunnett's LSD test; # $p \leq 0.01$ level, Dunn Rank Sum Test

In both sexes, there was a linear, but less than proportionate increased in both unchanged tetraniliprole and the major metabolite, BCS-CQ63359.

The NOAEL for oral toxicity of tetraniliprole in a one-year study in dogs was 12 800 ppm (equal to 408 mg/kg bw per day) the highest dose tested (Kennel, 2016a).

(b) Dermal application

In a 28-day toxicity study, groups of 10 Wistar rats received tetraniliprole (purity 89.6%) dermally at concentrations of 0, 100, 300 or 1000 mg/kg bw per day for six hours per day. Animals were inspected daily for mortality, moribundity, clinical signs of toxicity. Also, dosing sites were inspected daily for signs of irritation. Detailed clinical observations were performed weekly and an FOB was performed and smears for estrus cyclicity taken at the end of the treatment period. Body weight, body weight gain and food consumption were measured weekly. Ophthalmoscopic examinations were performed during the acclimatization period and prior to study termination. Haematological, clinical chemistry and urinalysis parameters were determined from samples taken prior to terminal kill. Gross examinations were performed on all animals, including those found dead, killed moribund or killed at the end of the study. Select organs were weighed and histopathological examinations performed.

There were no effects on mortality, clinical signs of toxicity, body weight, food consumption, ophthalmology, estrus cyclicity or FOB parameters. There were slight, non-adverse increases in ALP, liver weight and hepatocellular hypertrophy at the high dose in males, and non-adverse increases in adrenal weight in females in the absence of histopathological changes.

The NOAEL for dermal toxicity of tetraniliprole in a 28-day study in rats was 1000 mg/kg bw per day, the highest dose tested (Toeroek-Bathó, 2015).

(c) Exposure by inhalation

No inhalation toxicity studies were submitted.

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

In a 19-month toxicity and carcinogenicity study, groups of 50 mice (C57BL/6J) received tetraniliprole (purity 89.6%) in the diet at a concentration of 0, 260, 1300 or 6500 ppm (equal to 0, 32.9, 166 or 825 mg/kg bw per day for males, 0, 43.1, 225 or 1073 mg/kg bw per day for females). In addition, 10 mice per sex were added to all groups to assess changes at 52 weeks. Animals were inspected daily for mortality, moribundity and clinical signs of toxicity. Detailed clinical observations were performed weekly. Body weights and food consumption were measured weekly for the first 13 weeks of the study, then approximately monthly thereafter. Haematology parameters were measured at months 13 and 19 and blood samples were analysed for plasma concentrations of the test compound at months 4, 12, and at the end of the study. Where possible, blood smears were prepared from moribund animals just before sacrifice. All animals were subjected to necropsy, with selected organs weighed at scheduled interim or final sacrifice. Designated tissues were fixed, and those from the carcinogenicity phase were examined microscopically.

In the 12-month groups there were no treatment-related effects on mortality, clinical signs of toxicity, body weight, food consumption or haematological parameters. There were no gross pathological or organ weight findings. Histopathological examination was not performed on this group.

In the 19-month groups, there were no treatment-related effects on mortality, clinical signs of toxicity, body weight, food consumption or haematological parameters. Liver weights were increased in males and adrenal weights were increased in females in the high-dose group. Despite this, in the absence of associated gross or histopathological changes, the organ weight increases were not considered adverse.

There was no evidence of oncogenicity.

At all time points tetraniliprole and metabolite BCS-CQ63359 were detected in the plasma of all treated groups and increased with dose. However, plasma concentrations were neither linear nor proportional to the administered dose. Plasma concentrations were higher in females than males at all time points and for all doses.

Table 16. Plasma concentrations of tetraniliprole and metabolite BCS-CQ63359 in treated mice at 4, 12 and 18 months (mean values calculated from raw data)

	Sex and dose level (ppm)							
	Male				Female			
	0	260	1300	6500	0	260	1300	6500
Month 4								
Tetraniliprole (mg/L)	<LOQ	0.454	0.601	0.704	<LOQ	0.556	0.982	1.182
BCS-CQ63359 (mg/L)	<LOQ	0.051	0.121	0.349	<LOQ	0.041	0.121	0.501
Month 12								
Tetraniliprole (mg/L)	<LOQ	0.419	0.584	0.758	<LOQ	0.600	0.951	1.158
BCS-CQ63359 (mg/L)	<LOQ	0.043	0.110	0.395	<LOQ	0.035	0.126	0.598
Month 18								
Tetraniliprole (mg/L)	<LOQ	0.345	0.541	0.681	<LOQ	0.607	1.168	1.524
BCS-CQ63359 (mg/L)	<LOQ	0.050	0.106	0.373	<LOQ	0.076	0.171	0.954

<LOQ: Below the limit of quantification, 0.01 mg/L; Source: p.38 of Kennel, 2016b

The NOAEL for oral toxicity of tetraniliprole in a 19-month study in mice was 6500 ppm (equal to 825 mg/kg bw per day), the highest dose tested (Kennel, 2016b)

Rat

In a two-year toxicity and carcinogenicity study, groups of 60 Wistar rats received tetraniliprole (purity 89.6%) in the diet at concentrations of 0, 900, 4000 or 18 000 ppm (equal to 0, 35.3, 159 and 741 mg/kg bw per day for males, 0, 51.2, 221, or 1052 mg/kg bw per day for females). In addition, 10 rats per sex were added to all groups to assess changes at 52 weeks. Animals were inspected daily for mortality, moribundity and clinical signs of toxicity. Detailed clinical observations were performed weekly. Body weight was recorded weekly for the first 13 weeks, then approximately every four weeks thereafter. Food consumption was recorded twice weekly for the first six weeks of the study, then approximately weekly up to week 13, then every four weeks thereafter. Ophthalmological examinations were performed on all animals during acclimatization and after approximately one year, then on all surviving animals at two years. Haematology and clinical chemistry determinations and urinalysis were performed during months 3–4, 6, 12, 18–19 and 24–25 on selected animals. At scheduled sacrifice for the chronic and carcinogenicity phase of the study, selected organs were weighed and tissues samples collected, fixed, and examined microscopically.

Among the 52-week chronic toxicity groups, there were no treatment-related effects on mortality, clinical signs of toxicity, body weight, food consumption, ophthalmoscopy, haematology, clinical chemistry, urinalysis or postmortem parameters.

In the carcinogenicity groups, there were no effects on food consumption, haematology, clinical chemistry or urinalysis parameters. In males, there were no effects on body weight, clinical signs of toxicity or postmortem findings. Mortality was decreased in high-dose females compared to controls, resulting in a larger number of aged female rats.

In high-dose carcinogenicity group females, there were decreases in body weight and body weight gain, a slight increase in prolapsed vaginas and an increase in the severity of corpora lutea depletion (Table 17a). Histopathological changes consisted of an increase in squamous cell hyperplasia in the cervix and vagina at the high dose. There was an apparent increase in epithelial tumours of the uterus at the same dose. However, occurrences of glandular polyps, endometrial adenocarcinomas and adenosquamous carinomas should not be combined due to differences in the histopathological progression of the lesions. Adenocarcinomas originate in the glandular epithelium and are a downgrowth lesion into the endometrium, developing from endometrial hyperplasia. As there is no evidence of endometrial hyperplasia in the study, the tumour was considered incidental to treatment. Adenosquamous carcinomas originate as a papillary growth as opposed to a downgrowth. Glandular polyps are composed of glands and stroma covered by a thin epithelium and are unrelated to either of the former tumours. The endometrial adenocarcinomas seen in the control and high-dose groups and the adenosquamous carcinoma seen in the high-dose group were all seen at 688 days and longer. Mortality was reduced in the high-dose group, therefore, the incidences were seen to reflect the ageing rat and were not related to treatment. There was an increase in hyperplasia of the pars distalis, however, this is a preneoplastic lesion for adenomas of the pituitary, a very common tumour in aging rats, which were less frequent at the high dose and not considered adverse.

Table 17a. Select findings in carcinogenicity phase female rats

	Dose (ppm)			
	0	**900**	**4000**	**18 000**
Number of animals in group, *N*	**60**	**60**	**60**	**60**
Mortality				
Killed for humane reasons	29	17	18	12
Found dead	10	11	8	12
Died during anaesthesia	-	1	-	1
Accidental trauma	-	1	-	-
Total number [% mortality]	39 [65.0]	30 [50.0]	26 [43.3]	25 [41.7]
Adjusted mortality rates [a]	65.0%	48.4%	51.4%	40.0%

	Dose (ppm)			
	0	**900**	**4000**	**18 000**
Body weight and body weight gain (g ± SD, [% of control])				
Initial body weight (day 1)	158 ± 10.0	158 ± 9.5 [100]	159 ± 10.8 [101]	158 ± 10.1 [100]
Body weight: week 2 (day 8)	187 ± 13.1	184 ± 12.3 [98]	186 ± 14.4 [100]	182 ± 12.4 [97]
week 26 (day 176)	328 ± 26.7	326 ± 23.9 [99]	329 ± 37.4 [100]	325 ± 22.4 [99]
week 54 (day 372)	375 ± 39.9	379 ± 49.7 [101]	368 ± 60.3 [98]	358 ± 34.3 [95]
week 78 (day 541)	432 ± 59.0	408 ± 66.4 [94]	411 ± 77.0 [95]	390 ± 50.6 [90]**
week 105 (day 729)	451 ± 86.8	420 ± 70.3 [93]	434 ± 90.1 [96]	382 ± 54.0 [85]**
Body weight gain:				
weeks 1–2 (days 1–8)	29 ± 5.8	26 ± 6.5 [90]*	27 ± 6.4 [93]	24 ± 5.8 [83]**
weeks 1–14 (days 1–92)	141 ± 17.0	138 ± 16.1 [98]	139 ± 22.5 [99]	137 ± 18.0 [97]
weeks 26–54 (days 176–372)	47 ± 23.4	53 ± 36.7 [113]	39 ± 31.1 [83]*	33 ± 22.8 [70]**
weeks 54–78 (days 372–541)	56 ± 34.1	37 ± 31.4 [66]**	43 ± 37.4 [77]	34 ± 24.4 [61]***
weeks 1–105 (days 1–729)	294 ± 85.0	263 ± 67.2 [89]	274 ± 85.4 [93]	224 ± 50.9 [76]**
Select clinical signs of toxicity				
Number of animals in group, *N*	58	59	60	58
Prolapsed vagina	1 (1.7%)	1 (1.7%)	1 (1.7%)	4 (6.9%)
Histopathology – non-neoplastic lesions				
Uterus				
Squamous cell metaplasia: endometrium: focal				
Minimal	13	14	13	20 (33%)
Slight	1	2	0	3 (5%)
Total	**14**	**16**	**13**	**23 (38%)**
In animals found dead or killed for humane reasons	9	5	5	7
Squamous cell hyperplasia: cervix: diffuse				
Minimal	12	12	13	13
Slight	4	8	4	17 (28%)
Moderate	1	0	0	3 (5%)
Total	**17**	**20**	**17**	**33**(55%)**
In animals found dead or killed for humane reasons	11	9	5	10

	Dose (ppm)			
	0	**900**	**4000**	**18 000**
Vagina				
Squamous cell hyperplasia: diffuse				
Minimal	13	11	16	22 (37%)
Slight	4	2	1	4
Total	**17**	**13**	**17**	26* (43%)
In animals found dead or killed for humane reasons	9	6	5	8
Ovary				
Corpora lutea depletion				
Minimal	5	3	1	1
Slight	3	2	5	1
Moderate	8	0	2	3
Marked	3	5	3	3
Severe	27	30	35	42
Total	**46**	**40**	**46**	**50**
Pituitary gland				
Pars distalis hyperplasia: diffuse	4	2	7	6 (2%)
Pars distalis hyperplasia: focal	12	16	12	21* (36%)
Historical control data	13%–53%			
Histopathology – Neoplastic lesions				
Uterus				
Glandular polyp	2 (3.4%)	0	0	3 (5.0%)
Historical control data for above	0.0–1.7; mean 0.4[b]		0.0–8.2; mean 0.7[c]	
Endometrial adenocarcinoma	1 (1.7%)	0	0	2 (3.3%)
Historical control data for above	0.0–2.0; mean 0.7[b]		0.0–28.0; mean 4.8[c]	
Adenosquamous carcinoma	0	0	0	1 (1.7%)
Historical control data for above	0.0–0.0; mean 0.0[b]		0.0–2.0; mean 0.1[c]	

Week numbers quoted represent the start of the week; Source: pp.46, 47, 50, 56–58, 947 and 6109 of Odin, 2016c

[a] Unscheduled death: Kaplan–Meier, estimated rates after adjustment for censored animals (accidental trauma, died during anaesthesia); statistical analysis was conducted on mortality incidence only

[b] In-house historical control data

[c] RITA historical control data

* Statistically different from the control, $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Plasma concentrations of tetraniliprole and metabolite BCS-CQ63359 increased with dose in a non-linear and non-proportional manner at all time points. Plasma concentrations were higher in females than in males. In the low- and mid-dose males, metabolite concentrations were lower than unchanged parent, however in high-dose males and females metabolite concentrations were higher than those of the parent. At the mid dose in females, parent and metabolite plasma concentrations were roughly equal (Table 17b).

Table 17b. Concentrations in plasma of tetraniliprole and main metabolite BCS-CQ63359 (mean values calculated from raw data)

	Sex and dose level (ppm)					
	Male			Female		
Parameter	**900**	**4000**	**18 000**	**900**	**4000**	**18 000**
Month 3						
Tetraniliprole (mg/L)	0.33	0.37	0.54	0.63	0.68	0.96
BCS-CQ63359 (mg/L)	0.09	0.26	0.78	0.34	0.71	3.49
Month 12						
Tetraniliprole (mg/L)	0.30	0.41	0.90	0.70	1.2	2.3
BCS-CQ63359 (mg/L)	0.10	0.31	1.1	0.36	0.88	3.5
Month 23						
Tetraniliprole (mg/L)	0.38	0.65	0.75	1.1	1.5	1.7
BCS-CQ63359 (mg/L)	0.12	0.40	1.0	0.39	1.1	3.9

Limit of quantification = 0.01 mg/L; Source: pp.53–54 of Odin, 2016c

The NOAEL was 4000 ppm (equal to 221 mg/kg bw per day), based on decreased body weight, prolapsed vagina, increased squamous cell hyperplasia in the cervix and vagina, an increased severity of corpora lutea depletion at the LOAEL of 18 000 ppm (equal to 1052 mg/kg bw per day) (Odin, 2016c).

2.4 Genotoxicity

(a) In vitro studies

A range of in vitro studies was conducted to assess the genotoxicity of tetraniliprole at purities ranging from 89.0% to 97.2%, and to assess its potential for inducing chromosomal aberration and damage, and gene mutation (summarized in Table 18). There was no evidence of genotoxicity.

(a) In vivo studies

In addition to the in vitro human lymphocyte micronucleus assay, a micronucleus assay was performed in mice to assess the potential of tetraniliprole to damage chromosomes in vivo (summarized in Table 18). There was no evidence of genotoxicity.

Table 18. Overview of genotoxicity studies with tetraniliprole

End-point	Test system	Concentrations/doses tested	Purity	Results	Reference
		In vitro			
Reverse mutation	*Salmonella typhimurium*	***Test 1*** (± S9) Plate incorporation, all strains: 3–5000 µg/plate in DMSO; ***Test 2*** (± S9) Preincubation, all strains: 10–5000 µg/plate in DMSO	89.6%	Negative	Sokolowski, 2013a
Reverse mutation	*Salmonella typhimurium*	***Test 1*** (± S9) Plate incorporation, all strains: 3–5000 µg/plate in DMSO; ***Test 2*** (± S9) Preincubation: Strains TA 98 and TA 100; 3–5000 µg/plate in DMSO; Strains TA 1535 and TA 1537; 10–5000 µg/plate in DMSO;	97.2%	Negative	Chang, 2016a

Reverse mutation	*Salmonella typhimurium*	***Test 1*** (± S9) Plate incorporation, all strains: 3–5000 µg/plate in DMSO; ***Test 2*** (± S9) Preincubation, all strains: 10–5000 µg/plate in DMSO	89.0%	Negative	Chang, 2016b
Gene mutation	Chinese hamster lung cell (V79)	***Experiment 1*** (± S9) 4 h exposure, 10.2–1300 µg/mL in DMSO ***Experiment 2*** (+ S9) 4 h exposure, 10.2–325 µg/mL in DMSO ***Experiment 2*** (– S9) 24 h exposure, 10.2–325 µg/mL in DMSO	89.6%	Negative	Wollny, 2013a
Gene mutation	Chinese hamster lung cell (V79)	***Experiment 1*** (± S9) 4 h exposure, 8.8–210 µg/mL in DMSO ***Experiment 2*** (+ S9) 4 h exposure, 17.5–280 µg/mL in DMSO ***Experiment 2*** (– S9) 24 h exposure, 8.8–176 µg/mL in DMSO	89.0%	Negative	Wollny, 2016
Chromosomal aberration assay	Chinese hamster lung cell (V79)	**Pulse exposure method** ***Experiment I*** (± S9) 4 h exposure, 5.1–1300 µg/mL in DMSO ***Experiment IIA*** (+ S9) 4 h exposure, 25–600 µg/mL in DMSO ***Experiment IIB*** (+ S9) 4 h exposure, 50–600 µg/mL in DMSO **Continuous exposure method** ***Experiment IIA*** (– S9) 18 h exposure, 5.1–1300 µg/mL in DMSO	89.6%	Negative	Bohnenberger, 2013a
In vitro micronucleus assay	Human lymphocytes	***Trial IA*** (– S9) 4 h pulse, 5–2247 µg/mL in DMSO ***Trial IA*** (+ S9) 4 h pulse, 5–2247 µg/mL in DMSO ***Trial IB*** (– S9) repeat 4 h pulse, 10.4–300 µg/mL in DMSO ***Trial II*** (– S9) 20 h continuous exposure, 8–400 µg/mL in DMSO	89.0%	Negative	Chang, 2016c

		In vivo (oral)			
Mouse micronucleus[a]	NMRI mouse	2000 mg/kg bw in corn oil ***Main study***: 24-h post-treatment: 6/sex limit dose and ± controls; ***48-h post-treatment***: 6/sex limit dose; ***Positive control***: cyclophosphamide	89.6%	Negative	Dony, 2013

DMSO: Dimethyl sulfoxide

S9: 9000 × *g* supernatant fraction from liver homogenate from phenobarbital-treated and 5,6-benzoflavone-treated rats

[a] Bone marrow exposure was not demonstrated in this study; however, ADME studies indicate that the compound reached the bone marrow (see.Table 8, for example)

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a two-generation reproductive toxicity study, groups of 24 Wistar rats of each sex received tetraniliprole (purity 89.6%) at a dietary concentration of 0, 300, 600, 2700 or 12 000 ppm (equal to 0, 22, 44, 196 and 896 mg/kg bw per day in males, 0, 25, 41, 224 and 1032 mg/kg bw per day in females). In the lactation phase, dietary concentrations were decreased in females to 0, 150, 300, 1350 and 6000 ppm (equal to 0, 23, 47, 211 and 890 mg/kg bw per day). The F1 generation comprised two subsets (A and B) both including 24 male and 24 female progeny from each group, and these continued to receive the relevant diet, as per the F0 generation, throughout the study until termination (doses at premating: 0, 28, 57, 253 and 1138 mg/kg bw per day for males, 0, 30, 63, 266 and 1218 mg/kg bw per day for females). Only subset A was mated. The F2 generation received the relevant diet from weaning until termination on their postnatal day 70. During the study, the test animals were observed twice daily for clinical signs and physical examination was performed weekly on all adult animals. Body weights were measured weekly in males and premating females, and in females at gestation days (GDs) 0, 7, 14 and 20, then on lactation days (LDs) 0, 4, 7, 14, 21, 25 and 28. Feed consumption was measured weekly in males and premating females, and in females at GDs 0–6, 7–13, 14–19 and LDs 1–3, 4–6, 7–13 and 14–20. Animals were monitored for estrous cycles, mating performance, fertility, gestation length, parturition observations and reproductive performance. Sperm parameters, organ weight, macroscopic and microscopic pathology investigations were undertaken on the F0 and F1A generations. Organ weight and macroscopic pathology investigations were carried out on the F1B and F2 generations. For the offspring of each generation, clinical condition, litter size, survival rate, anogenital distance, sex ratio, sexual maturation (in selected F1 and F2 generation only), absolute body weight including body weight gain, organ weight, gross pathology were investigated.

In the parental animals, there were no effects on mortality, food consumption, no clinical signs of toxicity or effects on postmortem parameters. Both high-dose males and females of the F1 generation entered the premating period with reduced body weights. High-dose F1 males had body weights comparable to controls by week 6 and all decreases were less than 10% and therefore not considered adverse (Table 19a).

Table 19a. Effects of tetraniliprole on F1 parental animals in a reproductive toxicity study in rats

Dose level (ppm)	0	300	600	2700	12 000
			Males		
Body weight (g ± SD, [% change from control])					
Week 0[a]	84 ± 8.4	79 ± 10.9* [↓6]	82 ± 8.9* [↓2]	79 ± 8.3** [↓6]	77 ± 9.5** [↓8]
Week 1	131 ± 11.1	124 ± 14.8* [↓5]	126 ± 11.7* [↓4]	123 ± 11.1** [↓6]	120 ± 13.5** [↓8]
Week 2	175 ± 12.9	167 ± 16.5 [↓5]	171 ± 13.8 [↓2]	167 ± 13.0** [↓5]	163 ± 15.3** [↓7]
Week 3	219 ± 16.8	210 ± 20.4 [↓4]	213 ± 16.4 [↓3]	206 ± 15.9** [↓6]	205 ± 18.1** [↓6]
Week 4	263 ± 19.3	255 ± 23.4* [↓3]	255 ± 19.1* [↓3]	247 ± 17.6** [↓6]	244 ± 21.0** [↓7]
Week 5	297 ± 21.7	289 ± 26.0 [↓3]	291 ± 21.2 [↓2]	282 ± 20.4** [↓5]	281 ± 23.1** [↓5]
Week 6	324 ± 22.7	313 ± 27.2 [↓3]	313 ± 23.2 [↓3]	306 ± 23.3** [↓6]	303 ± 25.7** [↓6]
Week 7	348 ± 26.3	342 ± 31.0 [↓2]	340 ± 26.6 [↓2]	333 ± 22.0 [↓4]	335 ± 25.1 [↓4]
Week 18	470 ± 43.6	457 ± 46.8	456 ± 39.9	458 ± 39.0	462 ± 42.2
Body weight gain (g ± SD)					
Weeks 0–10	312 ± 29.3	311 ± 31.7	304 ± 30.6	304 ± 22.1	313 ± 32.1
Weeks 0–18	386 ± 40.8	379 ± 40.5	375 ± 37.1	380 ± 34.0	384 ± 41.5
			Females		
Body weight (g ± SD, [% change from control])					
Premating					
Week 0[a]	78 ± 7.0	75 ± 8.9* [↓4]	75 ± 8.2* [↓4]	73 ± 6.9** [↓6]	72 ± 8.3** [↓8]
Week 1	115 ± 7.7	111 ± 11.0	112 ± 9.7	107 ± 9.0** [↓7]	105 ± 10.2** [↓9]
Week 2	142 ± 8.5	137 ± 11.8* [↓4]	137 ± 9.0* [↓4]	134 ± 9.9** [↓6]	132 ± 9.5** [↓7]
Week 3	160 ± 10.0	156 ± 12.7* [↓2]	155 ± 10.4* [↓3]	151 ± 10.9** [↓6]	149 ± 10.0** [↓7]
Week 4	181 ± 11.5	176 ± 14.7* [↓3]	175 ± 10.4* [↓3]	170 ± 12.4** [↓6]	165 ± 10.6** [↓9]
Week 5	194 ± 11.6	190 ± 14.7	187 ± 10.9** [↓4]	184 ± 12.9** [↓5]	181 ± 11.5** [↓7]
Week 10	240 ± 14.5	227 ± 19.1* [↓5]	228 ± 16.4* [↓5]	225 ± 15.6** [↓6]	221 ± 15.6** [↓8]
Gestation					
Day 0	242 ± 13.9	227 ± 18.7* [↓6]	233 ± 16.1* [↓4]	232 ± 15.9* [↓4]	226 ± 15.0** [↓7]
Day 20	343 ± 24.9	324 ± 24.4* [↓5]	330 ± 25.2* [↓4]	328 ± 21.4* [↓4]	321 ± 25.0** [↓6]

Dose level (ppm)	0	300	600	2700	12 000
Lactation					
Day 1	265 ± 19.0	250 ± 19.5 [↓6]	258 ± 19.7 [↓3]	256 ± 17.8 [↓3]	250 ± 14.8** [↓6]
Day 7	287 ± 16.6	271 ± 21.5* [↓6]	278 ± 19.9* [↓3]	276 ± 15.1* [↓4]	272 ± 16.2** [↓5]
Day 21	288 ± 16.9	274 ± 18.6* [↓5]	278 ± 15.9* [↓3]	276 ± 15.2* [↓4]	274 ± 15.6** [↓5]
Day 28	264 ± 16.2	250 ± 19.2* [↓5]	257 ± 16.5* [↓3]	253 ± 15.0* [↓4]	250 ± 14.5** [↓5]
Body weight gain (g ± SD, [% change from control])					
Premating					
Weeks 0–10	162 ± 13.6	152 ± 17.9* [↓6]	152 ± 14.2* [↓6]	152 ± 14.5* [↓6]	150 ± 13.1** [↓7]
Gestation					
Days 0–7	18 ± 5.0	18 ± 5.6	17 ± 6.6 [↓6]	15 ± 5.7 [↓17]	17 ± 4.9 [↓6]
Day 7–14	23 ± 5.7	22 ± 3.2	25 ± 3.2 [↑9]	23 ± 4.5	21 ± 5.3 [↓9]
Day 14–20	60 ± 10.3	59 ± 9.9	56 ± 10.2 [↓6]	58 ± 7.8	57 ± 10.9 [↓5]
Day 0–20	101 ± 15.5	98 ± 12.8	98 ± 14.2	96 ± 10.9 [↓5]	96 ± 13.1 [↓5]
Lactation					
Days 1–4	11 ± 7.0	013 ± 6.8	13 ± 4.9	13 ± 6.4	16 ± 6.0* [↑45]
Day 7–14	11 ± 11.5	14 ± 8.9	9 ± 9.4	10 ± 8.7	10 ± 8.8
Day 14–21	–10 ± 9.7	–11 ± 11.8	–8 ± 13.3	–10 ± 9.2	–8 ± 11.1
Day 1–21	23 ± 9.6	24 ± 10.2	20 ± 11.5	20 ± 8.5	24 ± 8.8
Day21–28	–24 ± 9.6	–24 ± 7.8	–21 ± 9.7	–22 ± 9.3	–24 ± 8.0

[a] Start of direct dosing following weaning; Source: pp.144–148 of Patten, 2016

* Statistically different from control, $p < 0.05$, ** statistically different from control, $p < 0.01$

There were no effects on fertility or reproductive parameters. There was a statistically significant decrease in gestational length in the high-dose F0 pregnancies from 22.6 days to 22.3 days. It was eventually determined to be non-adverse due to the lack of effects in the F1 dams and lack of effect on survival rates. Additionally, the change was considered the result of two outliers in the control and low-dose groups.

In the offspring, there were no effects on clinical condition, litter size, survival rate, sex ratio or postmortem parameters. There were slight reductions in body weight in F1 and F2 pups (less than 10%) and a delay in the completion of vaginal opening in F1 females (Table 19b). Based on a covariant analysis (ANOVA) of the body weight and vaginal opening values, the delayed vaginal opening was considered to be secondary to decreased pup body weight.

Table 19b. Mean litter and pup body weights for F1 and F2 pups (g ± SD [% change from controls])

	Dose group (ppm)									
Time	**0**	**300**	**600**	**2700**	**12 000**	**0**	**300**	**600**	**2700**	**12 000**
	F1 litters – males and females combined					**F2 litters – males and females combined**				
PND 1	6.3 ± 0.5	6.4 ± 0.4	6.2 ± 0.4	6.4 ± 0.6	6.1 ± 0.5	6.3 ± 0.4	6.3 ± 0.6	6.5 ± 0.5	6.3 ± 0.4	6.1 ± 0.5
PND 4[b]	9.0 ± 0.9	9.0 ± 1.3	8.8 ± 0.8	9.0 ± 1.3	8.8 ± 1.0	9.4 ± 1.0	9.3 ± 1.4	9.7 ± 1.0	9.5 ± 0.9	9.1 ± 0.7
PND 4[c]	9.1 ± 0.9	9.0 ± 1.3	8.9 ± 0.8	9.1 ± 1.2	8.8 ± 1.0	9.4 ± 1.0	9.3 ± 1.4	9.7 ± 1.0	9.5 ± 0.9	9.1 ± 0.7
PND 7	14.5 ± 1.3	13.7 ± 2.0	14.2 ± 1.2	14.1 ± 2.0	14.0 ± 1.4	15.1 ± 1.3	14.6 ± 2.3	15.3 ± 1.4	15.2 ± 1.4	14.4 ± 0.9*
PND 14	30.9 ± 1.8	29.1 ± 2.9	30.1 ± 1.9	29.7 ± 3.0	29.1 ± 2.2* [↓6]	30.4 ± 2.4	29.8 ± 3.1	30.3 ± 2.7	29.6 ± 2.6	28.7 ± 1.9* [↓6]
PND 21	48.1 ± 3.4	45.8 ± 4.3	47.5 ± 2.8	46.4 ± 4.0	44.6 ± 2.8** **[↓7]**	46.9 ± 3.2	46.2 ± 4.4	46.4 ± 3.7	46.0 ± 3.9	43.8 ± 2.6** **[↓7]**
	F1 pups – male					**F2 pups – male**				
PND 1	6.5 ± 0.5	6.5 ± 0.4	6.4 ± 0.4	6.5 ± 0.6	6.2 ± 0.5	6.5 ± 0.4	6.5 ± 0.7	6.6 ± 0.5	6.5 ± 0.4	6.1 ± 0.5
PND 4[b]	9.2 ± 0.9	9.1 ± 1.3	9.1 ± 0.9	9.2 ± 1.4	8.9 ± 1.1	9.6 ± 1.1	9.4 ± 1.5	9.8 ± 1.1	9.7 ± 0.9	9.2 ± 0.7
PND 4[c]	9.2 ± 0.9	9.2 ± 1.3	9.1 ± 0.8	9.3 ± 1.4	8.9 ± 1.0	9.6 ± 1.1	9.5 ± 1.5	9.8 ± 1.1	9.7 ± 0.9	9.2 ± 0.7
PND 7	14.7 ± 1.3	14.0 ± 2.0	14.5 ± 1.3	14.5 ± 2.3	14.2 ± 1.4	15.5 ± 1.3	14.7 ± 2.5	15.5 ± 1.6	15.4 ± 1.5	14.7 ± 0.9*
PND 14	31.1 ± 1.9	29.5 ± 2.8	30.7 ± 2.3	30.3 ± 3.3	29.4 ± 2.2* [↓5]	31.0 ± 2.4	29.9 ± 3.2	30.6 ± 2.9	30.0 ± 2.8	29.1 ± 2.3* [↓6]
PND 21	48.7 ± 3.7	46.6 ± 4.1	48.5 ± 3.4	47.5 ± 4.4	45.3 ± 2.9** **[↓7]**	47.9 ± 3.7	46.6 ± 4.6	47.0 ± 4.2	47.0 ± 4.2	44.5 ± 2.9** [↓7]
	F1 pups – female					**F2 pups – female**				
PND 1	6.1 ± 0.5	6.3 ± 0.5	6.0 ± 0.5	6.2 ± 0.6	5.9 ± 0.5	6.1 ± 0.4	6.2 ± 0.6	6.3 ± 0.5	6.2 ± 0.5	5.9 ± 0.5
PND 4[a]	8.9 ± 1.0	8.9 ± 1.3	8.6 ± 0.9	8.8 ± 1.1	8.6 ± 1.0	9.2 ± 1.0	9.1 ± 1.3	9.5 ± 1.0	9.4 ± 0.9	8.9 ± 0.8
PND 4[b]	9.0 ± 1.0	8.9 ± 1.3	8.7 ± 0.9	8.8 ± 1.0	8.7 ± 1.0	9.1 ± 1.0	9.2 ± 1.4	9.5 ± 1.0	9.4 ± 0.9	8.9 ± 0.8
PND 7	14.3 ± 1.3	13.6 ± 2.0	13.9 ± 1.3	13.8 ± 1.7	13.9 ± 1.4	14.7 ± 1.3	14.5 ± 2.2	15.1 ± 1.4	15.0 ± 1.4	14.1 ± 1.2
PND 14	30.7 ± 1.8	29.0 ± 2.7*	29.6 ± 1.9*	29.0 ± 2.7*	29.0 ± 2.2* [↓5]	29.9 ± 2.5	29.7 ± 3.1	30.0 ± 2.6	29.3 ± 2.6	28.4 ± 1.9 [↓5]
PND 21	47.5 ± 3.4	45.4 ± 4.0	46.5 ± 2.6	45.3 ± 4.0*	44.0 ± 3.0** [↓7]	46.0 ± 2.9	45.7 ± 4.4	45.8 ± 3.6	45.2 ± 4.0	43.3 ± 2.7* [↓6]

	Dose group (ppm)									
Time	**0**	**300**	**600**	**2700**	**12 000**	**0**	**300**	**600**	**2700**	**12 000**
Vaginal opening – onset										
Age (days)	27 ± 1.2	27 ± 1.4	27 ± 1.4	27 ± 1.4	27 ± 1.5	-	-	-	-	-
Body weight (g)	72 ± 5.5	69 ± 7.9	70 ± 7.1	70 ± 6.5	68 ± 8.8* [↓6]	-	-	-	-	-
Vaginal opening – completion										
Age (days)	33 ± 2.3	33 ± 2.6	33 ± 2.8	34 ± 2.9##	36 ± 2.6**##	-	-	-	-	-
Body weight (g)	104 ± 11.1	102 ± 16.3	101 ± 13.7	100 ± 13.9	106 ± 11.3	-	-	-	-	-

PND: Postnatal day Source: pp.112–113, 154 and 163–164 of Patten, 2016

[a] Diet concentrations were halved during lactation, to maintain a more consistent dosage per body weight throughout the study

[b] Before standardization (culling)

[c] After standardization (culling)

* Statistically different from control, $p < 0.05$, ** $p < 0.01$; William′s test

[#] Statistically different from control, $p < 0.05$, [##] $p < 0.01$; ANOVA test (Wason, 2021)

The NOAEL for parental toxicity was 12 000 ppm (equal to 896 mg/kg bw per day), the highest dose tested.

The NOAEL for reproductive toxicity was 12 000 ppm (equal to 896 mg/kg bw per day), the highest dose tested.

The NOAEL for offspring toxicity was 2700 ppm (equal to 196 mg/kg bw per day), based on decreased pup body weights resulting in delayed vaginal opening at the LOAEL of 12 000 ppm (equal to 1032 mg/kg bw per day) (Patten, 2016).

(b) Developmental toxicity

Rat

In a developmental toxicity study, groups of 23 premated female Sprague Dawley (SD) rats were given tetraniliprole (purity 89.6%) in an aqueous solution of 0.5% methylcellulose 400 by gavage at doses of 0, 62.5, 250 or 1000 mg/kg bw per day from days 6–20 of gestation. Animals were monitored twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Body weight and body weight gain were measured on GDs 0, 6, 8, 10, 12, 14, 16, 18 and 21. Feed consumption was determined over the periods GD 1–6, 6–8, 8–10, 10–12, 12–14, 14–16, 16–18 and 18–21. All surviving animals were subjected to gross necropsy and uterine staining. All fetuses were examined for body weight, sex and external malformations. Half of each group was examined for visceral malformations, and the remaining half stained and examined for skeletal malformations.

There were no effects in maternal animals on mortality, clinical signs of toxicity, body weight, feed consumption, caesarian section parameters or postmortem findings.

In the fetuses, effects were limited to a nonadverse decrease in body weight and changes to ossification. Although the incomplete ossifications could be attributed to the decreased body weight, the increase in unossified fifth or sixth sternebrae were considered adverse as this is a relatively rare and serious variation (Table 20). There was no evidence of malformations.

Table 20. Cesarean section data

Fetal observations	Dose (mg/kg bw per day)				
	0	62.5	250	1000	HCD[a]
Number of litters [pups]	23 [177]	23 [156]	23 [167]	22 [168]	-
Body weights (g ± SD)					
Final dam weight	442.4 ± 26.42	434.2 ± 29.77	431.8 ± 42.94	442.5 ± 36.71	-
Mean fetal weight:					
Combined sexes [% diff. from controls]	5.53 ± 0.258	5.52 ± 0.255	5.44 ± 0.249	5.31 * ± 0.264 (↓4%)	5.30–5.61
Males [% diff. from controls]	5.69 ± 0.266	5.61 ± 0.252	5.55 ± 0.296	5.47 ± 0.328 (↓4)	5.45–5.75
Females [% diff. from controls]	5.36 ± 0.225	5.42 ± 0.260	5.32 ± 0.234	5.15 * ± 0.287 (↓4)	5.16–5.48
Ossification findings – fetus (litter); [percentage of fetuses (litters)] in square brackets					
5th or 6th sternebrae: incomplete ossification	18 (13) [10.12 (56.5)]	18(11) [11.5 (47.8)]	20 (12) [12.0 (52.2)]	31(14)* [18.5 (63.6)]	[3.1–16.8 (19.0–52.4)]
5th and/or 6th sternebrae: unossified	0 (0) [0.0 (0.0)]	3 (3) [1.9 (13.0)]	1 (1) [0.6 (4.3)]	7** (5)* [22.7 (4.2)]	[0.0–2.3 (0.0–17.4)]
Interparietal: split	0 (0) [0.0 (0.0)]	0 (0) [0.0 (0.0)]	0 (0) [0.0 (0.0)]	2 (2) [1.2 (9.1)]	[0.0–0.6 (0.0–4.8)]
Ischium (bi): incomplete ossification	0 (0) [0.0 (0.0)]	0 (0) [0.0 (0.0)]	2 (2) [1.2 (8.7)]	1 (1) [0.6 (4.5)]	[0.0–0.0 (0.0–0.0)]
Hyoid centrum: incomplete ossification	5 (3) [2.8 (13.0)]	16** (6) [10.3 (26.1)]	10 (6) [6.0 (26.1)]	2 (2) [1.2 (9.1)]	[0.0–3.9 (0.0–21.7)]
7th cervical centrum: unossified	5 (2) [2.8 (8.7)]	3 (3) [1.9 (13.0)]	5 (4) [3.0 (17.4)]	9 (6) [5.4 (27.3)]	[0.0–12.2 (0.0–41.7)]
1st metatarsal: unossified	2 (1) [1.1 (4.3)]	0 (0) [0.0 (0.0)]	2 (1) [1.2 (4.3)]	2 (2) [1.2 (9.1)]	[0.0–10.1 (0.0–36.0)]

Source: pp.30, 34–35 of Kennel, 2014

[a] In-house historical control data ranges, lowest%–highest%, for fetuses and litters; given for main uterine parameters in Attachment 3 of the Tables & Appendices section of the study report

The NOAEL for maternal toxicity was 1000 mg/kg bw per day, the highest dose tested.

The NOAEL for embryo/fetal toxicity was 250 mg/kg bw per day, based unossified fifth or sixth sternebrae at the LOAEL of 1000 mg/kg bw per day (Kennel, 2014).

Rabbit

In a developmental toxicity study, groups of 23 female premated New Zealand White rabbits were given tetraniliprole (purity 89.6%) at a concentration of 0, 62.5, 250 or 1000 mg/kg bw per day in 0.5% aqueous sodium methylcellulose 400 from days 6–28 of gestation. Animals were monitored twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Body weight and body weight gain were measured on GDs 3, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 29. Food consumption was determined over the periods GD 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. All surviving animals were subjected to gross necropsy and uterine staining. Blood samples were taken at terminal kill from at least five pregnant females per group to determine blood concentrations of tetraniliprole and metabolite BCS-CQ63359. All fetuses were examined for body weight, sex, and external and visceral malformations. Half of each group was stained and examined for craniofacial malformations, and all fetuses were stained and examined for skeletal malformations

There were no effects on maternal mortality, body weight, food consumption, clinical signs of toxicity or caesarian parameters nor any gross changes at necropsy.

There were no effects on embryo/fetal body weights, mortality or sex ratios nor evidence of any malformations.

Plasma concentrations of tetraniliprole and the major metabolite BCS CQ63359 were linear, but not proportional to dose. Parent compound was more abundant than the metabolite at the low dose, equal at the mid dose and less abundant than the metabolite at the high dose (Table 21).

Table 21. Mean concentrations of tetraniliprole and BCS CQ63359 in plasma at necropsy (24 hours after the last dose)

	Dose level of tetraniliprole (mg/kg bw per day)			
	0	**62.5**	**250**	**1000**
Number of pregnant animals	5	5	5	6
Tetraniliprole: mean conc. ± SD (mg/L)	<LOQ	0.240 ± 0.138	0.375 ± 0.122	0.574 ± 0.113
BCS-CQ63359: mean conc. ± SD (mg/L)	<LOQ	0.099 ± 0.033	0.327 ± 0.067	0.739 ± 0.211

LOQ = limit of quantification, 0.01 mg/L Source: p.34 of Kennel, 2015b

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 (Kennel, 2015b).

(c) Special studies on steroidogenesis

In an uterotrophic assay, groups of six immature (19-day-old) female Sprague Dawley rats were given tetraniliprole (purity 96.0%) at a concentration of 0, 100, 400 or 800 mg/kg bw per day in 0.5% aqueous methylcellulose 400 for three days. Estradiol benzoate was used as a positive control (at the three-day dosing only) for the induction of a uterotrophic response. Subsquently, groups of six immature female Sprague Dawley rats were given tetraniliprole (purity 96.0%) at a concentration of 0 or 600 mg/kg bw per day in 0.5% aqueous methylcellulose 400 for 20 days. The purpose of this study was to investigate the potential of tetraniliprole to interfere with uterine grown and/or vaginal opening (the three-day dosing) and the potential for anti-estrogenic effects. Animals were observed daily for clinical signs and checked twice each day for moribundity and mortality. Body weights were measured daily during the study. Vaginal opening was checked on day 4 of the treatment period and observations were recorded daily. For the groups designed to assess antiestrogenic activity, vaginal opening was recorded from day 10 until sacrifice on day 21. Uterine weights were recorded 24 hours after the end of the dosing period.

Under the conditions of the study, there were no effects on vaginal opening or uterine weight. The reliability of the antiestrogenic study is unclear due to the lack of a positive control group and the small group numbers (Blanck, 2016).

In a steroidogenesis assay, tetraniliprole and its main mammalian metabolite, BCS-CO63359, were evaluated for their effects on progesterone, testosterone, estradiol and cortisol secretion using the H295R cell line. Concentrations of test article were 0.1, 0.3, 1, 3, 10, 12, or 15 µM for tetraniliprole and 0.1, 0.3, 1, 2, 4, 8, or 12 µM for BCS-CQ63359, both in 0.1% DMSO. Forskolin (1 µM) and DMSO (0.1%) were employed respectively as positive and vehicle controls.

There were clear increases in estradiol and cortisol levels with tetraniliprole treatments at 3 µM and above. There were also slight increases in progesterone and testosterone levels at the same doses, but these did not show a dose–response relationship. With the metabolite, increases occurred in estradiol and cortisol at 1 µM and testosterone was increased at 8 µM. There were no apparent effects on progesterone. For all of the hormones tested increases were much lower when treated with tetraniliprole or its metabolite than with the positive control (Table 22; Tinwell, 2016).

Table 22. Steroidogenesis screen results

		Mean concentration (pg/mL) [% of control]			
Compound	**Concentration (μM)**	**Progesterone**	**Testosterone**	**Estradiol**	**Cortisol**
Tetraniliprole	0	3296	6757	411	33376
	0.1	3419 [104]	7056 [104]	393 [96]	40296 [121]
	0.3	3383 [103]	7509 [111]	400 [97]	45104 [135]
	1	3598 [109]	7550 [112]	392 [95]	44452 [133]
	3	4536 [138]	8921 [132]	563 [137]	62380 [187]
	10	4808 [146]	8998 [133]	809 [197]	107048 [321]
	12	4587 [139]	8423 [125]	853 [208]	112002 [336]
	15	4318 [131]	8019 [119]	927 [226]	109651 [329]
BCS-CQ63359	0	3234	7472	359	38913
	0.1	3062 [95]	6846 [92]	360 [100]	38105 [100]
	0.3	3166 [98]	7210 [96]	407 [114]	42176 [110]
	1	3736 [116]	8127 [109]	510 [142]	57846 [151]
	2	4247 [131]	9139 [122]	667 [156]	75703 [198]
	4	4028 [125]	8777 [117]	734 [204]	No data[a]
	8	3230 [100]	10299 [138]	778 [217]	65439 [171]
	12	3640 [82]	10839 [145]	649 [181]	46000 [120]
DMSO	0	3351	7396	402	31634
Forskolin	1	5210 [155]	11000 [149]	6398 [1591]	152233 [481]

[a] All values were off-curve high; Source: p.7 of Tinwell, 2016

Based on the study results tetraniliprole caused increased estradiol and cortisol secretion at concentrations of 3 μM and greater, as well as marginally increased testosterone and progesterone secretion at concentrations of 3 μM and greater. It was further determined that the primary mammalian metabolite, BCS-CQ63359 caused increased estradiol and cortisol secretion at concentrations of 1 μM and greater, as well as marginally increased testosterone secretion at concentrations of 8 μM and greater.

2.6 Special studies

(a) Immunotoxicity

No studies were submitted.

(b) Neurotoxicity

No studies were submitted.

(c) Studies on metabolites

The acute toxicity and genotoxicity of metabolite BCS-CR74541 (a carboxylic acid metabolite found in soil), the acute toxicity of metabolite BCS-CU81055 (the desmethyl-amide-carboxylic acid metabolite found in soil), the genotoxicity of metabolite BCS-CT30673 (the *N*-methyl-quinazolinone-carboxylic acid metabolite, found in soil) and the genotoxicity of metabolite BCS-CU81056 (the quinazolinone-carboxylic acid metabolite found in soil) are summarized in Tables 23 and 24.

Table 23. Acute toxicity study on metabolites of tetraniliprole

Test substance	Test system	Route	Purity	Results	Reference
BCS-CR74541	Rat, Wistar: female	Oral	93.4%	LD_{50} > 2000 mg/kg bw	Nagy, 2014a
BCS-CU81055	Rat, Wistar: female	Oral	95.2%	LD50 > 2000 mg/kg bw	Nagy, 2014b

Table 24. Genotoxicity study on metabolites of tetraniliprole

Test substance	End-point	Test object	Concentration	Purity (%)	Results	Reference
			In vitro			
BCS-CR74541	Reverse mutation	*Salmonella typhimurium*	3–5000 µg/plate (± S9)	95.3	Negative	Sokolowski, 2012
BCS-CR74541	Gene mutation	Chinese hamster lung (V79) cells	39.1–1250 µg/mL (+S9, 4 h) 78.1–1250 µg/mL (–S9, 4 h) 39.1–1250 µg/mL (–S9, 24 h)	95.3	Negative	Wollny, 2013b
BCS-CR74541	Chomosomal aberration	Chinese hamster lung (V79) cells	19.5–5000 µg/mL (± S9)	95.3	Negative	Bohnenberger, 2013b
BCS-CT30673	Reverse mutation	*Salmonella typhimurium*	3–5000 µg/plate (± S9)	96.9	Negative	Sokolowski, 2015a
BCS-CT30673	Gene mutation	Chinese hamster lung (V79) cells	15.9–508 µg/mL (± S9, 4 h) 31.8–1016 µg/mL (+S9, 4 h) 31.8–762 µg/mL (-S9, 24 h)	96.9	Negative	Wollny, 2015a
BCS-CT30673	Chomosomal aberration	Chinese hamster lung (V79) cells	7.9–2031 µg/mL (± S9)	96.9	Negative	Sokolowski, 2015b
BCS-CU81055	Reverse mutation	*Salmonella typhimurium*	3–5000 µg/plate (± S9)	99.4	Negative	Sokolowski, 2013b
BCS-CU81055	Gene mutation	Chinese hamster lung (V79) cells	93.8–3000 µg/mL	99.4	Negative	Wollny, 2013c
BCS-CU81055	Chomosomal aberration	Chinese hamster lung (V79) cells	11.7–3000 µg/mL (± S9, 4 h) 1500–5000 µg/mL (-S9, 4 h) 187.5–5000 µg/mL (+S9, 4 h) 1500–5000 µg/mL (+S9, 4 h)	99.4	Negative	Bohnenberger, 2014
BCS-CU81056	Reverse mutation	*Salmonella typhimurium*	3–5000 µg/mL (± S9)	95.4	Negative	Sokolowski, 2015c
BCS-CU81056	Gene mutation	Chinese hamster lung (V79) cells	9.7–310 µg/mL (± S9, 4 and 24 h)	95.4	Negative	Wollny, 2015b
BCS-CU81056	Chomosomal aberration	Chinese hamster lung (V79) cells	9.7–2470 µg/mL (± S9, 4 h) 9.7–617.5 µg/mL (+S9, 4 h) 9.7–2470 µg/mL (-S9, 18h)	95.4	Negative	Sokolowski, 2015d

In a 28-day toxicity study, groups of 10 Wistar rats received BCS-CR74541 (the carboxylic acid metabolite, purity 93.4%) in the diet at a concentration of 0, 900, 3000 or 10 000 ppm (equal to 0, 69, 233 and 775 mg/kg bw per day for males, 0, 72, 266 and 884 mg/kg bw per day for females). Animals were inspected daily for mortality, moribundity and clinical signs of toxicity Detailed clinical examinations were performed weekly and a neurological assessment was performed during week 4. An ophthalmological examination was performed during acclimatization and in week 4. Body weight and body weight gain were measured weekly and feed consumption was measured twice weekly. Haematological, clinical chemistry and urinalysis parameters were analysed from samples taken prior to terminal kill. All animals were necropsied, selected organs weighed, and a range of tissues collected, fixed and examined microscopically.

The NOAEL was 10 000 ppm (equal to 775 mg/kg bw per day), the highest dose tested (Totis, 2015).

In a 28-day toxicity study, groups of 10 Wistar rats received BCS-CU81055 (the desmethyl-amide-carboxylic acid metabolite, purity 95.2%) in the diet at a concentration of 0, 900, 3000 or 10 000 ppm (equal to 0, 69, 235 or 768 mg/kg bw per day for males, 0, 80, 269 or 845 mg/kg bw per

day for females). Animals were inspected daily for mortality, moribundity and clinical signs of toxicity Detailed clinical examinations were performed weekly and a neurological assessment was performed during week 4. An ophthalmological examination was performed during acclimatization and in week 4. Body weight and body weight gain were measured weekly and feed consumption measured twice weekly. Haematological, clinical chemistry and urinalysis parameters were analysed from samples taken prior to terminal kill. All animals were necropsied, selected organs weighed, and a range of tissues collected, fixed and examined microscopically.

The NOAEL was 10 000 ppm (equal to 768 mg/kg bw per day), the highest dose tested (Muhamedi, 2016).

3. Observations in humans

The Sponsor attested that during the development of tetraniliprole technical in laboratories and in small batch synthesis for a few months each year, no product-related adverse health effects had been observed or reported. No accidents with tetraniliprole technical have occurred and there have been no consultations of their Medical Department due to work or contact with tetraniliprole technical. Occupational medical surveillance of workers exposed to tetraniliprole technical, performed annually on a routine basis, had not revealed any unwanted effects amongst the workers (Steffens, 2019).

Comments

Biochemical aspects

Absorption, distribution, metabolism, excretion (ADME) studies were conducted in rats, using tetraniliprole labelled with ^{14}C at the pyrazole-carboxamide, pyridinyl-2, phenyl-carbamoyl or tetrazolyl positions. The absorption of tetraniliprole at a low dose of 2 mg/kg body weight (bw) was rapid (T_{max} < 1–7 hours), approximately 30–46% (based on bile, urine and carcass), and radioactivity was widely distributed to the tissues. The values for the area under the concentration–time curve for the interval 0 to 168 hours ($AUC_{0–168}$) in females were approximately double those for males: 1.21 g × hours/g in males and 2.36 g × hours/g in females. Absorption at 20 and 200 mg/kg bw, the mid- and high-dose levels, was very limited.

Elimination was rapid and nearly complete by 72 hours post dose. The half-life of elimination was slightly longer (less than two-fold) in males than in females at the low-dose level. Very low to nondetectable levels of radioactivity at the mid and high doses meant that the half-life could not be calculated for comparison. Elimination was predominantly via the faeces, bile being the secondary route with up to 39% of the administered radioactivity recovered in the bile. Radioactivity recovered in the bile and faeces accounted for 96–98% of the administered dose (AD). In proportional terms the highest levels of radioactivity recovered in the urine were at the low dose, with 7% of the AD based on the results from the pyridinyl labelled groups. In the mid- and high-dose groups, urinary excretion was negligible, suggesting a decrease in absorption with increased dose. There were no changes following repeat-dose treatment in males.

Seventy-two hours following dosing, trace amounts of radioactivity were found in the tissues, with higher concentrations in females than males. The highest concentrations were found in the liver, followed by the skin, with measurable amounts in the kidneys and in the ovaries and uterus of females. Relative residual concentrations were higher in the low-dose groups than in the high-dose groups (Bongartz & Miebach, 2016a).

A quantitative whole-body autoradiography study was performed and this confirmed the results of the main study. Absorption was rapid, though limited, with small amounts of radioactivity distributed to the liver and kidney and some distribution to the glandular organs and fatty tissues. There were no substantial sex-related differences (Koester, 2015).

Unchanged tetraniliprole was found in both the urine and faeces, though not the bile, and comprised 51–70% of the AD in the low-dose groups and over 90% of the AD in the mid- and high-dose groups. Hydroxylation was favoured in the males, however the metabolite profiles were essentially similar in

males and females. Other major metabolites comprised 1–9% of the AD and included the tetraniliprole-deshydrochloro-dihydrate, dihydroxy and hydroxy-*N*-methyl metabolites. Metabolites making up 1–4% of the AD included the benzyl alcohol glucuronide, hydroxypyridyl glucuronide, deschloro-desmethyl-amide-dihydroxy, despyridyl, benzylalcohol, pyridinyl-pyrazole-5-carboxylic acid, and hydroxypyridine derivatives of tetraniliprole. The remaining identified metabolites accounted for most of the metabolites, and were present in quantities equal to or less than 2.5% of the administered dose.

The proposed metabolic pathway in rats involves hydroxylation in several positions including the pyridinyl moiety, the *N*-methyl moiety, and the methyl group of the phenyl moiety. Conjugation with glucuronic acid takes place following hydroxylation. Intramolecular condensation (cyclization) of the parent molecule also occurred, yielding quinazolinone compounds, one of which was identified as BCSCQ63359 (tetraniliprole-*N*-methyl-quinazolinone), a primary plasma metabolite. Also noted was cleavage of the phenyl moiety yielding an amide, followed by oxidation or methylation and further cleavage reactions involving the pyridine and tetrazole rings (Bongartz & Miebach, 2016a; Koester, 2015).

There were no significant differences in tissue distribution, biokinetics or metabolite profile due to the label position (Bongartz & Miebach, 2016b, c, d).

In several toxicity studies, the parent compound was measured in plasma. Concentrations were higher in females than in males. At the highest dose tested in the two-generation rat study, parental plasma concentrations were 1.7 mg/L, which is equivalent to around 3 µM (Odin, 2016c).

A series of in vitro liver microsome assays on [*pyrazole-carboxamide*-^{14}C]tetraniliprole, [*pyridinyl-2*-^{14}C]tetraniliprole and [*tetrazolyl-4*-^{14}C]tetraniliprole at concentrations of 10 µM indicated that there were no metabolites unique to humans detected. Metabolism in the rat and the male dog microsome assays were the lowest with 91–100% of the administered parent compound found in rat assays and 91–97% of the administered parent compound found in the male dog assays. Unchanged parent was detected at 88–94% of the starting concentrations in the case of the male rabbit microsomes. In the mouse microsome study, unchanged parent compound represented 73–96% of the relative percentage, and in the human assays unchanged parent amounted to 74–87% of the starting concentration. Of the five metabolites identified and labelled as BCS-1 through BCS-5, only BCS-03 occurred at relative percentages greater than 5%. Metabolite BCS-3 occurred at relative percentages between 13% and 25% in the mouse microsome assays, and at 11% to 21% in human microsome assays, while relative percentages were in the range of 1–7% in rats, male dogs and male rabbits. Metabolite BCS-4 was found in mouse, male rabbit and human microsome assays at between 0.5% and 2% and BCS-2 was found in the male rabbit and human microsome assays only. There were no substantial differences due to labelling position (Mura, 2015a, b; Sola, 2015).

Toxicological data

In rats, tetraniliprole had an acute oral and dermal median lethal dose (LD_{50}) greater than 2000 mg/kg body weight (bw) and an acute inhalation median lethal concentration (LC_{50}) greater than 5.01 mg/L (Matting, 2013a, b, 2014; Nagy, 2013). Tetraniliprole was minimally irritating to the skin and transiently irritating to the eyes of rabbits (Matting, 2013c, d) Tetraniliprole was a dermal sensitizer in the local lymph node assay (LLNA) in mice (Hargaitai, 2013; Varga-Kanizsai, 2016).

The rat was the species most sensitive to tetraniliprole, with decreased body weights and effects on the ovaries, uterus and sexual development in females occurring at or above the limit dose.

In a 28-day toxicity study, mice received tetraniliprole in the diet at a concentration of 0, 600, 3000 or 6000 ppm (equal to 0, 100, 523 and 1010 mg/kg bw per day for males, 0, 113, 576 and 1159 mg/kg bw per day for females). The NOAEL was 6000 ppm (equal to 1010 mg/kg bw per day), the highest dose tested (Blanck, 2011).

In a 90-day toxicity study, mice received tetraniliprole in the diet at a concentration of 0, 900, 2700 or 6000 ppm (equal to 0, 145, 426 or 973 mg/kg bw per day for males, 0, 180, 544 or 1224 mg/kg bw per day for females). The NOAEL was 6000 ppm (equal to 973 mg/kg bw per day), the highest dose tested (Odin, 2016a).

In a 28-day toxicity study, rats received tetraniliprole dermally at a concentration of 0, 100, 300 or 1000 mg/kg bw per day for six hours per day. The NOAEL for dermal toxicity was 1000 mg/kg bw per day, the highest dose tested (Toeroek-Bathó, 2015).

In a 28-day toxicity study, rats received tetraniliprole in the diet at a concentration of 0, 500, 2000 or 8000 ppm (equal to 0, 38, 148 and 599 mg/kg bw per day for males, 0, 43, 171 or 700 mg/kg bw per day for females). The NOAEL was 8000 ppm (equal to 599 mg/kg bw per day), the highest dose tested (Lasserre, 2011).

In a 90-day toxicity study, rats received tetraniliprole in the diet at a concentration of 0, 900, 3000 or 10 000 ppm (equal to 0, 55.5, 178 and 608 mg/kg bw per day for males, 0, 65.7, 213 and 723 mg/kg bw per day for females). The NOAEL was 10 000 ppm (equal to 608 mg/kg bw per day), the highest dose tested (Odin, 2016b).

In a 90-day toxicity study, dogs received tetraniliprole in the diet at a concentration of 0, 800, 3200 or 12 800 ppm (equal to 0, 25.6, 126 or 440 mg/kg bw per day for males, 0, 29.9, 138 or 485 mg/kg bw per day for females). The NOAEL was 12 800 ppm (equal to 440 mg/kg bw per day), the highest dose tested (Kennel, 2015a).

In a one-year toxicity study, dogs received tetraniliprole in the diet at a concentration of 0, 650, 2900 or 12 800 ppm (equal to 0, 19.8, 91.2 and 440 mg/kg bw per day for males, 0, 18.3, 88.8 or 408 mg/kg bw per day for females). The NOAEL was 12 800 ppm (equal to 408 mg/kg bw per day), the highest dose tested (Kennel, 2016a).

In a 19-month toxicity and carcinogenicity study, mice received tetraniliprole in the diet at a concentration of 0, 260, 1300 or 6500 ppm (equal to 0, 32.9, 166 and 825 mg/kg bw per day for males, 0, 43.1, 225 and 1073 mg/kg bw per day for females). The NOAEL was 6500 ppm (equal to 825 mg/kg bw per day), the highest dose tested. No tumours were observed that were considered to be related to treatment with tetraniliprole (Kennel, 2016b).

In a two-year chronic toxicity and carcinogenicity study, rats received tetraniliprole in the diet at a concentration of 0, 900, 4000 or 18 000 ppm (equal to 0, 35.3, 159 and 741 mg/kg bw per day for males, 0, 51.2, 221, and 1052 mg/kg bw per day for females). The NOAEL was 4000 ppm (equal to 221 mg/kg bw per day) based on decreased body weight, increased diffuse squamous cell hyperplasia in the cervix and vagina and increased severity of corpora lutea depletion at the LOAEL of 18 000 ppm (equal to 1052 mg/kg bw per day). No tumours were observed that were considered to be related to treatment with tetraniliprole (Odin, 2016c).

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

Tetraniliprole was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found.

The Meeting concluded that tetraniliprole 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 tetraniliprole is unlikely to pose a carcinogenic risk to humans.

In a two-generation reproductive toxicity study, rats received a dietary concentration of tetraniliprole of 0, 300, 600, 2700 or 12 000 ppm (equal to 0, 22, 44, 196 and 896 mg/kg bw per day in males, 0, 25, 41, 224 and 1032 mg/kg bw per day in females). In the lactation phase, dietary concentrations in females were decreased to 0, 150, 300, 1350 and 6000 ppm (equal to 0, 23, 47, 211 and 890 mg/kg bw per day). The NOAEL for parental toxicity was 2700 ppm (equal to 196 mg/kg bw per day), based on decreased body weights in F1 animals at the LOAEL of 12 000 ppm (equal to 896 mg/kg bw per day). The NOAEL for reproductive toxicity was 12 000 ppm (equal to 896 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 2700 ppm (equal to 196 mg/kg bw per day), based on decreased pup body weights resulting in delayed completion of vaginal opening at the LOAEL of 12 000 ppm (equal to 896 mg/kg bw per day) (Patten, 2016).

In a developmental toxicity study, female rats received a gavage dose of tetraniliprole at concentrations of 0, 62.5, 250 or 1000 mg/kg bw per day mixed in 0.5% aqueous sodium methylcellulose 400, from days 6–20 of gestation. The NOAEL for maternal toxicity

was 1000 mg/kg bw per day, the highest dose tested. The NOAEL for embryo/fetal toxicity was 250 mg/kg bw per day, based on lack of ossification in the fifth or sixth sternebrae at the LOAEL of 1000 mg/kg bw per day (Kennel, 2014).

In a developmental toxicity study, premated rabbits were given tetraniliprole in 0.5% aqueous sodium methylcellulose 400 at a concentration of 0, 62.5, 250 or 1000 mg/kg bw per day from days 6–28 of gestation. The NOAELs for maternal and embryo/fetal toxicity were both 1000 mg/kg bw per day, the highest dose tested (Kennel, 2015b).

In an uterotrophic assay, groups of six immature (19-day-old) female Sprague Dawley rats were given tetraniliprole in 0.5% aqueous methylcellulose 400 at a concentration of 0, 100, 400 or 800 mg/kg bw per day for three days. There were no effects on vaginal opening or uterine weight. The reliability of the study is unclear due to the lack of a positive control group in the antiestrogenic assay and the small group numbers (Blanck, 2016).

In a steroidogenesis assay, tetraniliprole and its main mammalian metabolite BCS-CQ63359, were evaluated for their effects on progesterone, testosterone, estradiol and cortisol secretion using the H295R cell line. Concentrations tested were 0.1, 0.3, 1, 3, 10, 12 and 15 µM for tetraniliprole and 0.1, 0.3, 1, 2, 4, 8, and 12 µM for BCS-CQ63359, all presented in 0.1% dimethyl sulfoxide (DMSO). There were clear increases in estradiol and cortisol levels with the tetraniliprole treatments at and above 3 µM. There were slight increases in progesterone and testosterone levels at the same doses, but these did not show a dose–response relationship. In the case of the metabolite, increases occurred in estradiol and cortisol at 1 µM, and testosterone was increased at 8 µM. There were no apparent effects on progesterone. Increases seen in any of the hormones were much lower when treated with tetraniliprole and its metabolite than with the positive control (Tinwell, 2016).

The Meeting concluded that tetraniliprole is not teratogenic.

No specific studies on neurotoxicity were submitted. No evidence of neurotoxicity was reported in routine toxicological studies with tetraniliprole.

The Meeting concluded that tetraniliprole was unlikely to be neurotoxic.

No specific studies on immunotoxicity were submitted. No evidence of immunotoxicity was reported in routine toxicological studies with tetraniliprole.

The Meeting concluded that tetraniliprole was unlikely to be immunotoxic.

Toxicological data on metabolites and/or degradates

BCS-CR74541 (tetraniliprole-carboxylic acid)

The acute oral LD_{50} of BCS-CR74541, a soil metabolite, was greater than 2000 mg/kg bw (Nagy, 2014a) and it tested negative in reverse mutation, gene mutation and chromosomal aberration assays (Sokolowski, 2012; Wollny, 2013b; Bohnenberger, 2013b). In a 28-day dietary toxicity study in rats, the NOAEL was 10 000 ppm (equal to 775 mg/kg bw per day), the highest dose tested (Totis, 2015). BCS-CR74541 was considered to be covered by the toxicity of the parent compound.

BCS-CU81055 (tetraniliprole-desmethyl-amide-carboxylic acid)

The acute oral LD_{50} of BCS-CU81055, another soil metabolite, was greater than 2000 mg/kg bw (Nagy, 2014b) , and it tested negative in mutation, gene mutation and chromosomal aberration assays (Sokolowski, 2013b; Wollny, 2013c; Bohnenberger, 2014). In a 28-day dietary toxicity study in rats, the NOAEL was 10 000 ppm (equal to 768 mg/kg bw per day), the highest dose tested (Muhamedi, 2016). BCS-CU81055 was considered to be covered by the toxicity of the parent compound.

BCS-CT30673 (tetraniliprole-N-methyl-quinazolinone-carboxylic acid)

BCS-CT30673, a third soil metabolite, tested negative in mutation, gene mutation and chromosomal aberration assays (Sokolowski, 2015a, c; Wollny, 2015). BCS-CT30673 was considered to be covered by the toxicity of the parent compound.

BCS-CU81056 (tetraniliprole-quinazolinone-carboxylic acid)

BCS-CU81056, a fourth soil metabolite, tested negative in mutation, gene mutation and chromosomal aberration assays (Sokolowski, 2015d, e; Wollny, 2015b). BCS-CU81056 was considered to be covered by the toxicity of the parent compound.

BCS-CQ63359 (tetraniliprole-N-methyl-quinazolinone amide)

BCS-CQ63359 was the major plasma metabolite; it gave rise to no alerts for genotoxicity following QSAR analysis using OECD QSAR ToolBox, Version 4.5. In addition it is of similar structure to the parent. BCS-CQ63359 was considered to be covered by the toxicity of the parent compound.

BCS-CZ91631 (tetraniliprole-benzyl alcohol)

BCS-CZ91631 produced no alerts for genotoxicity following QSAR analysis using OECD QSAR ToolBox, Version 4.5. In addition it is of similar structure to the parent. BCS-CZ91631 was considered to be covered by the toxicity of the parent compound.

Tetraniliprole-despyridyl-N-methyl-quinazolinone

Tetraniliprole-despyridyl-*N*-methyl-quinazolinone gave rise to alerts for genotoxicity using OECD QSAR ToolBox, Version 4.5. This metabolite is not covered by the toxicity of the parent. It is a Cramer class III compound and a TTC of 0.002 5 µg/kg bw per day was recommended.

Microbiological data

No data for antimicrobial activity or impact of tetraniliprole on the human gut microbiome are available.

Human data

In reports on manufacturing plant personnel, no adverse health effects had been noted (Steffens, 2019).

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

Toxicological evaluation

The Meeting established an ADI for tetraniliprole of 0–2 mg/kg bw per day, from the two-year dietary toxicity study in rats and the two-generation reproductive toxicity study in rats, on the basis of the NOAEL of 221 mg/kg bw per day in the two-year rat study, and 196 mg/kg bw per day for offspring in the two-generation rat reproductive toxicity study. Findings at the LOAEL consisted of decreased body weight and changes to cervix, vagina and ovaries in the two-year study, and decreased body weight resulting in delayed vaginal opening in the two-generation study. A safety factor of 100 was applied.

The Meeting concluded that it was not necessary to establish an ARfD for tetraniliprole 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 tetraniliprole

Species	Study	Effect	NOAEL	LOAEL
Mouse	28-day oral toxicity study[a]	Toxicity	6000 ppm, equal to 1010 mg/kg bw per day	-
	90-day oral toxicity study[a]	Toxicity	6000 ppm, equal to 973 mg/kg bw per day[b]	-
	Eighteen-month study of carcinogenicity[a]	Toxicity	6500 ppm, equal to 825 mg/kg bw per day[b]	-
Rat	28-day oral toxicity study[a]	Toxicity	8000 ppm, equal to 599 mg/kg bw per day[b]	-
	90-day oral toxicity study[a]	Toxicity	10 000 ppm, equal to 608 mg/kg bw per day[b]	-
	Two-year study of toxicity and oncogenicity[a]	Toxicity	4000 ppm, equal to 221 mg/kg bw per day (females)	1052 mg/kg bw per day (females)
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	12 000 ppm, equal to 896 mg/kg bw per day[b]	-
		Parental toxicity	12 000 ppm, equal to 896 mg/kg bw per day[b]	-
		Offspring toxicity	2700 ppm, equal to 196 mg/kg bw per day	12 000 ppm, equal to 896 mg/kg bw per day
	Developmental toxicity study[c]	Maternal toxicity	1000 mg/kg bw per day[b]	-
		Embryo/fetal toxicity	250 mg/kg bw per day	1000 mg/kg bw per day
Rabbit	Developmental toxicity study[c]	Maternal toxicity	1000 mg/kg bw per day[b]	-
		Embryo/fetal toxicity	1000 mg/kg bw per day[b]	-
Dog	90-day study of toxicity[a]	Toxicity	12 800 ppm, equal to 440 mg/kg bw per day[b]	-
	One-year study of toxicity[a]	Toxicity	12 800 ppm, equal to 408 mg/kg bw per day[b]	-
Metabolite BCS-CR74541				
Rat	28-day study of toxicity[a]	Toxicity	10 000 ppm, equal to 755 mg/kg bw per day[b]	-
Metabolite BCS-CU81055				
Rat	28-day study of toxicity[a]	Toxicity	10 000 ppm, equal to 755 mg/kg bw per day[b]	-

[a] Dietary administration [b] Highest dose tested [c] Gavage administration
[d] Two or more studies combined [e] Lowest dose tested

Acceptable daily intake (ADI), applies to tetraniliprole, BCS-CQ63359 and BCS-CZ91631 expressed as tetraniliprole

0–2 mg/kg bw

Acute reference dose (ARfD), applies to tetraniliprole, BCS-CQ63359 and BCS-CZ91631 expressed as tetraniliprole

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 tetraniliprole

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid: T_{max} = 1 hour for all doses, except 20 mg/kg bw in females, where T_{max} = 7 hours Oral absorption: 46% (male), 30% (female), based on bile, urine and carcass (rat)
Dermal absorption	No data
Distribution	Extensive; highest concentrations in liver and kidneys
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	Relatively rapid; (complete within 48–72 hours in rats, mainly in faeces)
Metabolism in animals	Major metabolic reactions: demethylation of the *N*-methyl group and deschlorodination in the pyridine ring, two separate conjugations of tetraniliprole (after deschlorodination) with glutathione occurred, followed by degradation of one of the glutathione groups to the mercapto-alcohol (rat)
Toxicologically significant compounds in animals and plants	Tetraniliprole, BCS-CQ63359, BCS-CZ91631
Acute toxicity	
Rat, LD_{50}, oral	> 2000 mg/kg bw
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.01 mg/L
Rabbit, dermal irritation	Minimally irritating
Rabbit, ocular irritation	Minimally irritating
Mouse, dermal sensitization	Sensitizer (LLNA)
Short-term studies of toxicity	
Target/critical effect	Decreased body weight/body weight gain (dog)
Lowest relevant oral NOAEL	408 mg/kg bw per day, the highest dose tested (dog)
Lowest relevant dermal NOAEL	1000 mg/kg bw per day, the highest dose tested (rat)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Decreased body weight, prolapsed vagina, squamous cell hyperplasia of cervix/vagina, increased severity of corpora lutea depletion (rat)
Lowest relevant NOAEL	221 mg/kg bw per day (female rat)
Carcinogenicity	No evidence of carcinogenicity[a]
Genotoxicity	Unlikely to be genotoxic[a]
Reproductive toxicity	
Target/critical effect	Decreased pup body weight resulting in delayed vaginal opening
Lowest relevant parental NOAEL	896 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	196 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	896 mg/kg bw per day, the highest dose tested (rat)

(*Continued on next page*)`

Developmental toxicity	
Target/critical effect	Unossified fifth or sixth sternebrae
Lowest relevant maternal NOAEL	1000 mg/kg bw per day, the highest dose tested (rat)
Lowest relevant embryo/fetal NOAEL	250 mg/kg bw per day (rat)
Neurotoxicity	
Acute neurotoxicity NOAEL	No evidence of neurotoxicity
Subchronic neurotoxicity NOAEL	No data
Developmental neurotoxicity NOAEL	No data
Immunotoxicity	No data
Studies on toxicologically relevant metabolites	
BCS-CR74541	Acute oral LD_{50}: >2000 mg/kg bw (rat) 28-day NOAEL: 775 mg/kg bw per day, highest dose tested (rat) Not genotoxic: mutation, gene mutation and chromosomal aberration
BCS-CU81055	Acute oral LD_{50}: >2000 mg/kg bw (rat) 28-day NOAEL: 768 mg/kg bw per day, highest dose tested (rat) Not genotoxic: mutation, gene mutation and chromosomal aberration
BCS-CT30673	Not genotoxic: mutation, gene mutation and chromosomal aberration
BCS-CU81056	Not genotoxic: mutation, gene mutation and chromosomal aberration
Microbiological data	No data
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	0–2 mg/kg bw[a]	Two-year toxicity (rat) and two-generation reproductive toxicity (rat)	100
ARfD	Not necessary		

[a] Applies to tetraniliprole, BCS-CQ63359 and BCS-CZ91631 expressed as tetraniliprole

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