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Prepared by the ninety-fourth meeting of the
Joint FAO/WHO Expert Committee
on Food Additives (JECFA)

Toxicological evaluation of certain veterinary drug residues in food

Food and Agriculture Organization of the United Nations

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PREFACE

The monographs contained in this volume were prepared at the ninety-fourth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met online in a virtual format on 16–27 May 2022. These monographs summarize the data on the safety of residues in food of selected veterinary drugs reviewed by the Committee.

The ninety-fourth report of JECFA has been published by WHO as WHO Technical Report No. 1041. Reports and other documents resulting from previous meetings of JECFA are listed in Annex 1. The participants in the meeting are listed in Annex 3 of the present publication. A summary of the conclusions of the Committee is given in Annex 4.

JECFA serves as a scientific advisory body to FAO, WHO, their Member States and the Codex Alimentarius Commission, primarily through the Codex Committee on Food Additives, the Codex Committee on Contaminants in Food and the Codex Committee on Residues of Veterinary Drugs in Foods, regarding the safety of food additives, residues of veterinary drugs, naturally occurring toxicants and contaminants in food. Committees accomplish this task by preparing reports of their meetings and publishing specifications or residue monographs and toxicological monographs, such as those contained in this volume, on substances that they have considered.

The toxicological monographs contained in this volume are based on working papers that were prepared by WHO experts. A special acknowledgement is given at the beginning of each monograph to those who prepared these working papers. The monographs were edited by Dr Russell Parry, Shrewsbury, United Kingdom of Great Britain and Northern Ireland. Toxicological monographs were not prepared for all of the substances listed in Annex 4.

Many unpublished proprietary reports are submitted to the Committee by various producers of the veterinary drugs under review and in many cases represent the only data available on those substances. The WHO experts based the working papers they wrote on all the data that were submitted, and all these reports were available to the Committee when it made its evaluations.

The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of the World Health Organization (WHO) concerning the legal status of any country, territory, city or area or its authorities, or concerning the delimitation of its frontiers or boundaries. The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by the WHO in preference to others of a similar nature that are not mentioned.

Any comments or new information on the biological or toxicological properties of the compounds evaluated in this publication should be addressed to: Joint WHO Secretary of the Joint FAO/WHO Expert Committee on Food Additives, Department of Nutrition and Food Safety (NFS), World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland.

RESIDUES OF VETERINARY DRUGS

Imidacloprid

First draft prepared by

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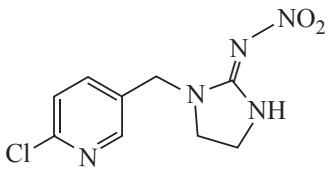
1. Explanation

Imidacloprid is the ISO-approved common name for (*E*)-1-(6-chloro-3-pyridinylmethyl)-*N*-nitroimidazolidin-2-ylideneamine (IUPAC), for which the Chemical Abstracts Service Number is 138261-41-3; see Fig. 1).

Figure 1
Structure of imidacloprid



It is a chloronicotinyl compound belonging to the neonicotinoid group, and it is used as a systemic insecticide. Its insecticidal effects are exerted on the central nervous system (CNS) by causing prolonged activation and desensitization of the postsynaptic insect nicotinic cholinergic receptor, interfering with the transmission of nerve impulses, which results in insects' paralysis and consequent death (Casida, 1998). The selective toxicity of imidacloprid to target species is attributed to differences in the binding affinity or potency at the nicotinic acetylcholine receptor (nAChR) compared to mammals (Chao & Casida, 1997). Specifically, imidacloprid binds avidly to insect nAChR, and relatively poorly to mammalian nAChRs, including the major neuronal subtypes α4β2 and α7. By comparison, nicotine itself binds poorly to insect nAChR and more avidly to mammalian nAChRs (Matsuda et al., 2001). Moreover, whereas nicotine penetrates the blood–brain barrier easily in mammals, imidacloprid has poor penetration. Thus, imidacloprid is much more toxic to insects than to mammals (Yamamoto et al., 1995; Chao & Casida 1997; Elbert, Nauen & Leicht, 1998; Tomizawa & Casida 2005).

Imidacloprid is used on a variety of agricultural commodities, to control sucking insects and some chewing insects (Gervais et al., 2010). It is also employed to protect seedlings from early-season pests that feed on roots and leaves, as well as in later season foliar treatments (US EPA, 2003; Morrissey et al., 2015). In residential environments, imidacloprid is applied to control grubs in lawns and as a termiticide (Sheets, 2014).

In veterinary medicine, imidacloprid is used primarily in dogs and cats to kill all life stages of fleas, and to prevent and control lice infestation on dogs. It is also used in combination with other medications to treat a range of parasites such as ticks, mosquitoes, chewing lice, heartworms, hookworms, roundworms, whipworms, scabies, cheyletiellosis, demodex and ear mites. Imidacloprid is

currently authorized in Norway for use in *Salmonidae* (which includes salmon and trout) being indicated for the treatment of pre-adult and adult salmon lice (*Lepeophtheirus salmonis*).

Imidacloprid was previously evaluated by Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 2001 (JMPR, 2002), and by other scientific or regulatory bodies: United States Environmental Protection Agency (U.S. EPA, 2003), California Environmental Protection Agency (CalEPA, 2006), European Food Safety Authority (EFSA, 2008, 2013) and the European Medicines Agency (EMA, 2021). However, imidacloprid had not previously been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The Committee evaluated imidacloprid at the 94th meeting at the request of the twenty-fifth session of the Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF) virtual meeting (12–20 July 2022) with the aim of assessing the health risk, establishing relevant health-based guidance values (HGBVs) and recommending maximum residue limits (MRLs) for fin fish, taken as muscle and skin in natural proportions.

For this assessment, a data package was submitted by the sponsor. Additionally, the following databases of published literature were searched using the search term:

"imidacloprid AND *toxic*/pharmacokinetics AND rats/rabbits/mice/humans/dogs/mammals".

Numbers of articles retrieved were as follows:
Aquaculture Compendium (69 articles retrieved), Agris (24), CAS (816), CAB abstracts (1122), Embase (626), FSTA (136), PubMed (362), Scopus (489) and WOS (328).

In total 3972 articles were retrieved, of which 2779 were removed as duplicates; the titles and abstracts of the remaining 1193 articles were screened to determine their relevance. Articles that investigated the pharmacodynamics, pharmacokinetics, short-term and long-term toxicity, genotoxicity, reproductive and developmental toxicity, or carcinogenicity of imidacloprid in laboratory animals or humans were considered pertinent to the current evaluation. Of these, 32 papers were considered relevant to this assessment. The following sources of information were also scrutinized and information considered relevant included in this assessment: evaluations from JMPR 2001, US EPA 2003, CalEPA 2006, EFSA 2008 and 2013, and the 2021 report from EMA (see all citations above). Many of the studies were not according to good laboratory practice (GLP) and where this was the case it has been indicated in this monograph.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution, and excretion

In a GLP-compliant study, imidacloprid (99.9% purity), labelled with ^{14}C in either the methylene or the imidazolidine ring, was administered in a physiological saline solution at concentrations of 0.1–2 mg/L to a total of 50 male and 20 female Wistar rats. Groups of rats (five of each sex) were given a single dose of 1 mg/kg body weight (bw) intravenously or were orally administered [^{14}C]imidacloprid as a single dose of 1 or 20 mg/kg bw. The oral dosing method was not specified. Another group (five of each sex) were administered unlabelled compound orally at a dose of 1 mg/kg bw per day for 14 days, then 24 hours after the final dose the animals were given orally a single dose of the [^{14}C]imidacloprid at 1 mg/kg bw. Radioactivity was determined in plasma and excreta as a function of time and after 48 hours the rats were sacrificed and total radioactivity measured in organs and tissues. An additional group of five male rats received orally a single dose of 20 mg/kg bw of [^{14}C]imidacloprid and the [^{14}C]CO_2 expired measured for 48 hours. A further study was performed with four groups of five male rats which were given orally a single dose of 20 mg/kg bw of [^{14}C]imidacloprid after which groups of rats were sacrificed after 40 minutes, one, five, three and six hours, and radioactivity determined in individual organs. In a supplementary assessment, a group of five bile duct-fistulated male rats were given a single intraduodenal dose of 1 mg/kg bw of radiolabelled imidacloprid, to examine the rate and extent of biliary excretion.

Following oral administration of 1 or 20 mg/kg bw of [^{14}C]imidacloprid, the radiolabel was extensively absorbed from the intestinal lumen and readily distributed from the plasma into the body. After intravenous and oral dosing, about 92% of the recovered radiolabel was excreted in urine and faeces within 48 hours. Most of the radiolabel was excreted via the kidneys, with an average ratio in urine: faeces of 4:1. More than 90% of the renal radiolabel was excreted during the 24 hours after dosage. No sex-related differences were observed, nor any due to route of administration or dose level. The average residual radiolabel in the body at sacrifice (not including the gastrointestinal tract), was about 0.5% of administered dose (AD), and that in the gastrointestinal tract was about 0.06% of AD. For both sexes, irrespective of route of administration or dose level, major sites for radioactive accumulation were liver, kidney, lung, skin, and plasma; minor sites were brain and testis. Time was not a significant factor when considering tissue distribution of radioactivity. The bile duct-fistulation experiment demonstrated that a major part of the faecal radioactivity originated in the bile since the rats with bile duct fistulas excreted only 4.7% of AD versus approximately 37% in the bile. These findings indicate the existence of

enterohepatic circulation of the radiolabel. The experiment examining expired air indicated that no significant amount of [^{14}C]CO_2 was expired by rats over 48 hours; this indicated that the labelling position of the molecule was stable in vivo. Elimination half-lives, calculated from two exponential terms, were 2.6–3.6 hours and 26–118 hours (Klein, 1987a).

ln a separate study employing whole-body autoradiography on X-ray film (GLP-compliant), male Wistar rats (number of animals not specified) were investigated 1, 4, 8, and 48 hours after oral administration, or five minutes after intravenous administration, in all cases administering 20 mg/kg of [*methylene*-^{14}C]imidacloprid.

The radiolabel was readily absorbed and rapidly distributed to the tissues and organs. The pattern of distribution showed that the radiolabel could readily permeate tissues. The relative tissue distribution did not change significantly over time, although the amount of radioactivity rapidly diminished. Estimated from the radiograph, major accumulations of radioactivity were detectable in the liver, kidney, adrenal, muscle, skin, walls of the aorta, stomach and small intestine, connective tissue attached to the spinal cord, and salivary, bulbourethral, and thyroid glands, within the first hour. Minor accumulation was noted in the lungs, fat, brain, testis and the mineral part of the bones. After 24 hours no radioactivity was observed in the stomach, but radioactivity was accumulating in the intestine. After 48 hours the only tissues above the detection limit were skin, nasal mucosa, liver, kidney, thyroid, walls of the aorta, and the connective tissue attached to the spinal cord. Overall the results of this qualitative experiment agreed with the quantitative tissue distribution studies (Klein, 1987b).

In a GLP-compliant study, male and female Wistar rats (number of animals not specified) were given oral doses of 1 mg/kg bw of imidacloprid labelled with ^{14}C at the 4 and 5 positions of the imidazolidine ring. Additionally, a group of male rats (number of animals not specified) were given a dose of 150 mg/kg bw, also orally. Radioactivity was determined in plasma and excreta and in organs and tissues at sacrifice 48 hours after administration. Absorption was rapid and distribution followed a similar pattern to that noted for the methylene-labelled compound described in the previous study (Klein, 1987b). Excretion was rapid and mainly by the renal route (Klein & Brauner, 1991).

A study (not GLP-compliant) was performed to assess the distribution of imidacloprid along with two of its metabolites: 6-chloronicotinic acid (6-CNA) and 6-hydroxynicotinic acid (6-HNA). Twenty female Wistar rats received by oral gavage, a single dose of 20 mg/kg bw imidacloprid (purity not specified) in corn oil. At each of 6, 12, 24 and 48 hours after administration five animals were necropsied. At each time point, blood and urine were collected from the five euthanized animals, and brain, liver, kidney and ovary were collected. The faeces were pooled for the periods 0–24 hours and 24–48 hours.

The maximum concentrations of imidacloprid, 6-CNA and 6-HNA were attained at 12 hours after dosing in both the organs (liver, brain, kidney, and ovary) and bodily fluids (blood, faeces, urine). However, the pattern of distribution of imidacloprid and its metabolites in different organs and bodily fluids varied. Maximum concentrations (C_{max}) of imidacloprid were found to be in the following order:

- For imidacloprid: urine > blood > brain > faeces > liver > kidney > ovary.
- For 6-CNA: urine > kidney > blood > faeces > brain > liver > ovary.
- For 6-HNA: urine > blood > faeces > kidney > liver > ovary > brain.

The areas under the concentration–time curve (AUC) for imidacloprid, 6-CNA and 6-HNA at 12 hours were in order as follows.
For imidacloprid: urine> blood > faeces > brain > liver > kidney > ovary.
For 6-CNA: urine > brain > blood > faeces > kidney > liver > ovary.
For 6-HNA: urine > blood > faeces > brain > liver > ovary > kidney.
(Kapoor et al., 2014)

2.1.2 Biotransformation

2.1.2.1 In vivo

(a) mouse

In a non-GLP study, C57BL/6J male mice (four per group) were exposed or not for 24 weeks to 0.6 mg/kg bw per day of imidacloprid via a powdered diet. At the end of the study mice were killed. At necropsy, blood, lung, liver, testis, brain, kidney, pancreas inguinal white adipose tissue, mesenteric white adipose tissue, and gonadal white adipose tissue, were collected and processed accordingly for chemical analysis. Sample analysis was carried out by liquid chromatography with tandem mass spectrometry (LC-MS/MS) using a triple quadrupole system. Imidacloprid and five of its metabolites were detected in mice. It was found that the brain, testis, lung, kidney, inguinal white adipose tissue, and gonadal white adipose tissue mainly accumulated parent imidacloprid. Blood and mesenteric white adipose tissue mainly accumulated imidacloprid-olefin. Liver mainly accumulated desnitro-imidacloprid. The pancreas predominately accumulated 4-hydroxy-imidacloprid. Tissue–blood concentration ratios recorded for the desnitrodehydro-imidacloprid and desnitro-imidacloprid metabolites were 1.0 or greater for testis, brain, lung, and kidney. The cumulative levels of the six detected imidacloprid compounds were found in the decreasing order:

blood > testis > brain > kidney > lung > inguinal white adipose tissue > gonadal white adipose tissue > mesenteric white adipose tissue > liver > pancreas (Nimako et al., 2021).

(b) Rat

From those rats used in the main study to evaluate absorption, distribution and excretion of imidacloprid (section 2.1.1), urine was collected separately from each animal under cool conditions, during the intervals 0–4, 4–8, 8–24 and 24–48 h. Faeces were collected in the intervals 0–24 and 24–48 h following dosing. The cage rinsings were collected in the urine containers. Metabolites were extracted from the faeces, isolated by preparative high-performance liquid chromatography (HPLC) and then further purified by other HPLC methods. The metabolites were identified by chromatographic comparison with known reference compounds in at least two independent chromatographic systems or by proton nuclear magnetic resonance and mass spectroscopic techniques.

Metabolites were found in both sexes and all dose groups. No biologically significant differences were observed in the pattern of excretion with regard to sex, dose level or route of administration. Two major routes of biotransformation were detected. The first route included an oxidative cleavage of the parent compound to yield 6-chloronicotinic acid and its glycine conjugate, which were found only in urine. These two metabolites together represented most of the identified metabolite, amounting to about 30% of the recovered radiolabel. Dechlorination of 6-chloronicotinic acid produced 6-hydroxynicotinic acid and its mercapturic acid derivative, which however, was a minor metabolite and was converted to 6-methylmercapturic acid and finally to its glycine conjugate which accounted for 5.6% of the recovered radiolabel. The second route included the hydroxylation of the imidazolidine ring at the 4 or 5 position to yield 4- or 5-hydroxy-imidacloprid and this accounted for about 16% of the recovered radiolabel. The loss of water from the hydroxyl derivative yields the olefinic derivative of imidacloprid. These products of metabolism and the unchanged parent drug were found in urine and faeces. A very minor compound, a guanidine-type derivative, was found only in faeces.

Parent drug was eliminated to the extent of about 14%, as confirmed by the elimination of more than 90% of the recovered radiolabel within the 24 hours following administration (Klein, 1987a).

The nitroso metabolite of imidacloprid, 1-(6-chloro-3-pyridylmethyl)-*N*-nitroso(imidazolidin-2-ylidene)amine (WAK 3839), has been identified as a minor constituent of edible plant commodities. With the aim of verifying whether this metabolite is formed as a biotransformation of imidacloprid in rats, a high single dose of imidacloprid was administered to male Wistar rats. No nitroso metabolite was formed under these conditions. However, in a further study, rats (number of animals, gender nor breed specified) were given a ration containing 103 mg/kg bw per day of imidacloprid for one year and then one oral dose of [*methylene*-^{14}C]imidacloprid. The nitroso compound corresponded to 9.3% of the urinary radiolabel formed, equivalent to 6.8% of the administered dose.

To confirm this finding, rats, and mice (number of animals, gender nor breed specified) were given a diet containing 1800 mg/kg feed of imidacloprid for about one year. Urine of both species presented the nitroso compound (Klein, 1990a).

(c) Human

In a clinical study of a single male volunteer, non-smoker, aged 50 years, weighing 83 kg, imidacloprid (purity not specified) was orally administered as a single dose of 0.06 mg/kg bw (total dose of 5 mg) prepared in 1 mL ethanol, diluted with 5 mL water. The dose was administered in an edible chocolate cup in the morning together with a light breakfast. Consecutive post-dose urine samples were collected for 48 hours thereafter. Screening for possible metabolites was carried out by liquid chromatography–high-resolution mass spectrometry. The parent compound and three putative metabolites (hydroxylated imidacloprid, imidacloprid-olefin and 6-CNA-glycine), were detected in post-dose urine samples, imidacloprid-oleofin deriving from the dehydration of hydroxylated imidacloprid, the major metabolite of imidacloprid (Wrobel et al., 2022).

2.1.2.2 **In vitro**

An in vitro non-GLP metabolism study, performed using individual recombinant cytochrome P450 isozymes from human liver P450 Supersomes™ (baculovirus infected insect cells [BTI-TN-5B1-4] containing human P450 cDNA), and insect cell control. Supersomes™, were incubated with [^{3}H]imidacloprid (purity 98% or greater). Each incubation mixture contained a single isozyme of cytochrome P450-CYP450 (125 pmol P450, 0.4–1.0 mg protein) or flavin monooxygenase (312 mg protein), [^{3}H]imidacloprid (3 mM, 119 ng, 3×106 dpm) and NADPH (0 or 1.5 mM). This was all in 100 mM phosphate buffer at pH 7.4, final assay volume 155 mL. This human study showed some similarities with the metabolism of imidacloprid in the rat, demonstrating that the principal organo-extractable NADPH-dependent metabolites are the 5-hydroxy (major) and olefin (minor) derivatives from hydroxylation and desaturation of the imidazolidine moiety and the nitrosoimine (major), guanidine (minor) and urea (trace) derivatives from reduction and cleavage of the nitroimine substituent. A single isozyme, CYP3A4 was responsible for oxidation in imidacloprid of the imidazolidine moiety and reduction of the nitroimine moiety (Schulz-Jander & Casida, 2002).

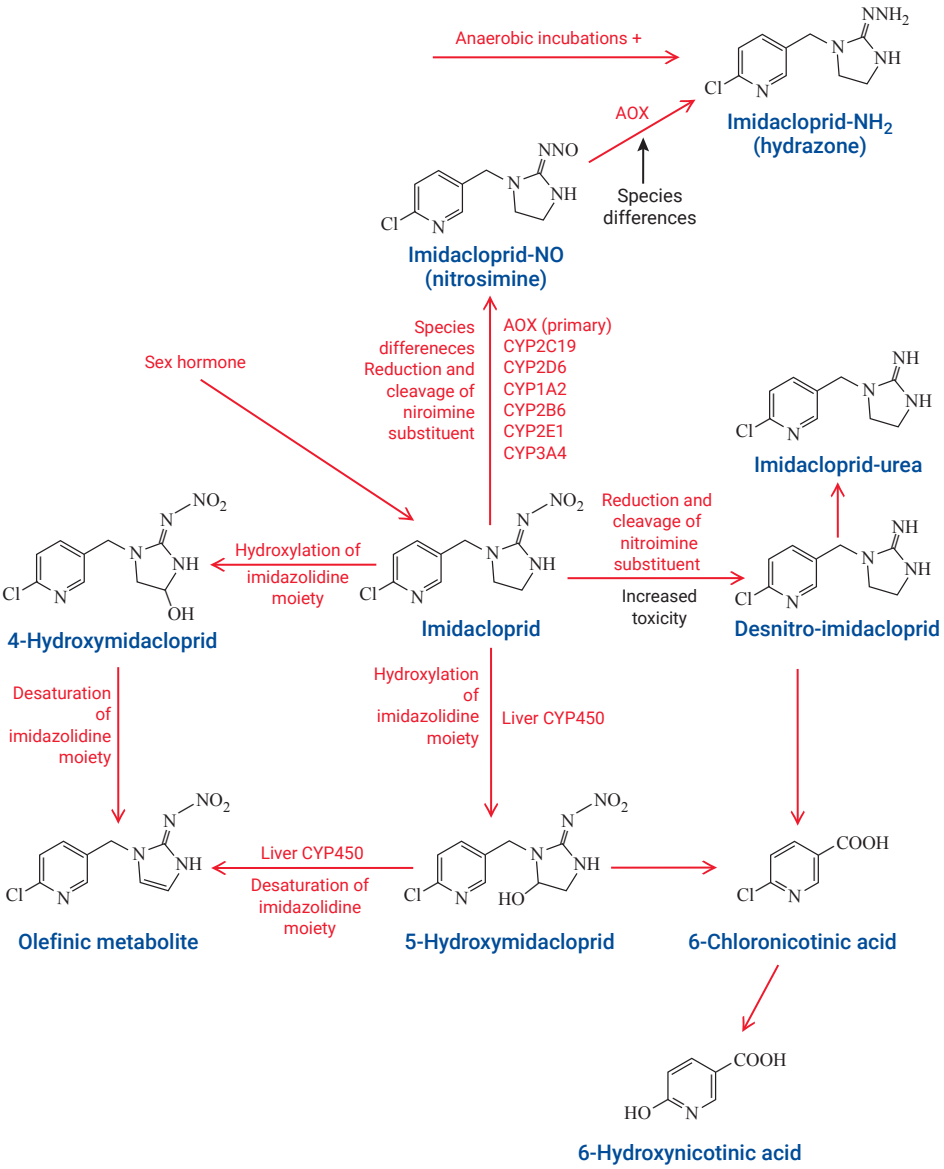
A comparative in vitro metabolism assay (not GLP-compliant) was conducted, aimed at assessing the formation of metabolites in the hepatic microsomal system of rats and rainbow trout exposed to imidacloprid. Four different rat and rainbow trout (sex not specified) microsome preparations were exposed to 3.125, 6.25, 12.5, 25, 50, 100, 150 and 200 µM imidacloprid (99.7% purity). Reaction mixtures composed of 100 mM potassium phosphate buffer (pH 7.4), trout microsomes 4 mg/mL, and NADPH 5 mM. Two replicates were

prepared for each treatment. Chemical analyses and metabolite characterization were performed on an ultra-high performance liquid chromatography system (UHPLC). Further metabolite identity validation was done by UHPLC coupled to a 6410 series triple quadrupole liquid chromatograph–mass spectrometer. A single metabolite, identified as 5-hydroxy-imidacloprid (IMI-1), was characterized for imidacloprid in the microsomal systems of both species (Kolanczyk et al., 2020).

An in vitro comparative metabolism study (not GLP-compliant) was carried out. Its aims were to investigate the interspecies differences in imidacloprid metabolite formation between cats, dogs, rats and humans following cytochrome P450 metabolism. Hepatic microsomes were taken from the following sources: male Sprague Dawley rats ($n = 3$), male dogs ($n = 3$), male cats ($n = 3$) and male and female humans ($n = 10$). For each species, hepatic microsome preparations were mixed with 0.1 M potassium phosphate buffer (pH 7.4), magnesium chloride, final concentration 3 mM, and glucose-6-phosphate final concentration 5 mM) all in 3% methanol. Imidacloprid (purity not specified) was added at final substrate concentrations of 10, 25, 50, 100, 200 or 400 µM) and the mixture pre-incubated at 37 °C for 5 min. A mixture of glucose-6-phosphate dehydrogenase, final concentration 2 IU/mL) and β-nicotinamide adenine dinucleotide phosphate (β-NADPH) final concentration 0.5 mM, was added to each sample to start the reaction. All assays were performed in duplicate for each sample. Liquid chromatography–mass spectrometry (1.7 µm Biphenyl 100 A LC column) was used to quantify the target metabolites of imidacloprid. The metabolites 4-hydroxy-imidacloprid, 5-hydroxy-imidacloprid, desnitrodehydro-imidacloprid, desnitro-imidacloprid and imidacloprid-olefin were detected in all selected species and other metabolites were detected in extremely low quantities and were only found in some reactions that employed a high substrate concentration. Among the four species, the kinetics of imidacloprid metabolism indicated that rats have the highest rate of oxidation of imidacloprid to 4-hydroxy-imidacloprid, while the fastest enzyme kinetics for conversion of imidacloprid to 5-hydroxy-imidacloprid were found in rats and humans (Khidkhan et al., 2021).

An extensive review provided by Wang et al. (2018) described the current state of understanding of imidacloprid's biotransformation pathways in mammals, and is shown in Fig. 2.

Figure 2.
Proposed metabolic pathway for imidacloprid



AOX: molybdo-flavoenzyme aldehyde oxidase; CYP450: cytochrome P450

(Redrawn from Wang et al., 2018)

2.2 Toxicological studies

2.2.1 Acute toxicity

2.2.1.1 Lethal doses

The results of studies in laboratory animals of the acute toxicity of imidacloprid with respect to the lethal dose, as reviewed by JMPR 2001 (JMPR, 2002) are summarized in Table 1. Extra information, such as details of the of imidacloprid administration procedures, clinical signs and gross pathology findings from these investigations were provided by CalEPA (2006).

The methods used in these studies complied with Organisation for Economic Co-operation and Development (OECD) guidelines and GLP.

Table 1.
Summary of acute toxicity studies with imidacloprid reviewed by JMPR (JMPR, 2002)

Imidacloprid purity	Species, strain	Route	LD_{50} (mg/kg bw), sex	LC_{50} (mg/kg bw), sex	Reference
94.2%	Mouse, BOR: NMRI	Oral	130, M	–	Bomann (1989a)
94.2%	Rat, Bor:WISW	Oral	420, M 450–480, F	–	Bomann (1989b)
96%	Rat, Bor:WISW	Oral	640, M 650, F	– –	Bomann (1991a)
94.3%	Rat, Bor:WISW	Oral	500, M 380, F	–	Bomann (1991b)
94.2%	Rat, Bor:WISW	Percutaneous	> 5000, M > 5000, F	–	Krotlinger (1989)
95.3%	Rat, Bor:WISW	Inhalation (aerosol, 4 hours)	–	>11[a], M >11[a], F	Pauluhn (1988a)
95.3%	Rat, Bor:WISW	Inhalation (dust, 4 hours)	–	> 848[b], M > 848[b], F	Pauluhn (1988a)
94.2%	Rat, Bor:WISW	Intraperitoneal	160 – 170, M 186, F	–	Krotlinger (1990)

bw: body weight; F: female; M: male; LD_{50}: median lethal dose; LC_{50}: median lethal concentration
[a] Aerosol concentration 69 mg/m^3; [b] Dust concentration 5323 mg/m^3;

To assess the acute oral toxicity, a single dose of imidacloprid (purity 94.2%) was administered by gavage as an aqueous suspension to Bor:NMRI - SPF mice (five of each sex per dose) at doses of 0, 10, 71 (males only), 100, 120, 140, 160 and 250 mg/kg bw. The animals were observed for clinical signs and gross pathology. Toxic signs were noted at doses higher than 10 mg/kg bw. All five males treated with 71 mg/kg bw of imidacloprid showed apathy and laboured breathing; decreased mobility was noted in two males, while one male had tremors. In addition to these signs of toxicity, staggering gait and severe trembling were also reported at higher doses (100–250 mg/kg bw). The lowest dose tested that caused

death was 100 mg/kg bw (20% of the male mice died) and 20% of the females died at 120 mg/kg bw. The toxicity was evident within 5–10 minutes of imidacloprid administration. The median lethal dose (LD_{50}) was calculated as 131 mg/kg bw and 168 mg/kg bw for male and female mice, respectively (Bomann, 1989a).

The acute oral toxicity of imidacloprid (purity 94.2%) was assessed in Wistar rats (Bor:WISW). Imidacloprid was administered in a single dose by gavage as an aqueous suspension to fasted rats (five of each sex per dose) at doses of 0, 50 (males only), 100, 250, 315, 400, 475 (females only), 500 and 1800 mg/kg bw. Clinical signs were evident within 15–40 minutes of treatment at doses higher than 50 mg/kg bw in males and 100 mg/kg bw in females. The main symptoms included apathy, laboured breathing, tremors, gait inco-ordination, decreased mobility, nasal and urine staining. The lowest dose at which mortality was 400 mg/kg bw (20% both sexes) and this increased abruptly to 100% at 500 mg/kg bw. Deaths occurred within 3–7 hours of treatment. The LD_{50} was calculated as 424 mg/kg bw and 450–475 mg/kg bw for male and female rats, respectively (Bomann, 1989b).

To evaluate acute dermal toxicity, imidacloprid (purity 94.2%) was applied as a paste in 0.9% sodium chloride to a shaven area of the back of Bor:WISW rats (five of each sex) at a dose of 5000 mg/kg bw. The treatment site was covered during the 24 hour exposure period. Imidacloprid did not cause signs of toxicity or any mortalities. The pathological evaluation did not reveal treatment-related changes. The acute dermal LD_{50} of imidacloprid in rats was greater than 5000 mg/kg bw (Krotlinger, 1989).

Bor:WISW Wistar rats (five of each sex) were exposed for four hours to 2.5% imidacloprid (95.3% active ingredient) in Lutrol® as vehicle, by inhalation at an aerosol concentration of 69 mg/m^3 (corresponding to 11 mg/kg bw) via head/nose only. This was reported as the highest concentration of imidacloprid attainable in the form of a liquid aerosol. Control animals received vehicle alone. Neither clinical signs nor mortalities were observed during the 14-day observation period (Pauluhn, 1988a).

To obtain sufficient concentrations to evaluate acute inhalation toxicity Bor:WISW Wistar rats (five of each sex per dose) were exposed by head/nose to imidacloprid (95.3% active ingredient) in the form of dust for four hours. Concentrations of imidacloprid were 1220, 2577 and 5323 mg/m^3, corresponding to 192, 412 and 848 mg/kg bw respectively. The control groups received air alone. Signs of toxicity were observed within 4–6 hours of treatment at concentrations higher than 1220 mg/m^3. Signs included difficulty breathing, reduced mobility, piloerection, and tremors. None of the animals died due to the treatment. The median lethal concentration (LC_{50}) was higher than 848 mg/kg bw (Pauluhn, 1988a).

Bor:WISW rats (five of each sex/dose) were administered imidacloprid (purity 94.2%) intraperitoneally as a single dose of 0, 10, 100, 160, 170, 180, 200,

250 or 500 mg/kg bw for males, 0, 10, 100, 150, 180, 200, 224 or 250 mg/kg bw for females. Tremors, apathy, reduced mobility, ptosis, and laboured breathing were observed within five minutes of treatment with doses higher than 10 mg/kg bw. Mortalities occurred from a dose of 170 mg/kg bw. Eight percent of males and 40% of females died within 2.5–5 hours of imidacloprid administration. The LD_{50} was 160–170 mg/kg bw and 160 mg/kg bw for male and female rats, respectively (Krotlinger, 1990).

In a GLP-compliant study, young females Han Wistar rats ($n = 9$) underwent an approximately 12-hour feed-and-fast cycle before being weighed and administered, by gavage, a single dose of the commercial formulation of imidacloprid (Ectosan, 98.9% purity) dissolved in 1% aqueous methyl cellulose in a dose volume of 10 mL/kg bw. A progression according to OECD Guideline 425 (up-and-down procedure) was followed, starting with a dose of 130 mg/kg bw in one animal, and and then progressing to doses of 410, and 1300 mg/kg bw until three animals were treated with each dose. Just one animal was treated with the top dose, 2000 mg/kg bw. The animals were observed at approximately 15 and 30 minutes post-dose, hourly between one and four hours post-dose, twice daily on days 2, 3 and 4, then once daily from the fifth to last day of the observation period. . Animals that died during the observation period were subject to necropsy, as were surviving animals that were killed on day 15.

No clinical signs were seen in animals treated at 130 mg/kg bw. Clinical signs of toxicity noted in one animal treated at 410 mg/kg bw were twitching, decreased activity, noisy breathing and hunched posture, which developed from two hours after dosing and lasted up to four hours after dosing. No alterations were observed in the other two animals treated at this dose level. Clinical signs of toxicity were noted in three of four animals treated at 1300 mg/kg bw. These signs were decreased activity, twitching, hunched posture, ptosis, laboured or noisy breathing and prone posture. Three of four animals treated with 1300 mg/kg bw were found dead one to six hours after dosing. Clinical signs of toxicity noted in the single animal treated at 2000 mg/kg bw were twitching and decreased activity 30 minutes after dosing, with clonic convulsions noted before death one hour after dosing. No abnormalities were noted at necropsy except for dark and inflated lungs which were seen in one animal treated at a dose level of 1300 mg/kg. Analysis using software (AOT425StatPgm) determined an LD_{50} of 1300 mg/kg bw, with a 95% confidence interval of between 494 and 1740 mg/kg bw (Dreher, 2018).

2.2.1.2 **Dermal irritation**

To access the potential acute irritation on the skin, 500 mg of imidacloprid (94.2% active ingredient), mixed with water to form a paste, was applied to three HC New Zealand rabbits (gender not specified). The treatment site was covered during four-hour exposure period. The skin was investigated for erythema

and edema during 14 consecutive days post-treatment. Apart from very slight erythema at one hour in one animal (which was cleared within 24 hours), imidacloprid did not cause irritation to the skin under the conditions of the test (Pauluhn, 1988b).

2.2.1.3 Ocular irritation

The eye irritating potential of imidacloprid (94.2% active ingredient) was investigated in three HC New Zealand rabbits (gender not specified). It was reported that the animals received about 60 mg of imidacloprid per eye in 0.1 mL in 0.9% sodium chloride solution. Conjunctival irritation was graded as 2 on a scale of 1 to 3 following one hour of treatment, but this alteration was completely reversed within 24 hours, indicating that under the conditions of the test imidacloprid did not possess a local irritant potential to the eye (Pauluhn, 1988c)

2.2.2 Short-term studies of toxicity

2.2.2.1 Oral administration

(i) Mouse

In a study (not GLP-compliant) imidacloprid (purity 92.8%) was administered, for 107 days, in the diet to B6C3F1 mice (10 of each sex per dose). The dietary levels of imidacloprid were 0, 120, 600 or 3000 mg/kg feed (equivalent to 0, 17, 86 and 430 mg/kg bw per day).

A total of 14 animals (seven of each sex) from the group treated with 430 mg/kg bw per day died before the end of the experiment. Amongst the survivors from this group, several animals were in poor general condition, had rough coats and markedly lower body weights. The average body weight of the males and females in this group was 15% and 27% lower than the control ($p \leq 0.01$), respectively. Body weight gain was also reduced (5%, $p \leq 0.05$) in males at 86 mg/kg bw per day, however food consumption was increased in males at 86 mg/kg bw per day and females at 430 mg/kg bw per day. At the higher dose of imidacloprid, clinical chemical tests showed significantly decreased urea and cholesterol concentrations in males and lowered alanine transaminase (ALT) activity and glucose concentration in females. Alkaline phosphatase (ALP) activity was significantly increased in both sexes at 430 mg/kg bw per day and in females at 17 and 86 mg/kg bw per day. Reduction in the weights of the liver, heart, spleen, kidneys, testes and adrenals was observed in the 430 mg/kg bw per day group. A no-observed-adverse effect level (NOAEL) of 17 mg/kg bw per day was identified, based in reduced body weight in males, and increased ALP activity in females and males treated with 86 mg/kg bw per day (Eiben, 1988a).

In a study (not GLP-compliant), male C57BL/6J mice (eight per dose) were orally administrated imidacloprid (purity 98%) in drinking water at doses

of 0, 3, 10 or 30 mg/L consuming an average of about 0, 0.5, 1.67 and 5 mg/kg bw per day respectively, for 70 days. Mice were fasted for 12 hours, euthanized and samples of serum, liver, ileum, and colonic contents collected.

Mice from the three imidacloprid-treated groups showed a significant decrease in relative liver weight. Animals treated with the highest dose of imidacloprid (5 mg/kg bw per day) displayed increased mucus in the colon, elevated AST and ALP activities, a decrease in serum and hepatic total bile acids (TBAs), and an increase in the faecal TBA levels. taking into account a number of deficiencies and inconsistencies, this study was not considered adequate for risk assessment of imidacloprid (Yang et al., 2020).

(ii) Rat

In a range-finding study (not GLP-compliant), imidacloprid (purity 92.8%) was administered in the diet for 98 days to Bor:WISW rats (10 of each sex per dose). Males were given doses of 0, 11, 57 or 410 mg/kg bw per day and females 0, 14, 78 or 510 mg/kg bw per day.

Mortality was not evident at any dietary level. Food intake was increased in animals at the highest dose in males (410 mg/kg bw per day) and females (510 mg/kg bw per day), and male body weight gain was decreased at 57 and 410 mg/kg bw per day. In those animals treated with the highest dose of imidacloprid the average body weight of the animals of both sexes was as much as 15–24% ($p \leq 0.01$) less than for the control. Significantly elevated ALP activity and depressed glucose concentration were found in males and females at 410 and 510 mg/kg bw per day, respectively, and the males also showed a reduced level of cholestero. Degenerative histological lesions in the epithelium of the testicular tubules was seen in five of the 10 males at 410 mg/kg bw, and multifocal group cell necrosis was diagnosed in the liver of one male at this dietary concentration. The NOAEL was 11 mg/kg bw per day, based on an 11% reduction in body weight of female rats at 78 mg/kg bw per day (Eiben, 1988b).

In a GLP-compliant oral toxicity study, imidacloprid (purity 95.3%) was administered through the diet to Bor:WISW rats (10 rats of each sex per dose), for up to 96 days. The concentration levels were 0, 150, 600 or 2400 mg/kg feed (equal to 0, 14, 61 and 300 mg/kg bw per day for males, 0, 20, 83 and 422 mg/kg bw per day for females). Satellite groups consisting of 10 male and 10 female rats received imidacloprid at a concentration of 0 or 2400 mg/kg feed (equivalent to 0 or 300 mg/kg bw per day) for the same period, but continued on the control diet during the subsequent period of four weeks, to explore the reversibility of any adverse effects.

Mortality was not evident at any dietary level. Body weights were reduced by about 8% ($p \leq 0.01$) in the males from the 61 mg/kg bw per day

group. The average body weight for the males and females at the higher dose of imidacloprid (300 and 422 mg/kg bw per day) was 14–16% less than in the control ($p \leq 0.01$) despite their increased food consumption. Slightly longer thromboplastin times and depressed thrombocyte counts were found in males and females treated with 300 and 422 mg/kg bw per day respectively, both effects being only partially reversible. Elevated ALP and ALT activities and depressed protein, albumin, cholesterol and triglyceride levels were found in males and females at 300 and 422 mg/kg bw per day respectively. In the case of males there was an increased incidence of focal necrosis (four of eight), single cell necrosis (eight of 10), swollen nuclei and cytoplasmic transformation (nine of 10) and round cell infiltration (all males). Round cell infiltration, necrosis of hepatocytes and cytoplasmic changes were also observed in three males at the mid-dose of 61 mg/kg bw per day. A NOAEL of 14 mg/kg bw per day was identified for males, based on liver toxicity and reduced body weight at 61 mg/kg bw per day (Eiben & Rinke, 1989).

In a gavage study (not GLP-compliant), female albino rats (10 per group) received imidacloprid (purity 96%) dissolved in corn oil, for 90 days at doses of 0, 5, 10 and 20 mg/kg bw per day. Body weight, food consumption and clinical signs of toxicity were recorded throughout the period of the experiment. It was reported that urine was collected at initial [sic], and at 90 days for urine analysis. Individual animals from each group were weighed weekly and body weight was recorded. Any signs of toxicity, such as salivation, lacrimation, piloerection diarrhoea, dyspnea, tremor, convulsion, paralysis or death, were inspected for once daily throughout the period of exposure. After 90 days of dosing rats were necropsied and blood collected for haematology and biochemical evaluation.

At 20 mg/kg bw per day a decrease in body weight gain was observed and at necropsy the relative body weights of liver, kidney and adrenal were also significantly increased. No mortality occurred during treatment period but food intake was reduced at the high dose. As to clinical chemistry parameters, the high dose of imidacloprid caused significant elevation of serum AST, ALT, glucose, and blood urea nitrogen (BUN) and decreased the activity of acetylcholinesterase (AChE) in serum and brain. Spontaneous locomotor activity decreased at the highest dose exposure, while there were no significant changes in haematological or urine parameters. The brain, liver and kidney of rats exposed to the high dose of imidacloprid showed mild pathological changes. The NOAEL was 10 mg/kg bw per day based on morphological, biochemical, haematological, and neuropathological alterations detected in female rats at 20 mg/kg bw per day (Bhardwaj et al., 2010).

In a study (not GLP-compliant) of female albino rats (five rats per group) animals received imidacloprid (purity 96%) dissolved in corn oil by gavage, at

doses of 0, 5, 10 or 20 mg/kg bw per day for 90 days. At the end of the experiment, females were euthanized and necropsied, and liver, kidney and brain collected for determination of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities. The level of glutathione (GSH) and lipid peroxidation (LPO) were also measured.

Imidacloprid did not cause any signs of toxicity or mortality. However, animals exposed to 20 mg/kg bw per day showed a significant reduction in body weight with mild diarrhoea, salivation, and piloerection during the 90 days of exposure. Doses of 5 or 10 mg/kg bw per day produced no effect on any of the parameters investigated. At 20 mg/kg bw per day lipid peroxidation was significantly increased in liver and kidney, SOD, CAT and GPx were increased in liver and brain, while GSH was increased in liver only. The NOAEL in this study was 10 mg/kg bw per day, based on clinical manifestations, reduced weight gain and the generation of oxidative stress produced by imidacloprid at 20 mg/kg bw per day (Kapoor et al., 2010).

In a study (not GLP-compliant) of female albino rats (10 rats per group) animals received imidacloprid (purity 96%) dissolved in corn oil by gavage, at doses of 0, 5, 10 or 20 mg/kg bw per day for 90 days. Clinical signs of toxicity were observed and recorded throughout the period of exposure. At the end of the experiment blood samples were collected for assay of serum follicle-stimulating hormone (FSH), luteinizing hormone (LH) and progesterone. Females were euthanized and necropsied, and ovaries were removed for evaluation of LPO, GSH, CAT, SOD and GPx.

There were no signs of toxicity due to imidacloprid at 5 or 10 mg/kg bw per day. Females given 20 mg/kg bw per day a showed significant reduction in weight gain, signs of piloerection, dyspnea, salivation, and diarrhoea. Similar observations were also made for animals receiving the highest dose, accompanied by decreased ovarian weight with cytoplasmic clumping and abundant lipofuscin in the granulosa cells of the ovarian follicles. No such abnormalities were detected in rats from the other two dose groups. In the same way, imidacloprid at 5 and 10 mg/kg per day did not produce any significant changes in hormones or the antioxidant status of the ovary. However, the 20 mg/kg bw per day dose, caused increased FSH, whilst LH and progesterone were decreased. Also observed in animals receiving the highest dose was an increase in ovarian lipid peroxidation, and decreases in GSH, SOD, CAT and GPx values. The NOAEL in this study was 10 mg/kg bw per day, based on clinical manifestations, reduced weight gain, ovarian morphology, hormone changes and the antioxidant status of the ovary, observed in females receiving 20 mg/kg bw per day (Kapoor, Srivastava & Srivastava, 2011)

In two dose range-finding, short-term studies (not GLP-compliant), imidacloprid (purity 98.5%) was administered orally to female and male

Han Wistar rats. In the first study, females (three per dose), were dosed by gavage with imidacloprid in 0.5% carboxymethylcellulose (CMC) at doses of 100, 150 or 200 mg/kg bw per day. Animals given 100 or 150 mg/kg bw per day were dosed for seven days, while animals given 200 mg/kg bw were only dosed for five days due to body weight losses seen early in the dosing period. There were neither deaths nor any clinical signs of toxicity. Minor weight losses occurred at 100 and 150 mg/kg bw per day, with a reduction in food intake at 150 mg/kg bw per day. At 200 mg/kg bw per day the body weight losses reduction in food intake were more marked, leading to the early termination of this group of females. At necropsy, abnormalities were confined to animals given 200 mg/kg bw per day and consisted of white raised areas on the gastric non-glandular mucosa in one rat, and two rats with stomachs distended with food.

In the second such feeding study (five of each sex per dose), males were given imidacloprid at 0, 113 or 137 mg/kg bw per day and females were dosed at 0, 97 or 170 mg/kg bw per day for 15 days. Other groups of males and females received imidacloprid at 151 and 198 mg/kg bw per day, respectively, for nine days.

No deaths occurred and there were no clinical sigs of toxicity. However, there were reductions in body weight gain and terminal body weight gain in all rats given imidacloprid. These effects were marked in males and females given the higher dose of imidacloprid (137 and 170 mg/kg bw per day, respectively) and this resulted in the early termination of this group after nine days. At 97 mg/kg bw per day there was a decrease in spleen weight in females. Males and females treated with 113 mg/kg bw per day and 97 mg/kg bw per day respectively, showed a decrease in thymus weight. In males treated with 137 mg/kg bw per day reductions in testes and liver weights were observed. Females treated at 170 mg/kg bw per day presented decreased liver weight and one female had a small thymus. Males from all treated groups showed decreases in prostate/seminal vesicle weights. No NOAEL could be identified in this study and a LOAEL of 97 mg/kg bw per day was based on a decrease in spleen weights in females (Chubb, 2018).

In a GLP-compliant, 52-week toxicity study, imidacloprid (purity 98.3%, 98.5% or 99.1% purity; three different batches) was administered to Wistar rats (10 of each sex per dose) at dietary levels of 0, 100, 300 or 1000 mg/kg feed (equal to 0, 5.6, 16,3 and 55.8 mg/kg bw per day for males, 0, 6.7, 19.5 and 63.7 mg/kg bw per day for females). Body weight and food intake were recorded periodically. An ophthalmoscopy study of control and high dose groups examined both eyes; before the start of treatment and at 52 weeks. Blood samples were taken during weeks 13, 26 and 52 and urine samples were taken during weeks 12, 25 and 51 from 10 animalsof each sex per dose; these were used for clinical pathology. All animals were subjected to a gross necropsy, specified organs were weighed and a full range of tissues was examined microscopically from control and high-dose

animals killed at the end of the treatment period.

No signs of toxicity or mortality were noted in animals treated with imidacloprid. Reduced food intake was seen in treated rats compared with controls. This was notable in males and females at 1000 mg/kg feed (55.9 and 63.7 mg/kg bw per day in males and females respectively). Body weights were lowered in a dose-related manner in males and females. These weight changes were seen largely during first 13 weeks of the study. However, reductions in body weight persisted for the duration of the study at 55.9 mg/kg bw per day in males and at 19.5 and 63.7 mg/kg bw per day in females. No imidacloprid-related effects were detected in ophthalmology, urinalysis, haematology and coagulation, clinical chemistry, organ weights, gross pathology or histopathology. The NOAEL for this study was 6.7 mg/kg bw per day, based on decreased body weight gains for females at the dose of 19.5 mg/kg bw per day (Britton, 2019).

In a gavage study (not GLP-compliant) of male Wistar rats (10 per dose), animals received imidacloprid (purity 99.8%) dissolved in 0.9% saline containing 0.1% dimethyl sulfoxide and 0.5% Tween 20, at doses of 0 or 0.06 mg/kg bw per day for 90 days. Body weight and food consumption were recorded throughout the period of the experiment. One day after the last imidacloprid administration, rats were fasted for three hours and then gavaged with fluorescein isothiocyanate-dextran (FITC-dextran) solution. After four hours, blood samples were taken, and serum isolated for measurement of FITC-dextran. Intestinal permeability was expressed as the amount of FITC-dextran exuded. Serum was also used to detect endotoxin (lipopolysaccharide), as well as tumour necrosis factor-alpha (TNF-α) and interleukin 1 beta (IL-1β). The number of animals assayed for serum detection (that is, FITC-dextran, lipopolysaccharide, TNF- α and IL-1β) was not stated.

Despite no alteration in food consumption or body weight gain, rats treated with imidacloprid showed a significant increase in intestinal permeability, accompanied by elevated serum levels of endotoxin and inflammatory biomarkers (tTNF- α and IL-1β). Because of some deficiencies, including the use of only one dose, this study was not considered adequate for risk assessment (Zhao et al., 2021a).

(iii) Dog

In a four-week study (not GLP-compliant) in beagle dogs (two of each sex per dose), animals received diets for 28 days containing imidacloprid (purity 98.2%) at concentrations of 0, 200, 1000 and 5000 mg/kg feed (equal to 0, 7.3, 31 and 49 mg/kg bw per day). Both males and females in the 49 mg/kg bw per day group died or were sacrificed before completion of the study. The first animal died after only two days of treatment; the other three dogs died on days 18 or 24 of

treatment. Symptoms observed in these dogs were a marked reduction in food intake and weight loss, ataxia, severe tremor and vomiting. Treatment-related hepatocellular atrophy was also diagnosed in the two surviving females from the 49 mg/kg bw per day group. Additional findings in dogs in the 49 mg/kg bw per day dose group were atrophy of the thyroid gland and bone marrow, advanced involution of the thymus (graded as moderate to severe) and testicular tubule degeneration.

Dogs treated at 31 mg/kg bw per day showed a decrease in food consumption (17% compared to the pretest level), however, their body weights were not affected. Hypertrophy of hepatocytes in one male and follicular atrophy of the thyroid in one female in this group were reported as morphological alterations produced by treatment. A NOAEL of 7.3 mg/kg bw per day was identified, based on hypertrophy of hepatocytes and atrophy of the thyroid at 31 mg/kg bw per day (Bloch et al., 1987).

In a 52-week GLP-compliant study, beagle dogs (four of each sex per dose), received diets containing imidacloprid (purity 94.9%) at 0, 200, 500 or 1250↑2500 mg/kg feed (equal to 0, 6.1, 15 and 41↑72 mg/kg bw per day). The concentration of 1250 mg/kg feed was increased from week 17 onwards.

Food consumption was decreased by 9–14% in females treated with 1250↑2500 mg/kg feed. Females receiving this highest concentration of imidacloprid also showed an increase in metabolic activity in the liver, as evidenced by the elevated levels of plasma cholesterol in females (up to 191% of control values) and in liver cytochrome P-450 enzymes in both sexes (151–193% of control values). The chronic oral NOAEL was 15 mg/kg bw per day, based on liver changes at 41 mg/kg bw per day (Allen et al., 1989).

In a GLP-compliant study, beagle dogs (four of each sex per dose), received diets containing imidacloprid (purity 95.3%) at 0, 200, 600 or 1800↓1200 mg/kg feed (equal to 0, 7.5, 24 and 67.5↓45 mg/kg bw per day) for 13 weeks (90 days). The highest dose (1800 mg/kg feed) was reduced at week 4 because of low food intake, resulting in an intake equal to 45 mg/kg bw per day.

There was no mortality or evidence of tissue damage. Vomiting of food or mucus were observed in all groups receiving imidacloprid, however, the number of vomiting incidents for any particular female never exceed one over the entire treatment. Trembling was observed in all males and females treated at 24 or 67.5↓45 mg/kg bw per day. Severe tremors were reported for all animals from the highest dose group (67.5↓45 mg/kg bw per day). The NOAEL was 7.5 mg/kg bw per day, based on tremors occurring in the first week in animals treated with 24 mg/kg bw per day (Ruf, 1990).

2.2.2.2 Dermal application

In a GLP-compliant dermal toxicity study, imidacloprid (purity 95%) was mixed to a paste in a physiological saline solution containing 2% Cremophor EL as vehicle. This was applied to a shaven area of the back and flank of New Zealand rabbits (five of each sex) at a dose of 1000 mg/kg bw. The vehicle control group were treated with 2% Cremophor EL in physiological saline solution. Sites, covered with a porous patch, were treated for six hours per day, five days per week for three weeks. The rabbits were observed for signs of general toxicity, behavioural alterations, and skin irritation. Clinical chemistry examinations were performed prior to, and at the end of, the treatment period, and pathological evaluations were made two or three days after the last dermal dose. Imidacloprid did not cause toxic signs, mortality, or any pathology changes in the organs examined. The NOAEL was 1000 mg/kg bw per day at the limit dose tested (Flucke, 1990).

2.2.2.3 Exposure by inhalation

In a GLP-compliant study imidacloprid (purity 95.3%) was administered to Wistar rats (10 of each sex per dose) in dust form through the nose alone. Exposure time was six hours per day, five days per week over a period of four weeks. The concentrations of imidacloprid were 0, 5.5, 30.5 and 191.2 mg/m^3 per day (approximately 0, 0.9. 5.2 and 33 mg/kg bw per day).

Male rats receiving the highest dose of imidacloprid (33 mg/kg bw per day) showed significant reductions in body weight gain (6–9% compared with controls), while females in the 33 and 5.2 mg/kg bw per day groups, presented a concentration-dependent increase of 7–14% in their absolute and relative liver weights. In these females also observed was hepatocellular damage and an increase in the activities of serum ALT, ALP and glutamate dehydrogenase (GLDH), by 25%, 70% and 732%, respectively. Also reported in females from the two higher concentration groups, were lengthening of coagulation time (10% higher than controls) and elevated urinary pH (from 6.5 in to 7.4). The no-observed-adverse-effect concentration (NOAEC) for this study was 5.5 mg/m^3 per day (approximately 0.9 mg/kg bw per day) based on changes in liver weight and alterations in liver function detected in females at 5.2 mg/kg bw per day (Pauluhn, 1989).

2.2.3 Long-term studies of toxicity and carcinogenicity

(i) Mouse

In a GLP-compliant study, imidacloprid (purity 95.3%) was administered in the diet for 24 months to B6C3F1mice (10 of each sex per dose) at concentrations of 0, 100, 330 or 1000 mg/kg feed (equal to 0, 20, 66 and 208 mg/kg bw per day for males, 0, 30, 104 and 274 mg/kg bw for females). In a supplemental study to determine the maximum tolerated dose, mice (25 of each sex per dose) were given diets

containing imidacloprid (purity 95.3%) at a concentration of 0 or 2000 mg/kg feed (equal to 0 and 414 mg/kg bw per day for male, 0 and 424 mg/kg bw per day for females) for 24 months. Ten additional mice of each sex were included per dose in both studies for interim sacrifice after 12 months of treatment.

At 2000 mg/kg feed, food intake was decreased by 24% in females compared with controls and water intake was also decreased in males and females at this concentration. Mice of both sexes treated with 1000 and 2000 mg/kg feed had significantly reduced weight gains which were dose-related, particularly during the latter half of the study. At 2000 mg/kg feed, lower leukocyte counts were registered in both sexes. Reduced blood cholesterol concentrations were observed at 2000 mg/kg feed after 52 weeks. An increased incidence of low-grade periacinar hepatic cell hypertrophy was found in males at 2000 mg/kg feed. The brains of animals ingesting the highest concentration showed more mineralization of the thalamus than those in the control groups. There was no evidence of carcinogenicity. The NOAEL was 330 mg/kg feed (equal to 66 mg/kg bw per day) based on reduction in body weight for male mice at a dose of 208 mg/kg bw per day (Watta-Gebert, 1991a, b).

(ii) rat

In a GLP-compliant two-year toxicity/carcinogenicity study, imidacloprid (purity 94.3–95.3%; mixed batch) was administered to Wistar rats (10 of each sex per dose) at dietary levels of 0, 100, 300 or 900 mg/kg feed (equal to 0, 5.7, 17 and 51 mg/kg bw per day for males, 0, 7.6, 26 and 73 mg/kg bw per day for females). Ten more rats of each sex per dose were used for interim examinations after one year of treatment. In a supplemental study to determine the maximum tolerated dose, male and female Wistar rats (25 of each sex per dose) were given diets containing imidacloprid for 24 months at concentrations of 0 or 1800 mg/kg feed (equal to 0 and 103 mg/kg bw per day for males, 0 and 144 mg/kg bw per day for females) . Ten additional rats per dose group and sex were included for interim tests after 12 months.

Rats exposed to the highest concentration of imidacloprid (1800 mg/kg feed) showed substantial reduction in body weight in both sexes at all periods evaluated. The weight decline reached maximum of 12% at week 10. A decrease in body weight was also observed in males and females at the 900 mg/kg feed level. The reduction in body weight was clearly treatment-related, because food intake of the animals from the 900 and 1800 mg/kg feed groups was similar to that of controls. Although no morphological changes were detected in liver, there were indications of liver toxicity for both sexes at 1800 mg/kg feed, based on alterations in serum chemistry: elevated serum ALP activity (by up to 37% compared with controls, $p \leq 0.01$) at 6, 12 and 18 months.

An increased incidence of mineralization in the colloid of the thyroid gland follicles, compared with such incidences in controls in previous studies, was observed in males at concentrations greater than 300 mg/kg feed and in females at 900 mg/kg feed. At 1800 mg/kg feed parafollicular hyperplasia and fewer colloid aggregation sites in the thyroid were diagnosed. At all doses the levels of thyroid hormones in plasma, triiodothyronine (T3), thyroxine (T4) and thyroid-stimulating hormone (TSH) in these rats were normal. There was no evidence of a carcinogenic effect. The NOAEL for this study was 5.7 mg/kg bw per day, based on an increase in incidence and severity of mineralized particles in the thyroid gland in male rats at 17 mg/kg bw per day (Eiben & Kaliner, 1991; Eiben, 1991).

2.2.4 Genotoxicity

A summary of the twelve in vitro and four in vivo studies on the genotoxicity of imidacloprid reviewed by JMPR (JMPR, 2002) is presented in Table 2. All studies had been conducted in compliance with GLP.

In vitro gene mutation assays with imidacloprid using bacterial and mammalian cells gave primarily negative results. Positive results were seen in one of the two in vitro sister chromatid exchange assays, but a negative result was obtained in the corresponding in vivo assay. In addition, imidacloprid caused in vitro chromosomal aberrations in human lymphocytes, but at concentrations where cytotoxicity was also evident. In vivo studies for chromosome aberration and micronucleus assays were negative. The 2001 Meeting concluded that imidacloprid is unlikely to be genotoxic (JMPR, 2002).

Tables 3 and 4 show subsequent genotoxicity studies on imidacloprid, both in vitro and in vivo, after revision by the 2001 Meeting (JMPR, 2002).

Of the various studies evaluating the genotoxic effects of imidacloprid, mostly from public literature, many were carried out with commercial products and for this reason were not considered in this evaluation since they did not include information on the composition of the test item, so the possibility could not be excluded that other substances in the commercial product had interfered with the results obtained. In addition, other studies, although they described the purity of imidacloprid, were also discarded as they did not reach the required procedural quality, or provided insufficient information to understand why the authors reached the conclusions stated (JECFA, 2016).

From the remaining reliable in vitro studies, two were conducted under GLP and in accordance with relevant OECD guidelines. Imidacloprid was tested in bacterial reverse mutation assay using *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* strain WP2 *uvrA* pKM101. The test was negative at all concentrations and in the presence or absence of the rat S9 metabolic activation system (Hargreaves, 2018a). In an in vitro micronucleus test,

imidacloprid was tested over concentration ranges using 3 + 21-hour exposures with and without metabolic activation, or with a 24 + 24-hour exposure without metabolic activation. Imidacloprid gave negative results in the 3 + 21-hour tests, with and without metabolic activation. However, without metabolic activation, positive results were obtained with the two highest concentrations (800 and 1300 µg/mL) used in the 24 + 24-hour tests (Hargreaves, 2018b).

In an in vitro study (not GLP-compliant), the genotoxicity of imidacloprid was evaluated by a series of three methods: micronucleus test, mutation assay and the comet assay, in human lymphoblastoid TK6 cells. No exogenous metabolic activation was used in any of the tests performed. Additionally, measurement of the formation of reactive oxygen species (ROS) was performed. Imidacloprid significantly increased micronucleus frequency, TK mutations and DNA damage in the absence of metabolic activation. On the other hand, ROS did not increase in TK6 cells after 24-hour exposures to IMI (Guo et al., 2020)

In a GLP-compliant, in vivo micronucleus test in the rat, four groups of six male Wistar rats were dosed orally by gavage with 0, 50, 100 or 200 mg/kg bw, on two successive days. There was no evidence of clastogenicity or aneugenicity from any of the doses tested (Dunton, 2019).

Table 2.
Studies of the genotoxicity of imidacloprid reviewed by JMPR, 2001 (JMPR, 2002)

Test system	Test object	Concentration or dose	Purity %	Results	Reference
In vitro					
Reverse mutation	*S. typhimurium* TA1535, TA100, TA1537, TA98	≤ 12 000 µg/plate	95.0	Negative[a]	Herbold, 1989a
Reverse mutation	*S. typhimurium* TA1535, TA100, TA1537, TA98	≤ 5000 µg/plate	96.0	Negative[a]	Herbold, 1991
Reverse mutation	*S. typhimurium* TA1535, TA100, TA1537, TA98	≤ 5000 µg/plate	96.3	Negative[a]	Herbold, 1991
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	≤ 5000 µg/plate	97.4	Negative[a]	Herbold, 1992
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2/uvrA	≤ 5000 µg/plate	93.7	Negative [NI]	Watanabe, 1991a
DNA damage	*B. subtilis* H17 (rec+), M45 (rec–)	≤ 5000 µg/plate	94.7	Negative [NI]	Watanabe, 1990a
Point mutation	hprt locus of Chinese hamster ovary cells	≤ 120, 1200 µg/ml	95.2	Negative[a]	Lehn, 1989a
Mitotic recombination	*Saccharomyces cerevisiae* D7	≤ 10 000 µg/ml	95.3	Negative [NI]	Herbold, 1988a
Unscheduled DNA synthesis	Rat primary hepatocytes	5.0–750 µg/ml	95.2	Negative [NI]	Cifone, 1988
Sister chromatid exchange	Chinese hamster ovary cells	≤ 5000 µg/ml	95.2	Positive[a]	Taalman, 1988
Sister chromatid exchange	Chinese hamster ovary cells	≤ 1200 µg/ml	95.2	Negative[a]	Putman & Morris
Chromosomal aberrations	Human lymphocytes	≤ 5200 µg/ml	95.2	Positive[b]	Herbold, 1989b
In vivo					
Micronucleus formation	Chinese hamster bone marrow	2000 mg/kg bw	94.6	Negative	Herbold, 1989c
Micronucleus formation	Mouse	80 mg/kg bw	95.3	Negative	Herbold, 1988b
Sister chromatid exchange	Chinese hamster bone marrow	500, 1000, 2000 mg/kg bw	95.0	Negative	Herbold, 1989d

Test system	Test object	Concentration or dose	Purity %	Results	Reference
Cytogenetic damage	Mouse germ cells	80 mg/kg bw	94.1	Negative	Völker, 1990

[a] With and without metabolic activation
[b] With metabolic activation
[c] Without metabolic activation
[NI] Metabolic activation status not indicated

Table 3.
Subsequent studies (2002–2022) of the genotoxicity of imidacloprid studies in vitro

Test system	Test object	GLP status	Concentration/dose	Purity (%)	Results	Reference
In vitro						
Micronucleus test	Human lymphocytes	Non-GLP	0.05, 0.1, 0.5 µg/mL	95	Positive[a, NI]	Feng et al., 2005
Sister chromatid	Human lymphocytes	Non-GLP	0.05, 0.1, 0.5 µg/mL		Positive[NI]	
Comet assay	Human lymphocytes	Non-GLP	0.05, 0.1, 0.2 and 0.5 µg/mL		Positive[NI]	
Micronucleus test	Human lymphocytes	Non-GLP	0.1, 1, 5, 10, 50,100 µg/mL	99.9	Negative[c]	Demsia et al., 2007
Sister chromatid	Human lymphocytes	Non-GLP	0.1, 1, 5, 10, 50,100 µg/mL		Negative[c]	
Micronucleus test	Human lymphocytes	Non-GLP	2 0 µM	99.9	Negative[c]	Stivaktakis et al. 2010
Chromosomal aberration	Chinese hamster ovary cells	Non-GLP	114.63 µM	98	Negative[a]	Al-Sarar et al., 2015
DNA damage					Negative[a]	
Micronucleus test					Positive[a]	
Reverse mutation	*Salmonella typhimurium* strains TA98, TA100, TA1535 & TA1537 *Escherichia coli* strain WP2 uvrA pKM101	GLP	**Experiment 1**: 5, 16, 50, 160, 500, 1600 and 5000 µg/plate **Experiment 2**: 39.6, 78.13, 156.3, 312.5, 625, 1250, 2500, and 5000 µg/plate	100.3	Negative[a]	Hargreaves, 2018a
Micronucleus test	Human lymphocytes	GLP	24 + 24 hours; 200, 800, 1200 µg/mL	100.3	Positive[a] (at 800 and 1200 µg/mL)	Hargreaves, 2018b
			3 + 21 hours; 500, 1000, 2000 µg/mL		Negative Negative[a]	
Gene mutation	TK6 cells	Non- GLP	0.01, 0.1, 1.0, 5.0, 10 µg/mL	99.5	Positive[c]	Guo et al., 2020
Micronucleus test	TK6 cells	Non- GLP	0.01, 0.1, 1.0, 5.0, 10 µg/mL		Positive[c]	
Comet assay	TK6 cells	Non- GLP	0.01, 0.1, 1.0, 5.0, 10 µg/mL		Positive[c]	

[a] With and without metabolic activation; NI: Metabolic activation status not indicated
[b] With metabolic activation
[c] Without metabolic activation

Table 4.
Subsequent studies of the genotoxicity of imidacloprid (2002 – 2022) - studies in vivo

Test system	Test object	GLP status	Concentration/dose	Imidacloprid Purity (%)	Results	Reference
In vivo						
Micronucleus test	Rat	Non-GLP	100, 200, 300 mg/kg bw	99.9	Positive (at 300 mg/kg bw)	Demsia et al., 2007
Micronucleus test	Rat	Non-GLP	5.5, 11, 22 mg/kg bw	98	Positive	Bagri, Kumar & Sikka, 2016
Chromasomal aberration	Rat	Non-GLP	5.5, 11, 22 mg/kg bw	98	Positive	
Micronucleus test	Rat	GLP	200 mg/kg bw	99.1	Negative	Dunton, 2019

2.2.5 Reproductive and developmental toxicity

(a) Reproductive toxicity

In a GLP-compliant study, Wistar rats (30 of each sex per dose) received diets containing technical grade imidacloprid (94.4–95.3% purity; mixed batch) at concentrations of 0, 100, 250 or 700 mg/kg bw in the feed (equal to 0, 6.6, 17 and 47 mg/kg bw per day), for 84 days before mating and throughout mating, gestation and lactation for breeding of the F1A and F1B litters. After weaning on day 21 post partum, selected F1B animals (26 of each sex per dose) were fed imidacloprid for 105 days prior to mating, during mating, gestation, and lactation for breeding of the F2 generation parental animals.

At 47 mg/kg bw per day, reduced food consumption and depressed body weight gain were observed in F0 males, reduced food intake in F0 and F1 females and reduced body weight gain in F0 females. Increased cytochrome P450, *O*-demethylase and *N*-demethylase activities were found in males at 47 mg/kg bw per day, and increased *O*-demethylase activity in F1 females at 17 and 47 mg/kg bw per day. Reduced body weights and body weight gains were observed in all generations (F1a, F1b, F2a and F2b) at 47 mg/kg bw per day during lactation. There were no effects on mating indices, fertility, gestation, litter size, mortality, and no evidence of pathology at any dose level. The NOAEL for parental effects was 6.6 mg/kg bw per day based on decreased premating body weights at 17 mg/kg bw per day. The NOAEL for reproductive effects was 47 mg/kg bw per day, the highest dose tested. The NOAEL for offspring effects was 17 mg/kg bw per day based on a decrease in pup body weight at 47 mg/kg bw per day (Suter et al., 1990).

In a GLP-compliant, extended one-generation reproductive toxicity study, Wistar rats (24 of each sex per dose) received diets containing technical grade imidacloprid (100.3% purity) in the feed at concentrations of 0, 100, 300 or 1000 mg/kg bw (equal to 0, 5.25, 15.35 and 48.4 mg/kg bw per day for males, 0, 10.4, 30.43 and 85.6 mg/kg bw per day for females). The F0 males were treated for 70 days, two weeks prior to pairing, through pairing to mating. The F0 females

were treated for up to 72 days, two weeks prior to pairing, during pairing and gestation and up to lactation day (LD) 21 (weaning of the F1 generation). Rats from the F1 generation (20 of each sex per dose) received diets containing the same concentrations of imidacloprid as the F0 had done, 0, 100, 300 and 1000 mg/kg in the feed. For F1 animals it was equal to 0, 11.4, 36.9 and 120.4 mg/kg bw per day for males, 0, 11.9, 35.5 and 121.0 mg/kg bw per day, for females. The regime continued up to 75 days (from maturation day 22 to 97) for cohort 1A, or up to 89 days (from maturation day 22 to 111) for cohort 1B.

Assessment of toxicity for the F0 generation was based on clinical observations, body weights, food consumption, estrous cycles, pregnancy indices, parturition, offspring development, and clinical pathology evaluations. The physical development of pups was evaluated using anogenital distance and nipple count (males only). F1 animals were assessed for food consumption, sexual maturation, and estrous cycles, ovarian follicle counts, seminology analysis, and clinical pathology.

One F0 female given 85.6 mg/kg bw per day was sacrificed in a moribund condition on LD 15. Reductions in body weight and food consumption were observed for males and females given the doses of 300 mg/kg feed (15.35 and 30.43 mg/kg bw per day for males and females respectively) and 1000 mg/kg feed (48.4 and 85.6 mg/kg bw per day for males and females respectively). There were no adverse effects on the numbers of estrous cycles or mean cycle length, however the mean cycle length in females given 30.43 or 85.6 mg/kg bw per day were higher, with statistical significance, compared with control values. Mating indices were reduced for males given 15.35 or 48.4 mg/kg bw per day (92% compared to 96% for controls) and for females given 85.6 mg/kg bw per day (92% compared to 96% for controls). Fecundity index was reduced for males given 15.35 mg/kg bw per day (86% compared to 100% for controls) and for females given 30.43 mg/kg bw per day (88% compared to 100% for controls). Fertility index was reduced for males given 15.35 or 48.4 mg/kg bw per day (79% and 92%, respectively when compared to 96% for controls) and for females given 30.43 or 85.6 mg/kg bw per day (88% and 92%, respectively compared to 96% for controls).

Decreased group mean absolute and relative heart weights were recorded at terminal sacrifice for females given 30.43 or 85.6 mg/kg bw per day. Increased group mean absolute and relative testis, liver and kidney weights were recorded for males given 48.4 mg/kg bw per day. Centrilobular hepatocellular eosinophilia/hypertrophy was recorded for males from all treatment groups and for females given 30.43 or 85.6 mg/kg bw per day. Aspartate transaminase (AST) activity (68 IU/L compared to 47 IU/L for controls), alkaline phosphatase (ALP) activity (86 IU/L compared to 55 IU/L for controls), and albumin (42 g/L compared to 39 g/L for controls) were increased (with statistical significance) in females given 85.6 mg/kg bw per day, compared to controls. An increased

incidence and severity of physiological hyperplasia of the mammary glands was noted for females in all treatment groups, compared to control females. Thymic atrophy was recorded in both sexes at terminal sacrifice, in males given 48.4 mg/kg bw per day and in females receiving 85.6 mg/kg bw per day, and this correlated with the decrease in thymic weights and the macroscopic observation where the thymus appeared reduced in size.

The duration of gestation was not affected. No effects related to the administration of the test article were noted on the number of females with live pups or the gestation index. Stillbirths were noted in females given 10.4, 30.43 or 85.6 mg/kg bw per day (13.0%, 15.0% and 9.5%, respectively compared to 0% for controls). The day 4 viability index was lower in litters of dams given 10.4 or 85.6 mg/kg bw per day (95% and 90%, respectively, compared to 96% for controls) and the mean percentage of male pups in each litter was lower on post-natal days (PNDs) 1 and 21 for litters of dams given 85.6 mg/kg bw per day, when compared with control. An imidacloprid-related, statistically significant reduction in live pups per litter was noted on PNDs 1, 4, 7, 14 and 21 for litters of dams given 85.6 mg/kg bw per day. A reduction in body weight was noted on PNDs 4, 7, 14, and 21 for pups of litters given 1000 mg/kg feed, when compared with controls. A statistically significant reduction in male pup body weight was also noted at weaning for litters of dams given 30.43 mg/kg bw per day. A statistically significant increase in mean T4 concentration was noted in male pups of dams given 30.43 or 85.6 mg/kg bw per day, and in female pups of dams given 85.6 mg/kg bw per day. Mean T4 concentration was also increased in pups (male and female combined) of litters given 30.43 or 85.6 mg/kg bw per day. Statistically significant reductions in the mean number of pups delivered (9.05 compared to 11.26 for controls) and live born pups (8.95 compared to 11.26 for controls) were noted in litters of dams given 85.6 mg/kg bw per day. The number of implantation sites was lower (with statistical significance) in females given 30.43 mg/kg bw per day (10.29 ± 3.05) or 85.6 mg/kg bw per day (9.86 ± 1.68) when compared with controls.

No test article-related mortalities occurred in F1 animals. Reductions in body weight and food consumption were observed in F1 animals given 300 mg/kg feed (equal to 36.9 and 35.5 mg/kg bw per day for males and females respectively) or 1000 mg/kg feed (equal to 120.4 and 121.0 mg/kg bw per day, for males and females respectively). No imidacloprid-related delayed sexual maturation in males or females, number of estrous cycles nor mean cycle length were observed. Compared to controls there was a statistically significant increase in AST in animals given 1000 mg/kg feed (equal to 120.4 and 121.0 mg/kg bw per day, for males and females respectively); the value for males was 42 IU/L compared to 36 IU/L for controls, in females 36 IU/L compared to 26 IU/L for controls. These changes were considered proportionate to the centrilobular hepatocyte hypertrophy/eosinophilia observed microscopically in the livers of animals given

imidacloprid at 1000 mg/kg feed (equal to 120.4 and 121.0 mg/kg bw per day for males and females respectively). Organ weights and organ weight ratios were significantly reduced in multiple tissues in animals given 1000 mg/kg feed (equal to 120.4 and 121.0 mg/kg bw per day for males and females respectively), and in occasional tissues from animals given 300 mg/kg feed (36.9 and 35.5 mg/kg bw per day for males and females respectively). There was an increase in organ weight for the reproductive tissues of F1 males from all treatment groups when compared with F1 controls; in the absence of organ:brain weight ratios, these were attributed to decreased terminal body weights.

The NOAEL for parental toxicity was 100 mg/kg feed (equal to 5.25 mg/kg bw per day), based on reduced body weight in males observed at 300 mg/kg feed (equal to 15.35 mg/kg bw per day) and above. Although physiological hyperplasia of the mammary gland was observed in all females exposed to imidacloprid, this NOAEL identified from males could be assumed as protective since the lowest dose used in females (10.4 mg/kg bw per day) is twice the dose used to identify the NOAEL. The NOAEL for offspring toxicity was 100 mg/kg feed (equal to 10.4 mg/kg bw per day) based on reduced pup body weights at 300 mg/kg feed (equal to 30.43 mg/kg bw per day). The NOAEL for reproductive toxicity was 1000 mg/kg feed (equal to 48.4 mg/kg bw per day), the highest dose tested(Holalagoudar, 2019).

(b) **Developmental toxicity**

(i) Rat

In a GLP-compliant study imidacloprid (purity 94.2%) was administered daily by gavage to a group of mated Wistar female rats (25 per group) from gestation day (GD) 6 to GD 15. The respective doses were 0, 10, 30 and 100 mg/kg bw per day. The rats were killed on day 21 post coitum, and the fetuses removed surgically by cesarean section and examined for developmental abnormalities.

Females from the highest dose group (100 mg/kg bw per day) presented toxicity and delay in embryo development. The maternal toxicity was evidenced by the reduced body weight gain (up to 43% reduction). Food consumption was also decreased (up to 35%) during the treatment period. At 10 and 30 mg/kg bw per day, a decrease in body weight gain in the dams (4% and 11% respectively) was observed. The NOAEL for maternal effects was 10 mg/kg bw per day, based on weight gain; the NOAEL for developmental effects was 30 mg/kg bw per day, based on delayed embryo development at 100 mg/kg bw per day (Becker, Vogel & Terrier, 1988a).

In a dose range-finding, prenatal developmental toxicity study (GLP-compliant), pregnant Wistar rats (seven perdose) were dosed with imidacloprid (purity 98.5%) once a day by gavage with 0, 50, 100 or 150 mg/kg bw per day in 0.5 % w/v CMC, from GD 6 to GD 19. All females were observed daily

from the start of dosing, and body weight and food intake were recorded at regular intervals. The animals were killed on GD 20, a necropsy was performed and the internal organs were examined for macroscopic abnormalities. Progress and outcome of pregnancy were assessed, and maternal dead body weight, gravid uterus and placenta weights were recorded. The fetuses were removed from the uterus, weighed, sexed and examined for external abnormalities.

Animals given 150 mg/kg bw per day were terminated on GD 10 due to excessive weight loss, inappetence and a decline in clinical condition. At necropsy they had dark livers, small spleens, and brown fluid in the caecum, colon, ileum and jejunum. One animal given 100 mg/kg bw per day showed piloerection on GD 11 and two animals given this dose displayed decreased activity on GDs 12 to 16. Two animals given 50 mg/kg bw per day displayed decreased activity on GDs 11 to 13. Administration of imidacloprid at 50 or 100 mg/kg bw per day caused initial body weight losses and marked reductions in food intake until GD 9, after which an improvement was seen. Overall body weight gain and food intake were lower than for controls. Gravid uterus weights were reduced relative to controls in animals given 50 or 100 mg/kg bw per day, and animals given 100 mg/kg bw per day had a lower terminal body weight adjusted for gravid uterus weight when compared with controls. Two females given 100 mg/kg bw per day underwent total resorption of their litters. Individual numbers of corpora lutea were comparable across the groups. There was an equivocal increase in incidence of postimplantation loss (by fetus) as a result of higher numbers of early intrauterine deaths in animals given the test item at 50 or 100 mg/kg per day, however this showed no dose–response relationship. Hence, it was concluded that the NOAEL for embryo/fetal toxicity was 150 mg/kg bw per day, the highest dose tested (Britton, 2018a).

In a GLP-compliant prenatal development toxicity study, pregnant Wistar rats (22 per dose) were administered imidacloprid (purity 98.5%) in 0.5% w/v CMC once daily by gavage at 0, 5, 15 or 50 mg/kg bw per day, from GDs 6 to 19. All females were observed daily from the start of dosing and body weight and food intake were recorded at regular intervals. The animals were killed on GD 20, necropsy was performed and internal organs were examined for macroscopic abnormalities. Progress and outcome of pregnancy were assessed, and the maternal dead body weight, gravid uterus and placenta weights recorded. Fetuses were removed from the uterus, weighed, sexed and examined for external abnormalities.

Animals given 50 mg/kg bw per day showed reduced food intakes and this persisted until termination when body weights were around 10% lower than control values. Food intake was unaffected at 5 and 15 mg/kg bw perday. There were no mortalities in this study and no clinical observations. Gross examination revealed no adverse effects. Mean fetal and placental weights were the same across

all groups and there were no effects on fetal sex ratios. There were no external fetal abnormalities and no skeletal or visceral anomalies.

The NOAEL in this study was 15 mg/kg bw per day for maternal effects, based on weight loss and reduced food intake. The NOAEL for embryo/fetal development was 50 mg/kg bw per day, the highest dose tested (Britton, 2018b).

(ii) Rabbit

In a GLP-compliant developmental toxicity study, imidacloprid (purity 94.2%), was administered by gavage to 16 mated chinchilla rabbits at doses of 0, 8, 24 or 72 mg/kg bw per day, on GDs 6–18. Cesarean sections and examination of the dams and fetuses were performed on GD 28.

Severe maternal toxicity was observed at 72 mg/kg bw per day. Two dams from this dose group died on GDs 19 and 28, one dam aborted, and two dams had had complete resorptions when examined at terminal necropsy. The females in the 72 mg/kg bw per day dose-group showed higher postimplantation losses (32%; $p \leq 0.01$) relative to control animals. Consequently, there was a statistically significant reduction in the number of live fetuses per dam. Food consumption for the surviving females was reduced up to 66% ($p \leq 0.01$) compared to controls. In turn, there was an overall weight loss over the entire treatment period. The weight loss trended significantly lower than in the controls within five days of treatment (8–11%; $p \leq 0.01$). The fetuses from these dams showed reduced body weight (10%; $p \leq 0.01$) and delayed ossification. Dams from the 24 mg/kg bw per day group presented decreased food intake (16%; $p \leq 0.01$) and body weight gain (33%; not statistically significant). The NOAEL for maternal toxicity was 8 mg/kg bw per day, based on reduced food consumption and body weight gains observed at 24 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 24 mg/kg bw per day based on increased postimplantation loss, reduced body weight and delayed ossification at 72 mg/kg bw per day group (Becker, Vogel & Terrier, 1988b)

2.2.6 Special studies

(a) Immunotoxicology

(i) Mouse

In a non-GLP compliant study, female BALB/c mice (6–8 animals per dose) were administered imidacloprid (purity 98%) by oral gavage, at doses of 0, 2.5, 5 or 10 mg/kg bw per day, for 28 consecutive days. Positive controls received cyclophosphamide or dexamethasone for five days; negative controls received CMC for 28 days. Animals were weighed daily. Seven days before the end of the exposure period, sets of treated and control mice were immunized by intraperitoneal injection of 0.3 mL sheep red blood cell (sRBC) suspension

to evaluate the haemagglutinating antibody (HA) titre. On day 18, mice were sensitized by a subcutaneous injection of 50 µL of sheep red blood cell suspension. After 10 days these mice were challenged to evaluate the delayed-type hypersensitivity (DTH) response. Blood samples were collected at the end of the exposure period and mice were killed. Spleen, liver, kidney and lungs were dissected for evaluation.

All animals survived the experimental period. The highest dose group (10 mg/kg bw per day) showed decreased total leukocyte count, percent lymphocytes, and platelet count. The two lower dose groups (2.5 and 5 mg/kg bw per day) showed no changes. A slight body weight decrease (approximately 8%) was seen in the 10 mg/kg bw per day group. No significant body weight changes were seen in any other dose group. All treated groups showed a decrease in spleen weight. Animals from high- and low-dose groups (10 and 2.5 mg/kg bw per day, respectively) displayed no significant effects on serum anti-sRBC agglutinin titre; however females from the mid-dose group (5 mg/kg bw per day) showed a significant ($p \leq 0.01$) decrease in titre. Intensity of DTH response was inversely related to the dose of imidacloprid administered; marked suppression was evident in the high dose group (10 mg/kg bw per day). Mice receiving imidacloprid at 10 mg/kg bw per day also displayed a significant decrease in lymphocyte proliferation. An apparently dose-related depletion in lymphocytes in white pulp was observed in the spleen. At this high dose there was also the increased presence of neutrophils and reticuloendothelial cells, along with congestion. Histopathological analysis of footpad sections of mice revealed a dose-related suppression of the DTH response. Since inconsistencies were found in the results, the Committee was not able to identify a specific effect on which base a reliable NOAEL and concluded that the study was not suitable for the risk assessment of imidacloprid (Badgujar et al., 2013).

(ii) Rat

From an extended GLP-compliant, one-generation reproductive toxicity study in rats (the main toxicity study described above in Section 2.2.5), one group (cohort 3) of F1 animals was selected to evaluate developmental immunotoxicity. For immunotoxicity studies these F1 rats (10 rats/sex per group) were given the same dietary concentration levels of imidacloprid as the F0 groups, that is 0, 100, 300 or 1000 mg/kg feed (equal to 0, 11.4, 36.9 and 120.4 mg/kg bw per day for males, 0, 11.9, 35.5 and 121.0 mg/kg bw per day for females) up to 61 days after weaning (that is, the 84th day of life), when they were sacrificed. Macroscopic examinations were then conducted and their spleens were weighed and preserved in formalin. Keyhole limpet haemocyanin (KLH) was administered (2 mL/kg bw intravenously [bolus] via manual injection into the

lateral caudal vein, at approximately 4 mL per minute) at a final concentration of 300 mg/kg on PND 56 ± 3 and on PND 70 ± 3. Blood samples (0.5 mL into serum separator tubes) were withdrawn from the animals' jugular vein or abdominal aorta on PND 61 ± 3, PND 70 ± 3, PND 77 ± 3 and PND 84 ± 3. Additionally, immunophenotyping was performed on samples taken from 10 males and 10 females per dose group from cohort 1A, and the following cell populations were examined: natural killer (NK) lymphocytes (CD3−/CD161a+), total T lymphocytes (CD3+), helper T lymphocytes (CD3+/CD4+), Cytotoxic T lymphocytes (CD3+/CD8+), B lymphocytes (CD3−/CD45RA+).

A dose-dependent reduction in T cell-dependent antibody response was apparent in females at all dose levels, but did not reach statistical significance due to high variability in the results. In males there was a slight reduction of the group average mean cell counts for total T lymphocytes, helper T lymphocytes and cytotoxic T lymphocytes. In addition, moderate imidacloprid-related changes in organ weights, macroscopical and microscopical effects were observed in thymus, spleen and lymph nodes at doses of 120.4 and 121.0 mg/kg bw per day (males and females respectively) and red to dark pigmentation of the thymus in females from the 36.9 mg/kg bw per day group. Administration of imidacloprid did not result in any noteworthy differences in lymphocyte count or proportions in the spleen. The immunophenotyping evaluation did not reveal any marked changes associated with imidacloprid in spleen lymphocyte populations when treated animals were compared with controls. The NOAEL for immunotoxicity was 121 mg/kg bw per day, the highest dose tested (Holalagoudar, 2019).

(b) **Neurotoxicity**

The acute and short-term effects of imidacloprid in rats, on the nervous system, conducted by Sheets (1994 a,b,c) were reported by the 2001 Meeting (JMPR, 2002), CalEPA (2006) and EFSA (2008, 2013). Two other neurotoxicological studies were provided by the sponsor, both in rats: one in adult animals that was conducted in conjunction with a standard toxicity tests of the same duration (see section 2.2.3), and the other with the extended one-generation reproductive toxicity study detailed in section 2.2.5. In each of these studies a cohort of F1 animals was selected in which to evaluate developmental neurotoxicity and neuropathology. Neurotoxicity studies could be also identified in a search of the open literature.

Neurotoxicity studies in adult rats

In an acute neurotoxicity study (GLP compliance not specified) imidacloprid (purity 97.6–98.8%) was administered by gavage in a single dose to Sprague Dawley rats (18 of each sex per dose). The test substance was suspended in deionized water containing 0.5% w/v methylcellulose and 0.4% w/v Tween 80.

The doses were 0, 42, 150 or 310 mg/kg bw. The rats in the neurotoxicity group, (12 of each sex per dose) were evaluated for neurobehavioural signs using the functional observational battery (FOB). Changes in motor activity were assessed by the figure-8 maze. Additionally six rats of each sex per group were subjected to pathological evaluation. The following tissues were examined for pathology: brain, spinal cord, eyes, peripheral nerves (sciatic, sural and tibial), gasserian ganglion and gastrocnemius muscles. Based on preliminary findings in rats, the time to peak effect for imidacloprid-induced behavioural changes was reported to occur 90–120 minutes following treatment. In the current study, clinical observations and FOB evaluations were performed at pretest, at the time to peak effect (90 minutes after dosing), then seven and 14 days after treatment. Motor activity was assessed at pretest, from 2.5 to 4.5 hours after treatment and on days 7 and 14.

The highest tested dose (310 mg/kg bw) produced severe toxicity, including deaths. Four males and ten females in this group died within to 24 hours of treatment. All animals still alive four hours after dosing had severe tremors and appeared cool to the touch, their body temperatures being reduced by 2°C in males and 5.5°C in females. Some of the effects, such as nasal and urine stains and decreased activity of the surviving males at the 310 mg/kg bw group, persisted after seven to 14 days of treatment. A dose-related increase in the incidence and severity of clinical signs was seen, with treatment-related effects in males at 150 and 310 mg/kg bw, and in females at just the highest dose. In males at 150 mg/kg bw the effects were limited to tremors and nasal staining, while males at the highest dose also displayed unco-ordinated gait, decreased activity and urine staining and they were cool to the touch. The treatment-related effects in females at the highest dose consisted of tremors, unco-ordinated gait, decreased activity, increased reactivity and red nasal staining. Clinical signs of toxicity were generally observed on day 0 and resolved in surviving males and females in the 1–5 days following treatment.

Treatment-related effects in the FOB were observed in males and females at the two higher doses. Effects consisted of an increased incidence of recumbency, tremors and nasal staining in males and tremors in females at 150 mg/kg bw. At 310 mg/kg bw there were numerous treatment-related effects in males and females, consistent with the lethality of this dose within 24 hours of treatment. All the toxic effects had resolved in surviving animals by the next observation period, seven days after treatment. A dose-related decrease in motor and locomotor activity on day 0 was observed in both sexes, with reduced activity in males at the two higher doses and in females at all three doses. As the slightly reduced motor activity in females at 42 mg/kg bw was comparable to that seen before treatment and 14 days after treatment, this reduction was considered not to be an adverse effect. Habituation was not affected. All clinical signs and neurobehavioural effects

showed complete reversal within seven days of treatment at sublethal doses. Animals at 150 mg/kg bw showed a decrease in serum triglyceride concentration, and animals that survived the highest dose had decreased serum potassium and cholesterol concentrations (females) and decreased serum ALT activity (males and females). Haematological changes were found in females at the highest dose. A NOAEL of 42 mg/kg bw based on statistically significant decreases in motor and locomotor activity of female rats at the 151 mg/kg could be identified. However, for the purpose of risk assessment, a benchmark dose (BMD) analysis was performed by CalEPA (2006) which identified a $BMDL_{05}$ for acute neurotoxicity of 9 mg/kg bw based on the same end-point (Sheets, 1994a).

The purpose of a second, supplemental study was to better identify the NOAEL for acute neurotoxicity of imidacloprid in rats. The supplemental study was performed in the same laboratory six months after the main study (Sheets, 1994a) and used a similar protocol. Twelve rats of each sex were used as controls or treated with imidacloprid via gavage in a single dose at 20 mg/kg bw. Behavioural tests were carried out on day 0 of treatment at the time of peak plasma concentration. The results indicated that a dose of 20 mg/kg bw did not cause apparent toxicity, including a decrease in the motor activity, which was observed in females at the LOAEL of 42 mg/kg bw in the main study. However, there was substantial uncertainty associated with the reported dose of 20 mg/kg bw as a NOAEL for the acute oral toxicity, because this study did not include a high enough dose of imidacloprid to produce toxicity in the rats (Sheets, 1994b).

In a 13-week neurotoxicity study (GLP compliance not specified), imidacloprid (purity 98.8%) was administered to Fischer 344 rats (12 of each sex per dose) at dietary levels of 0, 150, 1000 or 3000 mg/kg feed (equal to 9.3, 63 and 196 mg/kg bw per day for males, 10.5, 69 and 213 mg/kg bw per day for females). The FOB and activity tests on the figure-8 maze were performed at pretest and at weeks 4, 8 and 13 of treatment.

During most of the exposure period, imidacloprid caused a reduction in body weights in both sexes at 1000 mg/kg feed (up to 5% in females; 8% in males; $p \leq 0.05$) and 3000 mg/kg feed (up to 9% in females; 17% in males; $p \leq 0.05$). This effect was due, at least in part, to a decrease in food consumption (up to 13% and 29% for the animals at 1000 and 3000 mg/kg feed respectively). Lower grip strength of the forelimbs was recorded in the males exposed to imidacloprid, and this became statistically significant after eight weeks of treatment (23%; $p \leq 0.05$). At the end of the study (13 weeks), a higher incidence of unco-ordinated righting response was reported for the males exposed to imidacloprid. While only one control male (of 12) displayed an unco-ordinated righting response, there were two males from the 9.3 mg/kg bw per day group, three males form the 63 mg/kg bw per day group and seven males from the 196 mg/kg bw per day group ($p \leq 0.05$) that displayed unco-ordinated landings

in the FOB. Females treated with imidacloprid at 213 mg/kg bw per day showed a decrease in locomotor activity in all tests (at weeks 4, 8 and 13; up to 21%). Although this effect was not statistically significant it was consistent with the results from the acute neurotoxicity study (Sheets, 1994a), in which the locomotor activity was reduced by 46% and 89% in the females exposed to single doses of 151 and 307 mg/kg bw per day, respectively. The NOAEL was 9.3 mg/kg bw per day, based on decreased body weights and food consumption in male rats at a dose of 63 mg/kg bw per day (Sheets, 1994c).

In a neurotoxicity study (not GLP-compliant), 40 adult Sprague Dawley rats (12 weeks old) and adolescents (three weeks old) were randomly distributed to four groups of 10 animals and orally dosed with imacloprid (purity 99.9%) by gavage at 1 mg/kg bw per day, or with just corn oil (control groups), for 60 consecutive days. The rats were observed during the experiment for signs of toxicity, sickness and death. After the period of exposure, ten animals per group were evaluated in the following behaviour assays: hole-board, inclined plane, postural reflex and tail suspension tests. After the neurobehavioural evaluation, all animals were sacrificed and specimens obtained for histopathological and immunohistochemical investigation.

Neither mortalities nor signs of toxicity were recorded in any of the tested groups throughout the experiment. The results obtained demonstrated that imidacloprid exposure resulted in less exploratory activity, deficit sensorimotor functions and high depression levels. Histopathological investigations of the brain tissues of the imidacloprid-treated group revealed varying degrees of degeneration of the neurons. The immunohistochemical evaluation revealed a strong presence of glial fibrillary acidic protein (GFAP) and BAX-positive cells, but a low expression of Bcl-2 protein. These effects were more prominent in the adult rats than in the adolescents (Abd-El Hakim, Mohhammed & Mohamed, 2018).

In a GLP-compliant, one-year toxicity study, imidacloprid (purity 98.3, 98.5 or 9.1%; three batches) was administered to Wistar rats (80 of each sex per dose) at dietary levels of 0, 100, 300 or 1000 mg/kg feed (equal to 0, 5.6, 16,3 and 55.8 mg/kg bw per day for males, 0, 6.7, 19.5 and 63.7 mg/kg per day for females). Standard arena observations were performed on all animals before the start of dosing, at the start of week 2, and approximately monthly thereafter. Ten animalsof each sex per dose were also assessed for FOB responses before the start of dosing and during weeks 12–13, 25–26, 37–38 and 51–52. Observations consisted of sensorimotor responses to auditory, visual and proprioceptive stimuli, measurement of grip strength and automated motor activity assessment in an infrared photobeam system.

There were no changes in observations or behaviour in the home cage or open-field arena throughout the study. There were no clear effects on sensorimotor responses, grip strength or motor activity associated with imidacloprid. Although

some statistically significant lower grip strength values were recorded in weeks 13 and 26, these were not present in both forelimb and hindlimb, or were not clearly related to dose. In weeks 38 and 52 no differences were observed. Motor activity measurements throughout the study tended to indicate higher activity compared with controls for females given the test item, but there was no dose relationship in the size of the difference. At all dose levels males given imidacloprid showed similar activity to controls throughout the study. The NOAEL for neurotoxicity was 55.8 mg/kg bw per day, the highest dose tested (Britton, 2019).

Developmental neurotoxicity – in vivo study

In a GLP-compliant developmental neurotoxicity study, imidacloprid (purity 98.2–98.4%) was administered in the diet to female Wistar rats (30 animals per dose) at concentrations of 0, 100, 250 or 750 mg/kg feed from GD 0 until PND 21, when pups were weaned. Based on the food consumption by the dams during gestation, the average daily intake of imidacloprid was reported as 0, 8, 19 and 54.7 mg/kg bw per day. Pups were fed untreated food (no imidacloprid) after weaning. On PND 4 offspring were culled to leave four females and four males and these were observed for clinical studies, assessment of motor activity, auditory startle response, habituation, learning and memory, and ophthalmology. Pup physical development was assessed by body weight, surface-righting, auditory startle, eye opening, pupillary constriction. Also observed was vaginal patency in females and balanopreputial separation in males. The FOB tests were conducted between days 4 and 60. Neural tissues of offspring were collected for evaluation from 10 of each sex per dose on PND 11 and at termination of the study (75 days of age).

No maternal deaths or treatment-related clinical signs were observed over the duration of the study. No treatment-related effects on the body weight of mothers were observed; food consumption decreased for dams in the high dose group (700 mg/kg feed) during the third week of gestation and first week of lactation. There were no treatment-related effects on the number of litters, live litter size, number of stillborn pups, live birth index or viability index. Treatment-related effects for offspring were limited to the high-dose group (54.7 mg/kg bw per day) where body weights of males and females were significantly ($p < 0.05$) decreased prior to and after weaning, and during lactation. Overall, there were decreases in motor activity in males on PND 17 and females on PND 21, but the decreases were not significant. At study termination (PND 75), high-dose females showed a statistically significant ($p < 0.03$) decrease in thickness of the corpus callosum and caudate putamen width compared to controls. Although morphometric brain measurements were not performed in the intermediate and low dose-groups, the Committee considered that these findings were of uncertain significance. An offspring NOAEL was identified at 19 mg/kg bw per day, based on a reduction in body weight and decreased motor activity from the

group treated at 54.7 mg/kg bw per day (Sheets, 2001).

In a neurotoxicity study (not GLP-compliant), timed pregnant Sprague Dawley rats (five per group) were treated with a single intraperitoneal injection of 337 mg/kg bw (75% of the acute oral LD_{50}) of imidacloprid (purity 99.5%) or just corn oil as controls. Animals were observed for overt signs of toxicity, such as weight change, changes in eating and drinking, locomotor changes, timing of birth and recorded seizures. On PND 30 all male and female offspring were evaluated for AChE and butyrylcholinesterase (BuChE) activity, ligand binding for nAChR and muscarinic acetylcholine receptors (M2 mAChR), sensorimotor performance (inclined plane, beam-walking, and forepaw grip), histopathology and brain and GFAP immunostaining. The offspring of treated mothers exhibited deficits in beam-walk time without a difference in beam-walk score. Deficits in the inclined plane test and grip time were also noted, along with increased plasma cholinesterase activity (F1 males only), and brain region-specific AChE activity in F1 males and females. Compared with vehicle controls, GFAP immunostaining was increased in motor cortex (layer III), CA1 layer, CA3 layer, and the dentate gyrus of the hippocampus of F1 animals, but without evidence of histopathological alteration in surviving neurons from the various brain regions. (Abou-Donia et al., 2008)

From an extended, GLP-compliant, one-generation reproductive toxicity study in rats (main toxicity study described in section 2.2.5), two cohorts of F1 animals were selected to evaluate developmental toxicity and developmental brain neuropathology. For neurotoxicity evaluations (cohorts 2A and 2B), F1 rats (10 rats/sex per group) were given the same dietary concentration levels of imidacloprid as the F0 animals, that is 0, 100, 300 or 1000 mg/kg feed (equal to 0, 11.4, 36.9, 120.4 mg/kg bw per day for males, 0, 11.9, 35.5 and 121.0 mg/kg bw per day for females). Treatment continued from maturation day 22 to 75 (53 days) or for one day on maturation day 22. Cohort 2A was assessed for acoustic startle, motor activity, FOB and histomorphometry assessments. Cohort 2B was assessed for brain histomorphometry. Acoustic startle was assessed once on maturation day 24 (one day) and FOB on one occasion between maturation days 66 and 73 for males and between maturation days 63 and 73 for females. Motor activities were assessed for 30 minutes between maturation days 63 and 73. Animals from cohort 2A and 2B were sacrificed on maturation day 76 and 22, respectively, and several parts of the brain were subject to macroscopic evaluation and microscopy.

In the acoustic startle test, although habituation was reduced in a statistical significant manner in males given 120.4 mg/kg bw per day, no clear dose–response relationship was observed. No effects were seen in females. There were no notable effects on motor activity. There were no adverse effects on quantitative assessments in the FOB. No compound-related effects on brain weights, macroscopic or microscopic findings were observed in F1 animals from

cohorts 2A or 2B. The NOAEL was 121.0 mg/kg bw per day, the highest dose tested (Holalagoudar, 2019).

Developmental neurotoxicity – in vitro study

The effect of imidacloprid on the nAChRs of primary cultures of cerebellar neurons from PND1 Sprague Dawley rats was evaluated. Imidacloprid was reported to be an agonist of nAChRs; expression of these receptors at the perinatal stage is important for brain development. At 14–16 days the in vitro cerebellar neurons were administered a single dose of 1–100 µM imidacloprid (purity > 98%). An excitatory calcium influx assay was employed as an indicator of neural physiological activity in these cerebellar cultures. A significant excitatory pattern of intracellular calcium ions was observed at all concentrations. The relevance of these findings is unknown (Kimura-Kuroda et al., 2012)

(c) **Studies on male reproduction**

Three studies evaluating the effects of imidacloprid on the male reproductive system in rats were retrieved from the literature and are summarized below. The Committee considered that these studies did not provide sufficient details and displayed inconsistent results, hence they could not be used for risk assessment.

In a male reproductive study (not GLP-compliant) adult Wistar rats (five animals per group) were orally dosed by gavage for 28 consecutive days. The test article was dissolved in 0.03% ethanol in distilled water, and imidacloprid (purity 98%) provided at doses of 0, 0.06, 0.8 or 2.25 mg/kg bw per day. During the experiment body weights were monitored weekly. All the animals were euthanized 24 hours after the last treatment, testes and epididymis were collected and weighted and antioxidant status was determined. Damage to DNA in the haploid and diploid testicular sperm cells and the epididymal sperm cells was also evaluated using the alkaline comet assay.

A dose-dependent effect on body weight gain was observed in rats during treatment with imidacloprid. Animals treated with higher doses of imidacloprid (2.25 mg/kg bw per day) had lower body weight gains and a lower testis weight compared to control animals. Treatment at 0.06 mg/kg bw per day increased the level of GSH in the epididymis (73%), while the activities of epididymal GPx and SOD significantly increased in all treated rats. Exposure to imidacloprid resulted in a low, but significant, level of DNA damage in testicular sperm cells regardless of the concentration applied. A NOAEL could not be identified. (Lovaković et al., 2021).

In a male reproductive study (not GLP-compliant), Wistar rats (10 animals per group; 7–8 weeks old), received imidacloprid (purity 99.8%) for 90 days by oral gavage, dissolved in 0.9% saline containing 0.1% dimethyl sulfoxide and 0.5% Tween 20. The dose levels were 0, 0.06 or 0.6 mg/kg bw per day. At the

end of dosing period rats were sacrificed and blood samples collected for testosterone, estradiol and gonadotrophin (LH and FSH) assays. Testes and cauda epididymides were collected for histopathologic evaluation and spermatogenesis and sperm analysis, respectively.

A significant decrease in epididymal sperm concentration, accompanied by an increase in the percentage of teratospermia was found in both imidacloprid-exposed rat groups compared with the control group. Imidacloprid exposure significantly decreased the level of testosterone; testosterone levels in animals treated with 0.06 mg/kg bw per day were less than half of those in the controls. The testosterone level in animals treated with 0.6 mg/kg bw per day of imidacloprid were also significantly decreased. Arrested spermatogenesis and spermatogonial cells with fewer germ cells in seminiferous tubules were observed in the imidacloprid-treated groups. In both treated groups it was also observed that rats showed increased interstitial oedema with vacuolation and cystic dilatation of the seminiferous tubules. Additionally, the seminiferous tubules of different groups were compared during the same period of sperm division. In both groups of imidacloprid-treated rats, at the preleptotene stage, their spermatogonia and spermatocytes in the seminiferous epithelium were sparse compared with those of control group animals (Zhao et al., 2021b).

In a male reproductive study (not GLP-compliant), Wistar rats (five rats per group; 6–7 weeks old), received imidacloprid (purity 97%) by oral gavage dissolved in corn oil, at the doses of 0 or 9 mg/kg bw per day (five times a week) for 30 days. Serum, sperm, testis and prostate gland samples were collected from both groups at the end of the dosing period. The body weight and weights of the epididymis, seminal vesicles and epididymal, sperm numbers, morphology, and spermatozoa parameters were determined, as well as testosterone, LH and FSH levels. Enzymatic and non-enzymatic antioxidants in the testis were also assessed.

The body weight of imidacloprid-exposed rats was significantly reduced by 2.4-fold compared to the control animals. Rats exposed to imidacloprid had significantly increased testis weights; 14.2% greater compared to controls. These animals also exhibited a significant rise in prostate gland weight, by 20%. The imidacloprid-exposed rats showed a significant decrease in sperm count (32.1%) compared to controls and sperm motility was severely reduced, by 51%. Likewise, sperm normality in these treated rats was lowered significantly (by 49.2%) compared to control animals. Percentage sperm viability was significantly depressed, by 45.8% compared to controls. A morphological study of semen samples revealed a substantially lower proportion of normal morphological spermatozoa compared to spermatozoa of controls. A substantial decrease in SOD and GSH activity (26.9% and 61.6%, respectively) was observed in treated rats compared to controls. The testis CAT activity of treated rats was significantly higher (7.7%) than that of untreated rats. (Abdel-Razik et al., 2021).

(d) **Studies on metabolites**

In 2001 JMPR (JMPR, 2002) reviewed GLP-compliant studies on the acute toxicity of imidacloprid's metabolites, considering lethal doses in laboratory animals (route of administration not specified)These are summarized in Table 5.

Table 5.
Summary of acute toxicity studies with metabolites of imidacloprid

Metabolite	Species	LD_{50} (mg/kg bw), sex	Purity (%)	Reference
1-(6-Chloro-3-pyridylmethyl)-2-imidazolidinone	Rat	4080, M; 1820, F	99.9	Ohta , 1991a
1-(6-Chloro-3-pyridylmethyl)-*N*-nitro(4- imidazolin-2-ylidene) amine: an olefinic metabolite	Rat	3500, M 1100, F	98	Ohta , 1991b
1-(6-Chloro-3-pyridylmethyl)-*N*-nitroso(imidazolidin-2-ylidene) amine: a nitroso metabolite	Rat	1980, M 3560, F	98.1	Ohta , 1991c
1-(6-Chloro-3-pyridylmethyl)-*N*-nitroso (imidazolidin-2-ylidene) amine: a nitroso metabolite	Rat	> 600, M > 600, F	Not specified	Nakazato, 1988a
1-(6-Chloro-3-pyridylmethyl)-*N*-nitroso(imidazolidin-2-ylidene) amine: a nitroso metabolite	Mouse	200, M 200, F	Not specified	Nakazato, 1988b
1-(6-Chloro-3-pyridylmethyl)imidazolidin-2-ylideneamine	Rat	300, M; 280, F	87	Nakazato, 1991

bw: body weight; m: Male; F: female; LD_{50}: median lethal dose;

In a short-term study reviewed by JMPR (JMPR, 2002), Wistar rats (15 of each sex per group), received in their drinking water for 12 weeks the nitroso metabolite of imidacloprid, 1-(6-chloro-3-pyridylmethyl)-*N*-nitroso (imidazolidin-2-ylideneamine), at concentrations of 0, 100, 300 or 1000 mg/L (equal to 0, 13, 35 and 110 mg/kg bw per day for males, 0, 13, 39 and 120 mg/kg bw per day for females).

Water intake was decreased in the groups at 1000 mg/L. At 300 mg/L, higher lymphocyte counts and lower numbers of polymorphonuclear cells were observed. The NOAEL was 100 mg/mL (equal to 13 mg/kg bw per day) (Krötlinger, 1992).

JMPR (JMPR, 2002) and CalEPA (2012) evaluated in vivo and in vitro studies on genotoxicity of imidacloprid's metabolites and these evaluations are summarized below in Table 6.

Table 6.
Summary of in vitro and in vivo genotoxicity studies with metabolites of imidacloprid

Metabolite	End-point	Test object	Concentration or dose	Purity (%)	Result	Reference
In vitro						
1, 1-(6-chloro-3-pyridylmethyl)-2-imidazolidinone	Reverse mutation	*S. typhimurium* TA1535, TA100, TA1537, TA98; *E. coli* WP2uvrA	< 5000 µg/plate	99.9	Negative	Watanabe, 1991b
1-(6-chloro-3-pyridylmethyl)-*N*-nitro(4-imidazolin-2-ylidene)amine	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2uvrA	< 5000 µg/plate	98.0	Negative	Ohta, 1991d
1-(6-chloro-3-pyridylmethyl) imidazolidi-2-ylideneamine	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2uvrA	< 2500 µg/plate	87.0	Negative	Watanabe, 1991c
1-(6-chloro-3-pyridylmethyl)-*N*-nitroso(imidazolidin-2-ylidene) amine	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2uvrA	< 5000 µg/plate	98.3	Negative	Watanabe, 1990b
	Point mutation	*hprt* locus of Chinese hamster ovary cells	< 2000 µg/mL	94.3	Negative	Lehn, 1989b
	Point mutation	*hprt* locus of V79 Chinese hamster cells	< 2000 µg/mL	98.9	Negative	Lehn, 1989c
	DNA damage	*B. subtilis* H17 (rec+), M45 (rec–)	< 2000 µg/plate	98.1	Negative	Watanabe, 1991d
	Unscheduled DNA synthesis	Rat primary hepatocytes	5.0–750 µg/mL	98.9	Negative	Fautz, 1989
	Chromosomal aberrations	V79 Chinese hamster cells	< 1000 µg/mL	98.9	Negative	Heidemann, 1989
	Chromosomal aberrations	V79 Chinese hamster cells	1.0–0.25 mg/mL	Not specified	Negative	Usami, 1988a
In vivo						
1-(6-chloro-3-pyridylmethyl)-*N*-nitroso(imidazolidin-2-ylidene) amine	Micronucleus formation	Male BDF1 mice	Oral; 0, 40, 80, 160 mg/kg bw	96.4	Negative	Usami, 1988b
	Micronucleus formation	Male BDF1 mice	Intraperitoneal; 20, 40, 80 mg/kg bw	96.4	Negative	Usami, 1988c
	Micronucleus formation	Mice	Oral; 100 mg/kg bw	98.9	Negative	Herbold, 1989e
	Micronucleus formation	Mice	Oral; 50 mg/kg bw	98.9	Negative	Herbold, 1989f

2.3 Microbiological effects

The impact of imidacloprid residues on the human intestinal microbiome was evaluated through a decision-tree approach adopted by the sixty-sixth meeting of the Committee (JECFA, 2006), which complies with VICH guideline GL36 R (VICH, 2013). The guideline concerns test systems that can be used to address this toxicological end-point of concern, considering the complexity of the human intestinal microbiome. Assessing the effect on the human intestinal microbiome is an important aspect of JECFA work evaluation.

The VICH approach can be used to determine if a microbiological acceptable daily intake (mADI) is necessary, based on an evaluation of whether drug residues reach the human colon and are microbiologically active. It entails answering three questions to determine the need for establishing a mADI. First, determine if the drug residues, and/or its metabolites, are microbiologically active against representatives of the human intestinal microbiota; second, if the drug residues enter the human colon; and third, if the residues entering the human colon remain microbiologically active. If the answer to any of these questions is "no," then there is no need to calculate a mADI, and the assessment does not need to be completed. However, if a mADI needs to be calculated, two end-points of concern for human health are considered for the assessment: disruption of the colonization barrier of the human intestinal microbiome and increases in populations of resistant bacteria in the human intestinal microbiome.

A publication by Yang et al. (2020) described the toxic effects of imidacloprid on the intestinal barrier, gut microbiota and liver of mice. The study included 32 male mice (strain C57BL/6J; six weeks old) randomly divided into four groups; three exposure groups and a control. Mice received 0, 3, 10 or 30 mg/L of imidacloprid in their drinking water for 70 days. Samples from euthanized mice were taking from serum, liver, ileum, colon and colonic contents. Quantitative real-time polymerase chain reaction (qPCR) analysis were performed to quantify gene expression in imidacloprid-treated groups. Total genomic bacterial DNA was extracted from faecal samples and qPCR amplification was performed.

While studying the effects of imidacloprid exposure on the colon gut barrier it was shown that the secretion of mucus increased significantly in the colon of mice after exposure to imidacloprid at 30 mg/L. Immunohistochemical staining of MUC1 in the colon showed that protein levels of colonic MUC1 were increased in the 30 mg/L group when compared with the control. The expression level of mucus secretion-related genes in the colon was downregulated with 70 days of imidacloprid exposure, with the exception of the increased expression of the *MUC1* gene. Other secretion-related genes, including *Defa3, Defa20, Lyz* and *Mmp-7*, where downregulated in the mice receiving the highest dose (30 mg/L) of imidacloprid.

Levels of mRNA representing the *ZO1* and *Claudin-1* genes were also significantly downregulated in the colon, and the level of protein ZO1 also decreased in the 30 mg/L group. Although the level of serum lipopolysaccharide (LPS) content showed a rising trend, it was not statistically significant.

The evaluation of the main constituents of the faecal microbiota showed a significant increase in the relative abundance of Betaproteobacteria, Gammaproteobacteria and Verrucomicrobia in high-dose mice. Sequencing of 16S RNA was performed of microbiomes in the colonic contents of the controls and the 30 mg/L group, identifying a total of 520 operational taxonomic units

(OTUs), from which 52 and 208 OTUs were detected only in the control and the treated group, respectively. The Chao index of mice showed an upward trend based on α-diversity. In addition, principal co-ordinate analysis (PCoA) of weighted UniFrac distance and analysis of similarity (ANOSIM) showed that the gut microbiota of the treated mice changed greatly in the colonic content due to imidacloprid treatment.

At phylum level it was observed that there was a higher abundance of Bacteroidetes, but lower abundances of Firmicutes, Cyanobacteria, Verrucomicrobia and TM7 in the mice receiving 30 mg/L. In this group, at the genus level, the abundance of *Akkermansia* increased significantly, while the abundance of *Allobaculum* decreased significantly. Additionally, the number of Gram-negative bacteria increased, whereas the number of Gram-positive bacteria decreased. However, the number of aerobic bacteria increased, while the number of anaerobic bacteria decreased in the microbiome of the high dose group.

The authors indicated that the relative abundances of some colonic caecal bacteria appeared to increase relative to other bacteria, and they speculated that changes in bile acids were the cause of alterations in the bacterial content of the gut. Imidacloprid exposure studies showed that the gut barrier function was greatly impaired, and the sequencing of colonic contents showed the balance of the gut microbiota was disrupted. Furthermore, they indicated that even subchronic exposure to imidacloprid interfered with the gut barrier function, concluding that low-dose exposure influences the composition of the gut microbiota. However, this publication does not address the direct impact of imidacloprid in the intestinal microbiome.

In view of the above the Committee considered that there was insufficient information available to assess whether residues of imidacloprid have a direct impact in the intestinal microbiome. As a result, it was not possible to determine whether calculation of the mADI for imidacloprid is necessary. This conclusion also applies to the need to determine a microbiological acute reference dose (mARfD).

2.4 Observations in humans

Data from the medical department at Bayer AG concerning employees exposed to imidacloprid (Faul, 1996) and presented to JMPR (JMPR, 2002), showed no evidence of ill-health during the production of the active ingredient or its formulations. It was also reported from biological testing and field tests with imidacloprid formulations that no adverse effects on the health of operators or workers were identified. There have been reported mild cases of human contact dermatitis in pet owners after use of a veterinary formulation of imidacloprid, however this effect has been attributed to excipients in the product since dermatitis

has not been reported in pesticide workers. Furthermore, a four-year-old child who ingested about 10 mg/kg bw of a veterinary preparation of imidacloprid showed no signs of poisoning or adverse health effects.

No well conducted epidemiological studies of the effects of imidacloprid were available. Some cases of intentional or accidental exposure to imidacloprid have been published in the open literature, however these reports lack qualitative evidence and quantitative information.

3. Comments

3.1 Biochemical data

The pharmacokinetics of imidacloprid have been well documented in rats. Imidacloprid was rapidly and almost completely absorbed (92–99%) from the gastrointestinal tract of rats and was rapidly eliminated from the organism in the excreta (90% of administered dose [AD] within 24 hours), with no biologically significant differences shown between gender, different dose levels or routes of administration. Elimination was mainly renal (70–80% of AD), with 17–25% of AD in the faeces. The majority of faecal radioactivity originated in the bile. The radiolabel was rapidly distributed from the intravascular space into almost all organs and tissues, with the liver, kidney, lung, skin, and plasma being the major sites of distribution. Imidacloprid penetrated the blood–brain barrier to only a very limited extent. The maximum plasma concentration of radiolabel was reached between 1.1 and 2.5 hours after dosing (Klein, 1987a, b; Klein, 1990a, b; Klein & Brauner, 1991).

Metabolism of imidacloprid in mice and rats was rapid, the amount of unchanged parent compound representing 10–16% of AD. The parent imidacloprid initially underwent P450-dependent oxidation. The metabolites identified were 4-hydroxy-imidacloprid, 5-hydroxy-imidacloprid, 6-hydroxy-imidacloprid (6-hydroxy nicotinic acid), 6-chloronicotinic acid (6-CNA), 2-imidazolidone, olefin, guanidine and urea derivatives (Klein & Brauner, 1991; Karl, Klein & Weber, 1991; Klein, 1992; Kapoor et al., 2014; Nimako et al., 2021). Based on a clinical study, in a single male volunteer, and two in vitro studies, the two metabolic pathways identified in the rat were confirmed in humans. One route is via imidazolidine hydroxylation and desaturation to give 5-hydroxy-imidacloprid and the olefins, respectively. The other route is via nitroimine reduction and cleavage to yield the nitrosoimine, guanidine, and urea derivative products (Schulz-Jander & Casida, 2002; Khidkhan et al., 2021; Wrobel et al., 2022).

3.2 Toxicological data

In the JMPR 2001 toxicological monograph (JMPR, 2002), the oral LD_{50} of imidacloprid in mice was 130–170 mg/kg bw, and in rats 380–650 mg/kg bw. Behavioural and respiratory signs, disturbances in motility, narrowed palpebral fissures, transient trembling and spasms were seen in rats treated orally at doses of 71 mg/kg bw and above, and in mice at doses of 200 mg/kg bw and above. The clinical signs were reversed within six days.

In an acute oral toxicity study in the rat (GLP-compliant), submitted by the sponsor (Dreher, 2018), no clinical signs occurred at 130 mg/kg bw and the oral acute LD_{50} was estimated to be 1300 mg/kg bw (494–1740 mg/kg bw).

In relation to the short-term toxicity studies, the Committee reviewed the JMPR monograph (JMPR, 2002) and relevant studies published in the scientific literature. Reduction in body weight was the most common toxic effect observed in oral studies with mice, rats and dogs. The liver was the principal target organ as demonstrated by hepatic necrosis or hypertrophy in rats and dogs, elevated activities of serum enzymes and alteration in clinical chemistry parameters.

In one study (not GLP-compliant), mice were fed diets containing imidacloprid for 107 days at doses of 0, 0.17, 86 or 430 mg/kg bw per day. A NOAEL of 17 mg/kg bw per day was identified, based on an increase in ALP activity and a reduction in body weight (in males) at 86 mg/kg bw per day (Eiben, 1988a), as reported by JMPR 2001 (JMPR, 2002).

In another study, rats were exposed daily for up to 98 days to imidacloprid at concentrations of 0, 120, 600 or 3000 mg/kg feed (equal to 0, 11, 57 and 410 mg/kg bw per day for males, 0, 14, 78 and 510 mg/kg bw per day for females). The NOAEL was 120 mg/kg feed (equal to 11 mg/kg bw per day) based on a reduction in body weight at 600 mg/kg feed (equal to 57 mg/kg bw per day) (Eiben, 1988b).

In a further study, rats received diets containing imidacloprid at concentrations of 0, 150, 600 or 2400 mg/kg feed (equal to 0, 14, 61 and 300 mg/kg bw per day for males, 0, 20, 83 and 420 mg/kg bw per day for females) for up to 96 days. Satellite groups received the test substance at concentrations of 0 or 2400 mg/kg feed over the same period, followed by a four-week observation period following cessation of treatment. The NOAEL was 150 mg/kg feed (equal to 14 mg/kg bw per day) based on liver toxicity and reduced body weight at 600 mg/kg feed (equal to 61 mg/kg bw per day). These liver effects were reversible after four weeks (Eiben & Rinke, 1989).

In a study retrieved from the literature, rats were administered imidacloprid orally by gavage for 90 days at doses of 0, 5, 10 or 20 mg/kg bw per day. The NOAEL was 10 mg/kg bw per day, based on morphological, biochemical, haematological, and neuropathological changes in the brain, liver and/or kidney

of females at 20 mg/kg bw per day (Bhardwaj et al., 2010).

In another published study carried out by the same authors, imidacloprid was administered orally by gavage for 90 days to female rats at doses of 0, 5, 10 or 20 mg/kg bw per day. The NOAEL was 10 mg/kg bw per day, based on clinical signs and reduced weight gain at 20 mg/kg bw per day (Kapoor et al., 2010).

In an additional study conducted by the same researchers, female rats received imidacloprid by gavage for 90 days at doses of 0, 5, 10 or 20 mg/kg bw per day. The NOAEL was 10 mg/kg bw per day based on clinical signs, reduced weight gain and changes in ovarian morphology and hormones at 20 mg/kg bw per day (Kapoor, Srivastava & Srivastava, 2011).

In a 13-week study in dogs, animals received diets containing imidacloprid at concentrations of 0, 200, 600 or 1800/1200 mg/kg feed (equal to 0, 7.5, 24 or 67.5/45 mg/kg bw per day). A NOAEL was identified at 200 mg/kg feed (equal to 7.5 mg/kg bw per day) based on tremors occurring in the first week, in animals treated with 600 mg/kg feed (equal to 24 mg/kg bw per day) (Ruf, 1990).

In a 52-week study, dogs received diets containing imidacloprid at 0, 200, 500 or 1250 rising to 2500 mg/kg feed on week 17 (equal to 0, 6.1, 15 and 41 rising to 72 mg/kg bw per day). The NOAEL was 500 mg/kg feed (equal to 15 mg/kg bw per day) based on liver changes at 1250 mg/kg feed (equal to 41 mg/kg bw per day) (Allen et al., 1989).

For evaluation of long-term toxicity and carcinogenicity, the studies provided to JMPR (JMPR, 2002) and a more recent one-year study submitted by the sponsor were assessed.

Imidacloprid was administered in the feed to mice for 24 months at concentrations of 0, 100, 330 or 1000 mg/kg feed (equal to 0, 20, 66 and 208 mg/kg bw per day for males, 0, 30, 104, 274 mg/kg bw for females). There was no evidence of a carcinogenic effect. The NOAEL was 330 mg/kg feed (equal to 66 mg/kg bw per day) based on reduction in body weight at 1000 mg/kg feed (equal to 208 mg/kg bw per day) (Watta-Gebert, 1991a, b).

In a one-year, repeat-dose, oral toxicity study rats received imidacloprid in the diet at concentrations of 0, 100, 300 or 1000 mg/kg feed (equal to 0, 5.6, 16.3 and 55.8 mg/kg bw per day for males, 0, 6.7, 19.5 and 63.7 mg/kg bw per day for females). The NOAEL was 200 mg/kg feed (equal to5.6 mg/kg bw per day) based on decreased body weight gain at 300 mg/kg feed (equal to16.3 mg/kg bw per day) (Britton, 2019).

In a two-year toxicity/carcinogenicity study, imidacloprid was administered to rats at dietary levels of 0, 100, 300 or 900 mg/kg feed (equal to 0, 5.7, 17 and 51 mg/kg bw per day for males, 0, 7.6, 26 and 73 mg/kg bw per day for females). In a supplemental study to examine the maximum tolerated dose, rats were given diets containing imidacloprid for 24 months at concentrations of 0 or 1800 mg/kg feed (equal to 0 and 103 mg/kg bw per day for males, 0 and

144 mg/kg bw per day for females). There was no evidence of a carcinogenic effect. The NOAEL was 100 mg/kg feed (equal to 5.7 mg/kg bw per day), based on an increase in incidence and severity of mineralized particles in the thyroid gland at 300 mg/kg feed (equal to 17 mg/kg bw per day) (Eiben & Kaliner, 1991; Eiben, 1991).

The Committee concluded that imidacloprid was not carcinogenic in rats or mice.

The genotoxicity of imidacloprid was assessed by JMPR in 2001 (JMPR, 2002) and the following stated:

> *"Imidacloprid gave negative results in an adequate range of assays for genotoxicity in vitro and in vivo. Weak induction of sister chromatid exchange was found in one test with Chinese hamster ovary cells in vitro, but not in vivo. The Meeting concluded that imidacloprid is unlikely to be genotoxic or to pose a carcinogenic risk to humans."*

Subsequently, three genotoxicity studies of imidacloprid were submitted by the sponsor. In these an Ames test was negative, an in vitro micronucleus test was negative following three hours incubation, but positive after 24 hours incubation (Hargreaves, 2018a, b) and imidacloprid was shown to be negative in an adequately conducted in vivo micronucleus study in rats (Dunton, 2019). A number of in vitro and in vivo genotoxicity studies were also found in the open literature, however most of them were performed using commercial formulations of imidacloprid or had experimental flaws resulting in inconsistent data.

The Committee concluded that imidacloprid is unlikely to be genotoxic in vivo at doses expected from the diet.

The Committee concluded that imidacloprid is unlikely to pose a carcinogenic risk to humans from the diet, given that it is unlikely to be genotoxic in vivo and it is not carcinogenic in rats or mice.

The evaluation of reproductive and developmental toxicity was based on data provided by JMPR 2001 (JMPR, 2002) and studies submitted by the sponsor.

In a two-generation study described in detail by CalEPA (2006), rats received diets containing imidacloprid at concentrations of 0, 100, 250 or 700 mg/kg feed (equal to 0, 6.6, 17 and 47 mg/kg bw per day). The NOAEL for parental effects was 100 mg/kg feed (equal to 6.6 mg/kg bw per day) based on decreased premating body weights at 250 mg/kg feed (equal to 17 mg/kg bw per day). The NOAEL for reproductive effects was 700 mg/kg feed (equal to 47 mg/kg bw per day), the highest dose tested. The NOAEL for offspring effects was 2500 mg/kg feed (equal to 17 mg/kg bw per day) based on a decrease in pup body weight at 700 mg/kg feed (equal to 47 mg/kg bw per day) (Suter et al., 1990).

In an extended one-generation reproductive toxicity study, rats received diets containing imidacloprid at concentrations of 0, 100, 300 or 1000 mg/kg feed (equal to 0, 5.25, 15.35 and 48.4 mg/kg bw per day for males, 0, 10.4, 30.43 and 85.6 mg/kg bw per day for females). The NOAEL for parental toxicity was 100 mg/kg feed (equal to 5.25 mg/kg bw per day) based on reduced body weight at 300 mg/kg feed (equal to 15.35 mg/kg bw per day). The NOAEL for offspring toxicity was 100 mg/kg feed (equal to 10.4 mg/kg bw per day) based on reduced pup body weights at 300 mg/kg feed (equal to 30.43 mg/kg bw per day). The NOAEL for reproductive toxicity was 1000 mg/kg feed (equal to 48.4 mg/kg bw per day), the highest dose tested (Holalagoudar, 2019).

In a prenatal study of developmental toxicity, imidacloprid was administered daily by gavage to mated female rats from GDs 6 to15, at doses of 0, 10, 30 or 100 mg/kg bw per day. The NOAEL for maternal effects was 10 mg/kg bw per day based on effects on weight gain. The NOAEL for embryo/fetal toxicity was 30 mg/kg bw per day based on a delay in embryo development at 100 mg/kg bw per day (Becker, Vogel & Terrier, 1988a).

In a study of developmental toxicity, rats were administered imidacloprid by gavage at doses of 0, 5, 15 or 50 mg/kg bw per day, from GDs 6 to 19. The NOAEL was 15 mg/kg bw per day for maternal effects based on weight loss and reduced food intake at 50 mg/kg bw per day. the NOAEL for embryo-fetal toxicity was 50 mg/kg bw/day, the highest dose tested (Britton, 2018b).

In a study of developmental toxicity, pregnant rabbits were administered imidacloprid by oral gavage at doses of 0, 8, 24 or 72 mg/kg bw per day, on GDs 6–18. The NOAEL for maternal toxicity was 8 mg/kg bw per day based on reduced food consumption and body weight gain observed at 24 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 24 mg/kg bw per day based on increased postimplantation loss, reduced body weight and delayed ossification at 72 mg/kg bw per day (Becker, Vogel & Terrier, 1988b).

The Committee concluded that imidacloprid is not teratogenic.

A study of the immunotoxicity of imidacloprid in mice was retrieved from the scientific literature and evaluated. Organ weights and a number of haematological and immunological parameters were measured. The Committee identified some inconsistencies in the findings, concluding that the study was not suitable for the risk assessment of imidacloprid (Badgujar et al., 2013).

The immunotoxic effects of imidacloprid were evaluated in F1 rats from the extended one-generation reproductive toxicity study described above (Holalagoudar, 2019). The F1 rats received imidacloprid in their feed as detailed earlier for up to 61 days, providing doses of 0, 11.4, 36.9 or 120.4 mg/kg bw per day for males, 0, 11.9, 35.5 or 121.0 mg/kg bw per day for females. The NOAEL for immunotoxicity was 121 mg/kg bw per day, the highest dose tested (Holalagoudar, 2019).

In a study of acute neurotoxicity, rats were administered imidacloprid by gavage at doses of 0, 42, 151 and 307 mg/kg bw. Both neurobehaviour and neuropathology were assessed. No NOAEL could be identified, as neurobehavioural effects were observed at all doses, although this is equivocal as effects observed at the lowest dose were not statistically significant (Sheets, 1994a). Using the data from this study, CalEPA calculated a $BMDL_{05}$ (lower confidence limit on the benchmark dose for a 5% response) of 9 mg/kg bw, based on decreases in the motor and locomotor activity CalEPA (2006).

In a 13-week neurobehavioural study of imidacloprid in adult rats fed 0, 140, 960 or 3000 mg/kg feed (equal to 0, 9.3, 63 and 196 for males, 0, 10.5, 69 and 215 for females), functional observational battery (FOB) changes were observed in males in the highest dose group. The NOAEL was140 mg/kg feed (equal to 9.3 mg/kg bw per day) based on decreases in body weight gain and food consumption at the LOAEL of 960 mg/kg feed (equal to 63 mg/kg bw per day) (Sheets, 1994c).

In a one-year toxicity study described above, imidacloprid was administered to male and female adult rats in the diet at levels of 0, 100, 300 or 1000 mg/kg feed (equal to 0, 5.6, 16.3 and 55.8 mg/kg bw per day for males, 0, 6.7, 19.5 and 63.7 mg/kg bw per day for females). Observations were made in the open-field arena and FOB responses recorded at various times. The NOAEL for neurotoxicity was 1000 mg/kg feed (equal to 55.8 mg/kg bw per day), the highest dose tested (Britton, 2019).

In a developmental neurotoxicity study, imidacloprid was administered to female rats in the diet at doses of 0, 8, 19 and 54.7 mg/kg bw per day, from GD 0 until PND 21. An offspring NOAEL was identified at 19 mg/kg bw per day based on reduction in body weight and decreased motor activity in groups treated with 54.7 mg/kg bw per day (Sheets, 2001).

The developmental toxicity and developmental brain neuropathology of imidacloprid was also evaluated in F1 rats from an extended one-generation reproductive toxicity study described above. Animals received the same dietary concentrations of imidacloprid as the parental animals, that is 0, 100, 300 or 1000 mg/kg feed (equal to 0, 11.4, 36.9, 120.4 mg/kg bw per day for males, 0, 11.9, 35.5 and 121.0 mg/kg bw per day for females).Two cohorts were examined, one for acoustic startle, motor activity and FOB assessments, the other for brain histomorphometric evaluation. The NOAEL was 1000 mg/kg feed (equal to 120.4 mg/kg bw per day), the highest dose tested (Holalagoudar, 2019).

Three studies evaluating the effects of imidacloprid on the male reproductive system in rats were retrieved from the literature. The Committee considered that these studies did not provide sufficient details and displayed inconsistent results, hence they could not be used for risk assessment. It was

further noted that no effects indicative of male reproductive toxicity were identified in multigeneration or repeat-dose toxicity studies.

Studies on the acute oral toxicity and genotoxicity of several of imacloprid's metabolites were assessed by JMPR in 2001 (JMPR, 2002). The metabolites examined were*:*

1-(6-chloro-3-pyridylmethyl)-2-imidazolidinone;
1-(6-chloro-3-pyridylmethyl)-*N*-nitro(4-imidazolin-2-ylidene)amine;
1-(6-chloro-3-pyridylmethyl)imidazolidi-2-ylideneamine;
1-(6-chloro-3-pyridylmethyl)-*N*-nitroso(imidazolidin-2-ylidene)amine).

These metabolites were found to be less acutely toxic than the parent compound, and showed no evidence of genotoxicity.

Table 7.
Studies relevant to the risk assessment

Species/study type (route of administration)	Doses (mg/kg bw per day)	Critical end-point	NOAEL (mg/kg bw per day)	LOAEL (mg/kg bw per day)
Mouse				
Two-year toxicity study (dietary)	Male: 0, 20, 66 and 208 Female: 0, 30, 104 and 274	Reduction in body weight	66	208
Rat				
Acute neurotoxicity study (gavage)	0, 42, 151, 307 mg/kg bw	Decreased locomotor activity in females	9** (BMDL)	42
One-year toxicity study (dietary)	Male: 0, 5.6, 16.3 and 55.8 Female: 0, 6.7, 19.5 and 63.7	Reduction in body weight	5.6	16.3
Two-year study (dietary)	Male: 0, 5.7, 17 and 51 Female: 0, 7.6, 26 and 73	Increased incidence and severity of mineralized particles in the thyroid gland in male rats	5.7	17
Extended one-generation study (dietary)	Male F0:0: 5.25, 15.35 and 48.4 Female F0: 0, 10.4, 30.43 and 85.6	Reproductive toxicity: cycle length, mating, fecundity and fertility index	85.6	
		Parental toxicity: lower mean body, decrease food consumption	5.25*	15.35
		Offspring toxicity: decrease in pups delivered, increased stillborn, decrease in live pups, increase T4 concentration, reduced weights, lower spleen and thymus weights	10.4	30.43
Developmental toxicity study (gavage)	0, 5, 15 and 50	Maternal toxicity: reduced body weight and decreased food consumption	15	50
		Developmental toxicity: Nil	50	
Dog				
90-day (dietary)	0, 7.5, 24 or 67.5/45	Tremors	7.5	24

LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level.
* Pivotal study for the derivation of the toxicological ADI (Holalagoudar, 2019)
** Pivotal study for the derivation of the toxicological ARfD (Sheets 1994a)

3.3 Microbiological data

The impact of imidacloprid residues on the human intestinal microbiome was evaluated through a decision-tree approach adopted by the sixty-sixth meeting of the Committee (JECFA, 2006), which complies with the VICH guideline GL36(R) (VICH, 2013). This entails answering three questions to determine the need for establishing an mADI. Determine first if the drug residue, and/or its metabolites, are microbiologically active against representatives of the human intestinal microbiota. Second determine if the drug residues enter the human colon, and third, if the residues entering the human colon remain microbiologically active. If the answer to any of these questions is "no", then there is no need to calculate a mADI and the assessment does not need to be completed. However, if a mADI needs to be calculated, two end-points of concern for human health are considered for the assessment: disruption of the colonization barrier of the human intestinal microbiome and increases in populations of resistant bacteria in the human intestinal microbiome.

A published, non-GLP study in mice (Yang et al., 2020) examined imidacloprid's effects on the intestinal microbiome. Mice were fed imidacloprid in drinking water at 0, 3, 10 or 30 mg/L (equivalent to 0, 0.5, 1.67 and 5 mg/kg bw per day) for 70 days. Results indicated that relative abundances of some colonic caecal bacteria appeared to increase relative to others in the treated group. The authors concluded that following imidacloprid exposure studies showed that the intestinal barrier function was greatly impaired, and that the sequencing of colonic contents revealed the balance of the gut microbiota was disrupted. However, this publication does not address the direct impact of imidacloprid on the intestinal microbiome. Thus, there was insufficient information for the Committee to assess whether residues of imidacloprid have direct impact in the intestinal microbiome. As a result, it was not possible to determine if there is a need for the calculation of a mADI for imidacloprid. This conclusion also applies to the need to determine a mARfD. The Committee was aware of an ongoing GLP study to elucidate the direct effects of imidacloprid on some human intestinal microbiome representative bacteria that would assist in the evaluation.

4. Evaluation

In view of the absence of a study to assess the impact of imidacloprid on representative human intestinal microbiota, it was not possible to determine an mARfD or an mADI, thus the Committee was unable to establish an ARfD or an ADI for imidacloprid.

The Committee established a toxicological acceptable daily intake (tADI) of 0–0.05 mg/kg bw on the basis of a NOAEL of 5.25 mg/kg bw per day

for decreased body weight gain in the extended one-generation reproduction study, with the application of a safety factor of 100 to allow for interspecies and intraspecies differences.

The Committee established a toxicological acute reference dose (tARfD) of 0.09 mg/kg bw based on a $BMDL_{05}$ of 9 mg/kg bw reported by CalEPA for acute neurobehavioural effects in rats, and a safety factor of 100 to allow for interspecies and intraspecies differences. This value was supported by a NOAEL of 7.5 mg/kg bw per day for tremors occurring during the first week of treatment, in a 90-day toxicity study in dogs, although it is not known whether tremors occurred after the first dose

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Nicarbazin

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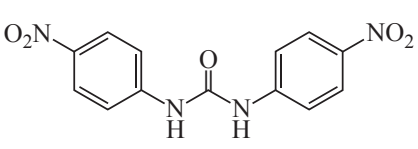
1. Explanation

Nicarbazin 1,3-*bis*(4-nitrophenyl)urea;4,6-dimethyl-*1H*-pyrimidin-2-one (IUPAC) Chemical Abstract Service number 330-95-0, is a carbanilide used for the prevention of faecal and intestinal coccidiosis in chickens, as well as in some other poultry species. Nicarbazin is used as a feed additive or as a veterinary drug for oral use in feed.

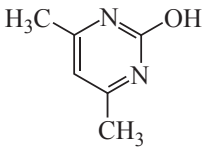
Nicarbazin is an equimolar complex of 4,4′-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP). The nicarbazin complex is almost completely insoluble, but will dissociate completely under acidic, aqueous conditions, such as the digestive tract, and the resulting components dissolve readily. The active anticoccidial component is DNC, while HDP has no anticoccidial activity. The absorption of DNC is greatly enhanced when the

two components are complexed together. The mode of action (MOA) of DNC is unclear but may involve the inhibition of mitochondrial electron transport.

Figure 1
Structure of nicarbazin; component parts DNC and HDP



DNC (4,4'-dintrocarbanilide)



HDP (4,6-dimethylpyrimidine)

Nicarbazin was evaluated for toxicology and residues by the Committee at its fiftieth meeting (JECFA, 1999). An ADI of 0–400 µg/kg bw nicarbazin (24 mg per person for a 60 kg person) was established. Maximum residue limits (MRLs) for chicken muscle, liver, kidney and skin/fat (in natural proportions) were recommended at 200 µg/kg nicarbazin, using DNC as the marker residue.

The Committee evaluated nicarbazin at the present meeting at the request of the twenty-fifth session of the Codex Committee on Residues of Veterinary Drugs in Food (CCRVDF) with a view to recommending MRLs for edible chicken tissues. A toxicological re-evaluation was also undertaken to establish health-based guidance values (HBGVs) due to the time that had elapsed since its last review. The sponsor provided unpublished proprietary studies as well as data from studies in the published literature to support the assessment.

The inclusion rate for nicarbazin provided by the sponsor was 125 mg/kg bw nicarbazin per day in complete feeding stuffs to be used in chickens for fattening. For this dose, withdrawal periods ranged from 1–10 days for edible tissues. The Committee noted that a higher inclusion rate of 200 mg/kg feed is approved in at least one Member State, with a withdrawal period of five days, but no residue data were provided for this dosing regimen. When used in combination with either narasin or monesin the nicarbazin inclusion rate is lower at 50 mg/kg feed and withdrawal periods range from 0–8 days. Products are not intended for use in animals producing eggs for human consumption.

Nicarbazin is not currently used as a plant protection product or as a human medicine.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

In studies conducted prior to the introduction of good laboratory practice (GLP), rats were given a single oral dose of 1, 5 or 10mg/kg body weight (bw) nicarbazin, and 6 or 18 hours after administration the animals were killed and blood levels of the DNC and HDP components determined (details not provided). The pyrimidone component was detected in considerably higher concentrations, which increased between six and 18 hours of exposure. Qualitatively similar findings were obtained in rats treated orally with 0.1, 1 or 5mg/kg bw per day for eight days and killed four or 24 hours after the administration of the last dose. Blood concentrations of HDP were dose-dependent, while those of the DNC remained unchanged. In the latter experiment, the urine at five hours after the last dose contained each of the nicarbazin components regardless of dose, but the concentration of HDP was an order of magnitude greater than that of DNC (Kuna et al., 1955).

The effect of HDP on the bioavailability of DNC was evaluated in a study conducted in accordance with GLP (Lloyd, 2009a). Forty-five male rats were assigned to the study, all jugular vein-cannulated. The medication was administered by oral gavage to three treatment groups. Equivalent doses between groups were selected based on the molecular weight of DNC. The first group received nicarbazin (the DNC and HDP complexed) at doses of 0, 50, 150 or 450mg/kg bw. The second group received a simple mixture of DNC and HDP at 0 + 0, 35 + 15, 106 + 44 or 319 + 131mg/kg bw. The final group received only DNC at 0, 150, 450 or 900mg/kg bw. Blood samples were collected via the cannula at 0.5, 1, 2, 4, 8, 12, 24, 36, 48 and 72 hours. Concentrations of DNC were quantified, and the pharmacokinetics (PK) for each group analysed. The addition of HDP as a simple admixture did not appear to alter the relative bioavailability of DNC.

The mean terminal half-life ($t_{½}$) of DNC was 5.98–13.8 hours when given as nicarbazin, 8.71–26.4 hours when given alone, and 6.49–17.3 hours when given as a simple mixture of DNC and HDP.

The total area under the concentration–time curve ($AUC_{0-\infty}$) of DNC when given as nicarbazin at a DNC-equivalent dose of 319mg/kg bw was 8518 hour . ng/mL. This was more than 20-fold higher than the $AUC_{0-\infty}$ for DNC administered alone (269 hour . ng/mL) or as a mixture of DNC + HDP (251 hour . ng/mL), as shown in Table 1 below.

Table 1
Summary of kinetic parameters for DNC alone, DNC with HDP and nicarbazin in rats, (means ± SD)

	Dose Level (mg/kg bw as DNC)	$AUC_{(0-\infty)}$ (hour.ng/mL)	Half-life (hours)	CL/F (mL/hr per kg bw)
DNC alone	106	209 ± 226	19.3 ± 15.5	911 123 ± 591 377
	319	269 ± 59.0	8.71 ± 3.69	1 244 967 ± 342 072
DNC + HDP	106	202 ± 90.8	9.53 ± 3.29	634 301 ± 319 596
	319	251 ± 83.0	17.3 ± 19.6	1 384 346 ± 462 185
nicarbazin	106	4140 ± 748	11.1 ± 13.7	14 626 ± 5273
	319	8518 ± 4621	5.98 ± 0.939	28 110 ± 16 427

$AUC_{(0-\infty)}$: Total area under the concentration–time curve; CL/F: Oral clearance, $AUC_{(0-\infty)}$ /dose ; Source: modified from Lloyd, 2009a

Taking into consideration bioavailability, PK parameters and the overall DNC concentrations, it was clear that the administration of nicarbazin results in a considerably higher absorption of DNC than when DNC is administered alone or as a simple mixture with HDP.

Rogers (1983) indicated that the dissolution of the DNC–HDP complex in the gut results in the formation of DNC crystals that are much smaller than if relatively insoluble DNC is administered alone. Hence, the presence of an intermolecular bonded moiety such as HDP, as in nicarbazin, appears to be necessary for the effective absorption of DNC from the gastrointestinal (GI) tract.

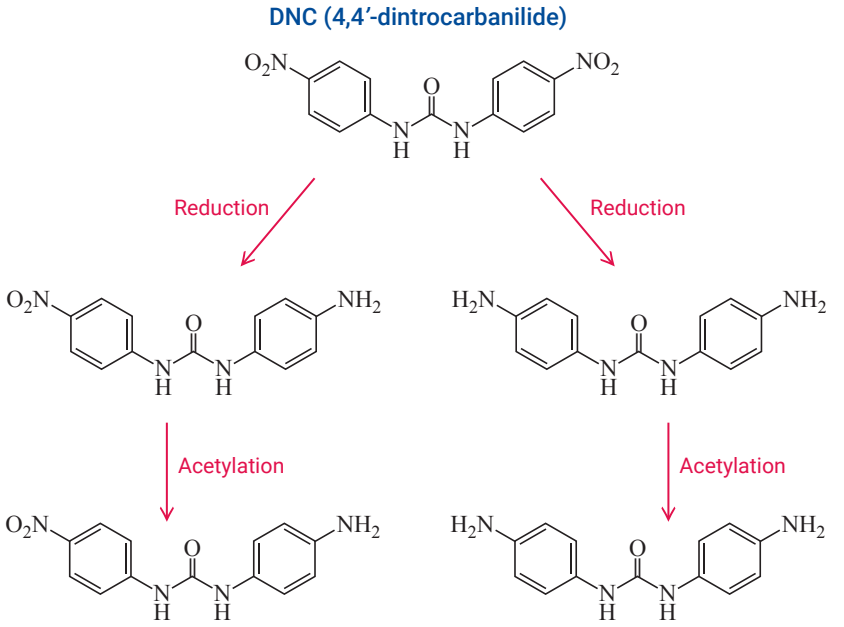
2.1.2 Biotransformation

Metabolic studies of nicarbazin show that it completely dissociates into DNC and HDP, which then behave independently. The DNC is excreted mainly (46% unchanged DNC) in faeces, whilst HDP is excreted mainly (90% unchanged HDP) in urine. The metabolic pathways of DNC are well established and its major metabolites in excreta have been identified. Major metabolites in bile and tissues are: monoacetylamino-DNC (M1, resulting from reduction and acetylation of one nitro group), and diacetylamino-DNC (M3, corresponding to the reduction and acetylation of both nitro groups). Metabolite *N*,*N′*-1,4-phenylene-*bis*(acetamide) is also produced by molecular splitting, reduction and acetylation. (EFSA, 2003).

Rats were administered nicarbazin radiolabeled with ^{14}C in the DNC moiety in their diet at about 100mg/kg feed (1mg/kg bw per day) for five days (Manthey, 1985). Urine and faeces were collected and analysed for metabolites. Parent DNC and metabolite M1 (DNC with one nitro group reduced and acetylated) were the major constituents found in the faeces. Metabolites M1 and M3 (DNC with both nitro groups reduced and acetylated) were the major constituents found in urine. Metabolites M1 and M3 were also found in chickens

and this finding indicates that the metabolic fate of nicarbazin is the same in rats as it is in chickens.

Figure 2.1
Metabolic pathways of DNC



Source: Manthey, 1985

2.1.3 Effects on enzymes and other biochemical parameters

No data was submitted.

2.2 Toxicological studies

2.2.1 Acute toxicity

(a) Lethal doses

Detailed information on the acute toxicity of nicarbazin was not available but a summary was available in the form of a report of a series of studies conducted in the 1950s (Kuna et al., 1955). These studies were conducted prior to the introduction of GLP or the development of guidelines for the conduct of toxicological studies. This information was considered only to be supportive. The acute oral toxicity of nicarbazin in rodents was low, the LD_{50} value being greater than 25 000 mg/kg bw

in mice (Table 2). No toxic signs were observed in mice in the 24 hours following administration of up 20 000mg/kg bw nicarbazin. At 25 000mg/kg bw there was 30% mortality. In the subsequent seven days after administration, one or two animals died in the groups that had received 15 000, 20 000 and 25 000mg/kg bw. The LD_{50} in rats was higher than 10 000 mg/kg bw (Table 2). There were no signs of acute or delayed toxicity, or of mortality over the seven days of observation in rats administered up to 10 000 mg/kg bw. The individual components of nicarbazin also displayed low acute toxicity. The individual components of nicarbazin also displayed low acute toxicity: the oral LD_{50} in mice for HDP was 4000 mg/kg bw and for DNC it was greater than 18 000 mg/kg bw (Kuna et al., 1955).

Table 2
Oral LD_{50} of nicarbazin

Species	Sex	LD_{50} (g/kg bw)
Mouse	Unspecified	> 25
Rat	Unspecified	> 10

The acute toxicity of nicarbazin via inhalation was determined in a study performed according to Organisation for Economic Co-operation and Development (OECD) guideline 403. A group of ten rats (five of each sex) was exposed, nose only for four hours, to an atmosphere with a mean achieved concentration of nicarbazin of 5.12 ± 0.20 mg/L, followed by observation for 14 days. No deaths or signs of toxicity, either on observation of the animals or macroscopically at necropsy, were observed. The acute inhalation median lethal concentration (four-hour LC_{50}) of nicarbazin in the rat was > 5.12 mg/L. No other details of this study were available (EFSA, 2017).

The acute dermal toxicity of a mixture of DNC and HDP was assessed in rats: for this study information on compliance with GLP or OECD guidelines was not available. Sprague Dawley rats (five animals per sex) were dosed dermally with a 3:1 mixture of DNC and HDP equal to 2000mg/kg bw. The test mixture was kept in contact with the skin for 24 hours. Dermal responses were recorded at 24 hours post dosing and on day 14. Animals were observed for mortality, toxicity and pharmacological effects one hour and four hours post dosing and then once daily for 14 days. Body weights were recorded on days 0, 7 and 14. On termination, animals were examined microscopically. There were no deaths. Clinical signs observed included chromorhinorrhea, chromodacryorrhea and wetness of the anogenital area. Two females lost body weight from day 0 to day 7 and one female from day 7 to day 14. All animals had gained body weight by the end of the study. There was no, or only very slight, erythema at the site of administration, but this showed test article staining which persisted until termination on day 14. There were no macroscopic changes evident at necropsy (EFSA, 2021). The dermal LD_{50} for a mixture of DNC and HDP was > 2000mg/kg bw.

(b) **Dermal irritation**

Nicarbazin's potential to cause skin irritation was assessed in rabbits according to OECD TG 404. Nicarbazin was very slightly irritant when applied dermally to the skin of rabbits. Only a brief summary of this report was available, so it was not possible to assess the data in any detail (EFSA, 2017).

According to a report by EFSA (2021), a mixture of DNC and HDP, in the proportion 3:1 respectively, was very slightly irritating to the skin of rabbits, when tested according to OECD TG 404.

(c) **Ocular irritation**

Nicarbazin's potential to cause eye irritation was assessed in rabbits following OECD guideline 405. Scores after 24, 48 and 72 hours were all close to zero for chemosis, conjunctival redness, iris lesions and corneal opacity following a single ocular administration of nicarbazin, hence nicarbazin was not considered to be irritating to the eyes of rabbits. Only a brief summary of this report was available, so it was not possible to assess the data in any detail (EFSA, 2017).

In a study conducted according to OECD TG 405 and reported by EFSA (2021), a mixture of DNC and HDP, in the proportion 3:1 respectively, was severely irritating and corrosive when applied to the eyes of rabbits. Only a brief summary of this report was available, so it was not possible to assess the data in any detail.

(d) **Dermal sensitization**

The skin sensitization potential of a commercial preparation containing nicarbazin was investigated in Duncan/Hartley Guinea pigs according to the Buehler method. The mixture tested consisted of 25% w/w nicarbazin, together with stearic acid (12.60%), polysorbate 20 (1.39%) and wheat middlings (61.01%). Nicarbazin was applied to the shaved skin of the left shoulder of twenty female Guinea pigs. The dose was 0.5 mL of a 40% w/v solution of the commercial preparation in water, which was equivalent to 50 mg of nicarbazin. Ten females serving as controls were treated with vehicle alone. Induction was for six hours under surgical gauze, after which the dressing was removed. Three applications per week were made for three weeks: a total of nine applications. The application site was assessed for erythema and oedema 24 hours after removal of the dressing. Two weeks after the last induction treatment, animals were challenged with the same dose of nicarbazin as used for induction, applied to the shaved flanks. After six hours under a surgical gauze the dressings were removed. The challenge sites were assessed 24 hours and 48 hours after removal of the dressings. Animals were observed throughout for clinical signs, and body weight was recorded on day 1 and on the day of study termination. The treated animals exhibited no mortality, clinical signs of toxicity or effects on body weight. There were no dermal reactions

in any of the animals. The positive control, hexyl cinnamic aldehyde, produced delayed contact hypersensitivity (skin sensitization) (Coleman, 2001).

In a study reported briefly by EFSA (2017), the skin sensitization potential of nicarbazin was assessed in a murine local lymph node assay, performed following OECD TG 429 (it is possible that this is the same study as reported below, for DNC and HDP). No mortality or clinical signs were observed during the study. There was no cutaneous reaction or increase in ear thickness following treatment with nicarbazin. No lymphoproliferation was observed. Nicarbazin showed no evidence of causing skin sensitization in this study. Only a brief summary of the report was available, so it was not possible to assess the data in any detail.

The skin sensitization potential of a mixture of DNC and HDP (ratio not stated) was determined in a murine local lymph node assay according to OECD TG 429. No evidence of skin sensitization by was observed. Only a brief summary of the report was available, so it was not possible to assess the data in any detail (EFSA, 2021).

2.2.2 Short-term toxicity

(a) Rats

Only summaries of the short-term studies on nicarbazin were available for rats, and these reports were inadequate for a detailed evaluation as they contained minimal detail of the protocols used, limited data on toxicological findings, and often took the form of progress reports. Therefore, the data were considered as supporting information only. These studies were conducted prior to the introduction of GLP or the development of guidelines for the conduct of toxicological studies.

In rats (10 per group) treated with nicarbazin at 0, 1 or 5 g/kg bw per day by gavage for up to 67 days, both treated groups showed an increase in water consumption (two-fold compared to that of the control group), and a reduction of 16% and 16.7% in body weight gain at doses of 1 and 5 g/kg bw per day, respectively (Stebbins, 1954). In all groups rats died prior to scheduled termination (one, three and five rats in the 0, 1 and 5 g/kg bw per day groups, respectively). In both groups of treated rats, haemoconcentration was observed initially, but animals were anaemic at the end of the treatment period. After 10 days, four animals per group were killed for macroscopic and microscopic examination. Both groups showed markedly enlarged, pale kidneys. Histopathologically, renal tubular dilatation with interstitial nephritis and the presence of crystals were observed in both groups (Kuna et al., 1955).

In another summary report, four groups of four immature rats were each fed 0, 5, 25 or 80mg/kg bw per day nicarbazin in the diet for 123 days (Kuna et al., 1955). There was no significant change in food or water consumption among treated animals, nor were any abnormal haematologic changes observed.

A three-month subchronic toxicity study (GLP compliant) of the drug combination narasin plus nicarbazin, administered orally to Fischer 344 rats, was available, but this study is not relevant to the current assessment for re-evaluation of nicarbazin due to the presence of narasin in the treatment, as narasin is more toxic than nicarbazin (Novilla & Usher, 1984). Hence, the findings from the following study are just supportive or supplementary to the existing data on nicarbazin. Narasin and nicarbazin were administered orally in equal parts to rats (20/sex per group) at 0, 7.5, 20, and 60mg/kg feed of each ingredient in the diet for 90–93 days. The mean body weight and body weight gain of males in the 0.006% group was reduced compared to controls on day 21, but then returned to normal. In females, the high-dose group (0.006%) showed a decrease in mean body weight and weight gain compared to the control group for the first 42 days, but then returned to normal. Males in the high-dose group showed a decrease in mean daily food intake for the first 35 days of treatment, which afterwards returned to normal. The average daily food intake of high-dose females was reduced compared to controls during the study period. No other compound-related effects were observed. Based on changes in body weight and body weight gain at the beginning of the study, a no-observed-adverse-effect level (NOAEL) of 0.002% narasin and 0.002% nicarbazin (equivalent to a time-weighted average daily dose of c 1.5 mg/kg bw per day of each component) was identified from this study.

More recently, 13-week oral toxicity studies in rats have been conducted to examine more closely the toxicity of nicarbazin and DNC. These studies were conducted in accordance with current OECD guidelines and according to GLP.

Nicarbazin was administered orally via the diet to provide doses of 0 and approximately 200, 600 or 1000mg/kg bw per day to groups of Crl:CD(SD) rats (15/sex per group) for 13 weeks (Lloyd, 2009b). Significant reductions in food consumption and body weight were noted during the first week of the study in the high- and mid-dose animals, therefore these doses were decreased to 400 and 600mg/kg bw per day, respectively. There were five unscheduled deaths during the study: one male in the 1000/600mg/kg bw per day group during the second week, two males in the 600/400mg/kg bw per day group and two females in the 1000/600mg/kg bw per day group in the final week of the study. At study termination it was clear that overt toxicity had occurred in all dose groups and that a NOAEL could not be identified. Although gross abnormalities were observed macroscopically in various tissues, microscopic examination was limited to the kidneys, testes and epididymis. Kidney toxicity was expressed in all dosed animals and was characterized by the presence of crystals associated with mild to severe tubular degeneration, fibrosis, and inflammation. There were significant changes in clinical chemistry and urinalysis parameters indicative of renal toxicity. Males from the 1000/600mg/kg bw per day group had seminiferous

tubule degeneration/atrophy of the testes and granulomas and oligospermia/germ cell debris in the epididymis.

Another 13-week toxicity study was conducted in order to identify a NOAEL for DNC alone (Lloyd, 2009c). The top dose level was chosen to be equivalent to DNC present when given as 1000 mg/kg bw per day nicarbazin. The DNC was administered by oral gavage at 106, 284 and 709 mg/kg bw per day for 91 days to Crl:CD(SD) rats (15/sex per group). Additional animals (four/sex per group) were included for toxicokinetic evaluations. Significant, dose-related, systemic exposure to DNC was demonstrated. Full evaluations were undertaken, including clinical chemistry, haematology, urinalysis, and microscopic assessment. There were no effects on any of the parameters evaluated, including histopathological changes in the kidneys. The NOAEL for DNC was 709mg/kg bw per day (the molar equivalent of 1000mg/kg bw per day nicarbazin), the highest dose tested.

In an OECD guideline study reported in summary by EFSA (EFSA, 2017), Sprague Dawley rats (10/sex per group) were fed for 13 weeks a diet containing nicarbazin that provided a dose of 100mg/kg bw per day, or an equimolar mixture of DNC and HDP to provide a dose of 71 + 29mg/kg bw per day. A range of histopathological effects were observed in the kidneys of nicarbazin-treated animals, with associated changes in clinical chemistry and urinalysis. By contrast, for the mixture of DNC and HDP the only dose tested, (71 + 29mg/kg bw per day), was the NOAEL for the mixture. The full study report was not available, so it was not possible to assess the data in detail.

In another OECD guideline study reported in summary by EFSA, a subchronic study was conducted in RccHan:WIST rats (EFSA, 2021). Groups of 13 rats of each sex were fed diets containing a 3:1 mixture of DNC and HDP at doses of 0 + 0, 50 + 17, 150 + 50 or 300 + 100mg/kg bw per day (DNC + HDP) for 13 weeks. Since there were no findings related to the test product in this study, the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) panel concluded that the NOAEL was 300 + 100mg/kg bw per day of DNC + HDP, the highest dose tested (EFSA, 2021). The full study report was not available, so it was not possible to assess the data in detail.

(b) **Dogs**

Summaries of two subchronic studies in dogs were available. The information in the summaries was insufficient to enable a detailed evaluation, as they contained minimal detail of the protocols used, limited data on toxicological findings, and took the form of progress reports. These data were considered only as supporting information. The studies were conducted prior to the introduction of GLP or the development of guidelines for the conduct of toxicological studies.

In the first study, six adult dogs were divided into three groups of two

animals each (Kuna, 1955). They received nicarbazin by oral gavage at doses of 0, 1 or 5 g/kg bw per day for 165 days. The stools of all test dogs were soft and yellow in co1our, similar to the colour of nicarbazin. There were no changes in body weight. There was a marked fall in red blood cell (RBC), haematocrit (Ht) and haemoglobin (Hb) after eight days in the dogs receiving 5 g/kg bw per day. One of these animals progressed to develop marked hypochromatic and hypocythemic anaemia. The other developed slight anaemia. Animals treated with 1 g/kg bw per day showed no evidence of haematological changes.

In a second study, dogs were fed diets containing nicarbazin at concentrations of 0, 100, 400 or 1600mg/kg feed (equal to 0, 7.8, 31.0 and 110mg/kg bw per day) for 141 days (Stebbins 1954). Yellow faeces were noted, attributed to the presence of nicarbazin itself. This effect was recorded as slight in the mid-dose group, and very marked with, in addition, a soft stool in the high-dose group, . There were no effects on haematological parameters.

An OECD guideline (TG 409) subchronic study conducted in beagle dogs was reported in summary by EFSA (2021). Groups of four dogs of each sex were orally administered nicarbazine (3:1 mixture of DNC and HDP) dissolved in a 0.5% methylcellulose solution at doses of 0 + 0, 60 + 20, 180 + 60 or 600 + 200 mg/kg bw per day (DNC + HDP) for 90 days, presumably by gavage but this was not stated. There were no differences between the control and treated groups with respect to body weight, clinical pathology findings (blood, coagulation, clinical chemistry, urine), organ weights, nor in gross or microscopic pathology findings. Since there were no observational findings related to nicarbazin in this study, the FEEDAP panel identified the NOAEL as the highest dose tested, 600 + 200mg/kg bw per day of DNC + HDP (EFSA, 2021). The full study report was not available, so it was not possible to assess the data in detail.

2.2.3 Long-term toxicity

(a) Rats

In a study based on OECD Guideline 452, groups of 20 Sprague Dawley rats of each sex were fed a control diet or a diet containing DNC/HDP mixture at 0 + 0, 20 + 8, 50 + 20.5 or 154 + 63mg/kg bw per day for 52 weeks (EFSA, 2017). Blood samples were taken from the last surviving 10 animals of each group at week 52 to confirm exposure to the test substance. Animals were divided into four groups, observed daily, and a detailed clinical examination performed weekly throughout the study period. Body weight and food intake were recorded weekly for the first 14 weeks and monthly thereafter. For haematological evaluation, blood was drawn from all animals at the end of the study after a 14-hour fast and serum, separated from blood, was submitted to clinical biochemical analysis. A cadaveric autopsy was performed on all animals, body and organ weights (adrenal glands, brain,

epididymis, heart, kidney, liver, ovary, spleen, testis, thymus, and uterus) were measured, and all tissues were preserved for microscopic examination. Males in the high-dose group gained less weight than males in other groups, especially in the second half of the study. However total weight gain was not significantly different from the control group. The only treatment-related difference in the blood, serum and urinalysis was the observation of crystals in the urine of males and females at the high and intermediate doses. This correlated with microscopic renal changes in both sexes, including tubular basophilia, interstitial chronic inflammation, mononuclear inflammatory infiltrate, tubular dilatation, cysts, intraductal inflammatory cells, hyaluronic acid clots, and papillary oedema. Males were more affected than females. Although changes at intermediate doses were slight, the 2017 EFSA panel concluded that the NOAEL in this study was 20 + 8mg/kg bw per day of DNC + HDP. The full study report was not available, so it was not possible to assess the data in detail.

In an OECD guideline (TG 452) and GLP-compliant study summarized and reported by EFSA (2021), RccHan:WIST rats (20 of each sex per group) received a 3:1 mixture of the two individual components (DNC and HDP) at doses of 0 + 0, 52.5 + 17.5, 150 + 50 or 300 + 100mg/kg bw per day in their feed (ad libitum) for up to 52 weeks. There were no deaths associated with the test product, no clinical or ophthalmologic observations, no changes in body weight or food intake, and no coagulation effects associated with the test product. Urea nitrogen and/or creatinine levels were mildly to moderately elevated at doses of 150 + 50 mg/kg bw per day and greater for males and 300 + 100 mg/kg bw per day and greater for females on day 365. These dose-related changes were more pronounced in males; haematological changes at day 365 in animals treated with 300 + 100 mg/kg bw per day were consistent with an inflammatory response associated with the test substance and microscopically correlated with chronic inflammation in the kidney. These findings included a minimal to mild increase in absolute neutrophil counts in both sexes, a minimal decrease in mean RBC count, Hb concentration, mean erythrocyte volume, and erythrocyte distribution width in males. Urinalysis showed only a slight decrease in urine pH on day 365 in males treated with 300 + 100 mg/kg bw per day, microscopically associated with increased incidence and severity of tubular degeneration and regeneration, chronic inflammation, and renal crystal accumulation. In the kidneys of animals receiving 150 + 50mg/kg bw per day or more, microscopic findings associated with the test substance were tubular degeneration/regeneration, chronic inflammation, and increased severity and/or incidence of crystal accumulation. Chronic inflammation of the kidneys approximetely correlated with surface roughness and brown discoloration in the animals receiving 150 + 50 mg/kg bw per day or more, and with increased kidney weight in males receiving 300 + 100 mg/kg bw per day. In general, the observed kidney changes were more prominent in males

than in females. Based on these observations, the FEEDAP committee identified a NOAEL of 52.5 + 17.5 mg/kg bw per day of DNC + HDP (EFSA, 2021). The full study report was not available, so it was not possible to assess the data in detail.

A group of FDRL rats were fed diets containing a 3:1 mixture of DNC and HDP (purity unknown) at concentrations calculated to be 0 + 0, 50 + 17, 150 + 50 or 300 + 100mg/kg bw per day for DNC + HDP for two years (Vogin, 1969a). The control and high-dose groups consisted of 50 males and 50 females, and the low-dose and intermediate groups consisted of 40 males and 40 females. Five animals of each sex in the control and high-dose groups were terminated after six and 18 months, and 10 animals of each sex in each group were terminated at 56 weeks, for interim assessment. Animal behaviour, physical appearance, and survival were observed daily. Food consumption and food utilization efficiency were assessed weekly for the first 12 weeks and periodically thereafter for 15 animals of each sex per group. Body weights were recorded weekly for the first 12 weeks, once every two weeks until week 26, and monthly thereafter. Water intake and urine output were measured for 10 rats of each sex per group for one week per month during the first three months. At months 3, 6, 9, 12, 18, and 24, limited haematological, clinical chemical and urinary parameters were assessed in 10 rats of each sex per group. Gross and microscopic examinations were conducted on a wide range of tissues from all rats. This study was conducted before guidelines for toxicological studies had been developed. No abnormal behaviour was observed and mortality was not affected by dosing. Food and water intake and weight gain were similar in all groups. Haemoglobin, Ht, leukocytes, blood urea nitrogen, serum alanine aminotransferase (ALT) activity, blood glucose, urinary parameters, organ weights, and gross pathological appearance were not affected by administration of the mixture. In the kidneys, changes such as calcification in the renal tubules, calcification of the renal pelvis, tubules, glomeruli and medulla, and stones were more common in the treated groups than in the controls at 56 weeks. However, the overall incidence of these effects at the end of the study was similar in all groups. There was a slight increase in the incidence of testicular atrophy in some treatment groups at week 104, but the difference was not significant (2/14, 4/16, 3/15 and 6/18 in the control, low-, mid- and high-dose groups respectively). In animals that died between 56 and 104 weeks, there was no indication of any effect on the incidence of testicular atrophy (2/14, 1/15, 3/16 and 0/9 in control, low-, mid- and high-dose groups respectively). Testicular weight was unaffected by treatment. Tumour incidence was unaffected by treatment. The authors concluded that the NOEL/ NOAEL was 400 mg/kg bw per day of a 3:1 mixture of DNC and HDP.

In this study administration of the DNC and HDP mixture did not affect the incidence of tumours and there were no signs of dose-related gross or histopathologic changes during the two-year study period, so the Committee

identified the NOAEL as 300+ 100 mg/kg bw per day for the DNC + HDP mixture, the highest doses tested.

(b) Dogs

Groups of five male and five female beagle dogs were fed diet containing DNC and HDP (purity unspecified) in a 3:1 ratio on six days per week for two years (Vogin, 1969b). Actual intakes were 0, 60, 180 or 600mg/kg bw per day for DNC and 0, 20, 60 or 200mg/kg bw per day for HDP. Two animals of each sex per group were terminated after one year. Clinical observations were made daily. Body weight, food intake and reflexes were measured weekly. Water intake and urine output were measured monthly. Haematological, clinical chemistry, and urinary parameters were measured before administration and at months 3, 6, 12, 18, and 24. A gross and microscopical examination was made of various tissues from all dogs. This study was conducted prior to the development of guidelines for the conduct of toxicological studies.

No abnormal behavioural or physical signs were observed, but one male dog on the medium dose died of unknown causes at 44 weeks. A greenish to yellowish hue was noted in the faeces of all treated dogs. Body weight gain, food intake, and blood and urine parameters were unaffected by the treatment. Serum ALT activity increased in several dogs at the highest dose and in one dog at both lower doses, but in most cases the effect was transient. The highest value was observed at approximately 12 months, and the increased activity persisted only in two dogs at the highest dose. Organ weights and gross pathological appearance showed no treatment-related changes. Histopathological appearance was unremarkable except for a slight bile duct proliferation in one dog that was terminated after one year of treatment at the high dose (no further information was provided in study report). This dog displayed elevated serum ALT activity.

Although the relationship between the hepatic findings and treatment was unclear, JECFA 1998 concluded that the conservative NOEL in this study was 240mg/kg bw per day (DNC + HDP). Correcting for duration of exposure on six days per week, the NOAEL was 154 + 51mg/kg bw per day for DNC + HDP, equivalent to 200mg/kg bw per day of nicarbazin.

2.2.4 Genotoxicity

The bacterial mutagenicity of nicarbazin and its individual components DNC and HDP was examined in an Ames test (Bradley & Cook, 1980). In this study, nicarbazin, HDP and DNC were tested using *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100 with and without metabolic activation by liver enzymes from rats pre-treated with Aroclor 1254 (S9 mix). The concentrations (in DMSO) of nicarbazin, HDP and DNC were in the ranges 100–2000 µg/plate, 200–2000 µg/plate and 100–2000 µg/plate, respectively. It was noted that

nicarbazin and DNC formed precipitates at concentrations higher than 500 and 300 µg/plate, respectively. None of the substances produced a mutagenic response in this test. However, only two replicate plates were employed per dose level and the test included only one phase and no confirmatory experiment was conducted. Hence, the study plan limits the reliance that can be placed on its results.

In another study, available only as a brief summary, the mutagenicity of nicarbazin was tested in *Salmonella typhimurium* strains TA1535, TA1537, TA1538, TA98 and TA100, and *Escherichia coli* strain WP2uvrA. There was no evidence of mutagenicity other than a weak effect at high concentrations in TA1538 and TA98, with and without metabolic activation (Ohta et al., 1980).

Another Ames test study was conducted in accordance with GLP as per OECD guideline 471 (Blackstock, 2004). Nicarbazin was tested in *S. typhimurium* strains TA1535, TA1537, TA98 and TA100 and *E. coli* strain WP2uvrA over a test concentration range of 3–1000 µg/plate, with and without metabolic activation system (S9) in two independent experiments. Precipitation of the test material was observed at 1000 µg/plate in both metabolic activation conditions, but not at concentrations up to 333 µg/plate. Slight increases in revertant colony rates (2.8-fold or less) were observed in frame shift strain TA98 in the presence and absence of S9 in both experiments. However, as increases greater than 2.0-fold were seen in this strain in repeat tests with and without S9 it was therefore concluded that nicarbazin is weakly mutagenic to *S. typhimurium* strain TA98.

Nicarbazin was negative in other in vitro tests: a DNA damage test (rec assay) (Ohta et al., 1980), two mouse lymphoma assays (CVM, 2018), and a chromosomal aberration test using human lymphocytes (EFSA, 2017).

With respect to in vivo evaluation, three bone marrow micronucleus tests (mouse and rat), a rat liver unscheduled DNA synthesis (UDS) test, and sister chromatid exchange (SCE) test in Chinese hamster were negative, as discussed below.

Both micronucleus assays were GLP-compliant. In a bone marrow micronucleus study, mice (10 per sex) were given two doses of nicarbazin 24 hours apart, at 2000 mg/kg bw and bone marrow was taken 24 hours after the last dose (Innes, 2001). A very slight depression in the polychromatic erythrocyte to normochromatic erythrocyte ratio (PCE : NCE ratio) was observed (0.88 compared to 0.98 in concurrent controls). Furthermore, an evaluation of nicarbazin concentrations in plasma from treated mice demonstrated systemic exposure to nicarbazin (concentration range 0.066–3.340 µg/mL; Airs, 2002). There were no increases in micronucleus number in mice treated with the maximum recommended dose of test article, 2000 mg/kg bw. A positive response was induced in a group of animals treated with the positive control chemical cyclophosphamide.

In accordance with OECD TG 474, another micronucleus study was

performed in bone marrow cells of male Wistar WI(Han) rats to evaluate the potential of nicarbazin (composition given as DNC 70.9%, HDP 28.7%) to induce chromosomal damage after oral administration. Animals were orally administered 500, 1000 and 2000mg/kg bw of nicarbazin at 0 and 24 hours, then killed 24 hours after administration. No clinical signs of toxicity or death were reported, except for weight loss associated with administration on days 1 to 3. Chemical analysis detected DNC and HDP in plasma, confirming that animals were systemically exposed to nicarbazin at all dose levels. For micronucleus analysis, 8000 polychromatic erythrocytes (PCEs) were scored for each animal. Micronucleus frequencies were significantly higher ($p < 0.01$) in the treated groups at 1000 and 2000mg/kg bw than in the control group. However, the increased frequencies were within the historical vehicle control range. The EFSA panel considered the in vivo micronucleus assay results "equivocal" because all of the criteria for a positive response were not clearly met (EFSA, 2021).

An assay evaluating unscheduled DNA synthesis in rat liver was available (Nesslany, 2007). This study was conducted according to OECD guideline 486, was GLP-compliant, and fulfilled the requirement at the time for a second in vivo study when positive or equivocal results had been generated in a first in vitro study. Groups of male rats (three per group) were given single oral doses of nicarbazin at 1000 and 2000mg/kg bw. Hepatocytes were isolated 2–4 hours or 12–16 hours later and assessed for the induction of unscheduled DNA repair. No increase in net nuclear grain count was seen at either dose level or time-point and this confirmed the absence of any potential DNA reactivity due to nicarbazin.

In a GLP-compliant study, crystalline nicarbazin (as well as crystalline narasin and EL-9193, a combination of the two drugs) was tested in vivo using a sister chromatid exchange (SCE) assay in the bone marrow of adult female Chinese hamsters (CVM 2018). Three animals per dose group were treated by oral gavage with 0, 200, 300, 400 or 500mg/kg bw nicarbazin in 10% aqueous acacia solution. Two animals formed the vehicle control group and one animal was the positive control, treated with cyclophosphamide at 50mg/kg bw. Animals were terminated 21 hours after administration of nicarbazin. Twenty-five representative metaphases from bone marrow samples taken from the femur were scored for SCEs and 100 metaphase figures were used to assess cytotoxicity. Nicarbazin treatment was not associated with any cytotoxicity and there were no signs of SCE induction in bone marrow samples from animals treated with nicarbazin at any dose. The positive control produced the expected response.

Based on the above findings, no evidence of genotoxicity was observed, including from in vitro mammalian gene mutation assays, other than a weak positive response, a two-fold increase in revertant colonies at 1000 µg/plate, in *S. typhimurium* TA98 with and without metabolic activation.

Nicarbazin can contain *p*-nitroaniline (PNA) and methyl-(4- nitrophenyl)

carbamate (M4NPC) as impurities (EFSA, 2017, 2018). In addition, when chicken meat is subjected to heat treatment, PNA can be expected as a product of DNC breakdown (Bacila et al., 2019). Negative or weakly positive results from the Ames test have been reported for PNA in strain TA98 (Josephy et al., 2016). A dose-dependent increase in revertant yield for *p*-nitroaniline in *S. typhimurium* strain TA98 was reported at doses above 300 µg/plate. In 2019, JECFA reviewed 4-chloroaniline (PCA), a close structural analogue of PNA, and concluded that PCA does not exhibit DNA-reactive genotoxicity in vivo (JECFA, 2020). In 2017 an EFSA panel concluded that no safety concern would arise from the impurity PNA if a maximum content in nicarbazin of 0.1% were respected (EFSA, 2017). The impurity M4NPC was also considered safe for the consumer provided a maximum concentration of 0.4% in nicarbazin were not exceeded.

Genotoxicity studies are summarized below in Table 3.

Table 3
In vitro genotoxicity studies

Assay type	Test item and dose	Test system	Noteworthy findings	Reference
Ames test	Nicarbazin: 100–2000 µg/plate; DNC 100–2000 µg/plate; HDP 200–2000 µg/plate Nicarbazin and DNC formed precipitates at concentrations of ≥ 1000 and 500 µg/plate, respectively.	*S. typhimurium* strains TA1535, TA1537, TA98 and TA100, with and without metabolic activation (± S9)	Negative Limitation in study design, so not considered for evaluation.	Bradley & Cook, 1980
Ames test	Nicarbazin: up to 10,000 µg/plate Four concentration steps (± S9)	*S. typhimurium* strains TA98, TA1538, TA100, TA1535, and TA1537 *E. coli* strain WP2*uvr*A	Weakly positive (TA98 and TA1538) with or without of S9 mix. Limitation in study design, so not considered for evaluation.	Ohta et al., 1980
Ames test	Nicarbazin: maximum level of ≤ 2500 µg per plate, based on level at which preciptation occurred	*S. typhimurium* strains TA1535, TA1537, TA98, TA100, and TA102	Positive, (TA98, ± S9) No details are available.	EFSA, 2017
Ames test (GLP)	Nicarbazin: gradient concentrations of 0.1–1.0 µg/mL, 1.0–10 µg/mL, 10–100 µg/mL, 100–1000 µg/mL, (± S9)	*S. typhimurium* strains *G46, TA1535, TA100, C3076, TA1537, D3052, TA1538 and TA98,* *E. coli* strain WP2*uvr*A	Negative Cytotoxic in presence of S9, particularly to TA1538 and TA98 at 500 µg/mL	CVM, 2018
Ames test (GLP)	Nicarbazin: 25–500 µg/plate, (± S9)	*S. typhimurium* strains *TA1535, TA1537, TA1538, TA98, TA100*	Negative	CVM, 2018

Assay type	Test item and dose	Test system	Noteworthy findings	Reference
Ames test (GLP)	Nicarbazin: 3–1000 µg/plate, (± S9) Precipitation observed at 1000 µg/plate with and without S9	*S. typhimurium* strains TA1535, TA1537, TA98 and TA100 *E. coli* strain WP2*uvr*A	Nicarbazin is weakly mutagenic to *S. typhimurium* TA98 (≤ 2.8-fold increase in revertants other than 5-fold in one test at 1000 µg/plate, associated with a low vehicle control count)	Blackstock, 2004
Ames test (GLP)	DNC: 0–500 µg/plate (maximum concentration limited by solubility) HDP: 5–5000 µg/plate (all ± S9)	*S. typhimurium* strains TA1535, TA1537, TA98 and TA100 *E. coli* strain WP2*uvr*A	DNC and HDP both negative	EFSA, 2021
Rec-assay	Nicarbazin: unknown (± S9)	*Bacillus subtilis strains* H17 (*rec*+) and M45 (*rec–)*	Negative Limitation in study design, so not considered for evaluation.	Ohta et al., 1980
Mouse lymphoma cell mutation assay (GLP)	Nicarbazin: the top concentration tested, 100 µg/mL, was based on compound solubility and induced cytotoxicity	Mouse lymphoma L5178Y cells	Negative Cytotoxic at concentrations as low as 0.4 µg/ml, depending on test conditions.	Riach, 2004
Mouse lymphoma cell mutation assay (GLP)	Nicarbazin: 1.0–1000 µg/mL without activation; 0.05–100 µg/mL with activation, with precipitation at the highest concentration	Mouse lymphoma L5178Y cells	Negative (three assays; only one resulted in a > 2-fold increase in mutation frequency (at 100 µg/mL), which did not meet the criterion for a positive response)	CVM, 2018
Human lymphoma cell mutation assay (GLP)	Nicarbazin: range of concentrations up to 125 µg/mL; ± S9	Human lymphoma cells	Negative Limited details provided	EFSA, 2017

Table 4
In vivo genotoxicity studies with oral adminstration

Assay	Test item and dose	Test System	Noteworthy Findings	Reference
Micronucleus study in bone marrow, (GLP)	Nicarbazin: two doses of nicarbazin at 2000 mg/kg bw, 24 hours apart	Mouse	No increases in micronuclei. Systemic exposure demonstrated by Airs (2002)	Innes, 2001; Airs, 2002
Micronucleus study in bone marrow, (GLP)	Nicarbazin: 500, 1000 and 2000 mg/kg bw	Rat	Negative Significantly higher frequency ($p < 0.01$) in treated animals at 1000 and 2000 mg/kg bw, however, increased frequencies were within the historical control range	EFSA, 2021
Unscheduled DNA synthesis in rat liver, (GLP)	Nicarbazin: (1000 and 2000 mg/kg bw)	Rat	Negative	Nesslany, 2007
Sister chromatid exchange test in bone marrow. (GLP)	Nicarbazin: 200, 300, 400 and 500 mg/kg bw	Female Chinese hamster cells	Negative	CVM, 2018

2.2.5 Reproductive and developmental toxicity

(a) Multigenerational reproductive toxicity

Groups FDRL rats, (12 of each sex) were fed diets containing 3:1 mixtures of DNC and HDP (purity unknown) at concentrations calculated to be 0 + 0, 50 + 17, 150 + 50 and 300 + 100mg/kg bw per day for DNC + HDP (Kirschner & Vogin, 1970). This study was conducted prior to the development of guidelines for conducting toxicity studies.

Treatment was administered continuously during the production of two litters per generation for three successive generations. The initial group of animals was paired 10 weeks after the start of treatment to produce F1a litters and paired again seven days after weaning of the first litter to produce F1b litters. At four weeks, 12 males and 12 females of the F1b litters were mated according to the above schedule to produce F2a and F2b litters, and the F2b litter was then used to produce the F3a and F3b litters. Animals were examined daily for survival, behaviour, and appearance. Body weight and food consumption were measured weekly in adults. After birth, each litter was limited to eight animals, weighed at birth and then at 4, 12, and 21 days old.

After the litters were weaned, adult rats were autopsied and the testes of F2 males in the control and high-dose groups were examined histopathologically. The liver, kidneys, heart, bladder, and gonads of five male and five female F3b weanling pups from each group were also examined microscopically. Adult rats of each generation showed no effect on survival, body weight gain, or food intake, and there were no abnormalities in the results of gross dissection or testicular examinations. Pregnancy rate and gestation period were also unaffected. Weight gain of F1b pups reared at the highest dose was slightly lower during lactation, but no similar effects were observed in the other pups. In subsequent generations, the F2a and F3a pups that were reared on high doses had slightly fewer litters, but this effect was not reproduced in the F2b and F3b pups. Histopathological examination of a limited number of organs from the F3b pups revealed no treatment-related abnormalities and no significant reproductive effects. The NOAELs for parental, reproductive and offspring toxicity were all 400mg/kg bw per day (300mg/kg bw DNC + 100mg/kg bw HDP per day), the highest dose tested (JECFA, 1998).

A two-generation reproductive toxicity study was reported by EFSA (2017). A 3:1 mixture of DNC and HDP was administered to male Sprague Dawley rats (details unknown, 24 per group) by mixed feeding (0 + 0, 100 + 33, 300 + 100 or 580 + 193mg/kg bw). Administration to parent animals (F0) started 10 weeks prior to mating and continued throughout the mating, parturition, and nursing period. The animals were then administered the mixture until they were weaned. The F1 animals were treated for 10 weeks and mated to produce F1 offspring.

One male and one female per litter was selected from both F0 and F1 offspring and necropsied at around 22 days old. The parental animals were necropsied after weaning of the offspring. Maternal animals and individual offspring of each generation were extensively examined, but no dose-related effects were observed in F0 and F1 offspring. No reproductive effects were observed in the parent animals, but renal crystal deposition, changes in kidney weight, and renal impairment were observed in all dose groups. No parental NOAEL could be identified, as the LOAEL was 100 + 33mg/kg bw per day, the lowest dose tested. The NOAEL for reproductive and offspring toxicity was 580 + 193 mg/kg bw per day for DNC + HDP, the highest dose tested. The full study report was not available, so it was not possible to assess the data in detail.

In a GLP-compliant dietary two-generation reproductive toxicity study based on OECD TG 416, four groups of Crl:CD(SD) female and male rats (25/sex per group) received either the basic diet or a 3:1 mixture of DNC and HDP in the diet.Target concentrations were 0 + 0, 52.5 + 17.5, 150 + 50 and 300 + 100mg/kg bw per day DNC + HDP, which were equivalent to 0, 70, 200 and 400mg/kg bw per day of nicarbazin, respectively. The mixture was administered daily for at least 70 days prior to mating. Offspring of the F0 animals (one of each sex per group) were selected on postnatal day (PND) 21 to yield the F1 generation, which received the test mixture from then onwards. F0 and F1 males continued to be treated throughout mating and until the day of termination. F0 and F1 females continued to receive the test diet throughout mating, gestation and lactation until termination. F1 and F2 litters were reduced to eight pups per litter (four of each sex where possible) on PND 4, to reduce interlitter variability. All animals were observed twice daily for mortality and morbidity. Clinical observations, body weights and food consumption were recorded in males and females at regular intervals throughout the study. Vaginal lavage was performed daily to determinatine estrous cyclicity, from 21 days prior to mating. All F0 and F1 females were allowed to deliver and rear their pups until weaning on lactation day (LD) 21. At regular intervals F1 and F2 pups were assessed for clinical observations, body weights, and the number of each sex was noted. Developmental landmarks (balanopreputial separation and vaginal patency) were evaluated in the F1 rats. Non-selected F1 pups and all surviving F2 pups were necropsied on PND 21. At necropsy on PND 21, selected organs from one pup of each sex per litter from both F1 and F2 generations were weighed. Surviving F0 and F1 parental animals underwent a comprehensive gross necropsy following weaning of the F1 and F2 pups, respectively, at which selected organs were weighed. Spermatogenic end-points (sperm motility, morphology and numbers) were determined for all F0 and F1 males, and ovarian primordial/primary follicle counts were recorded for F1 females in all groups. A histopathological examination was performed on a range of tissues from all F0 and F1 parental animals in the control and high-

dose groups, and from all parental animals that were found dead or terminated in extremis. Blood samples for bioanalysis were collected from F0 males and females (five per group) on study day 65 and from F1 pups culled on PND 4 (five litters per group). Since there were no findings associated with the test mixture in this study, the NOAELs for parental, reproductive and offspring toxicity were all 300 + 100 mg/kg bw/day DNC + HDP, the highest dose tested (EFSA, 2021). The full study report was not available, so it was not possible to assess the data in detail.

(b) **Developmental toxicity**

Results from a study investigating the effects of nicarbazin on embryo/fetal development in rats were reported (Tajima, 1979). This study was conducted prior to the introduction of GLP regulations and OECD test guidelines. Although detailed descriptions of the methods used were not provided, the apparent study design and analyses were sufficient to determine the teratogenic potential of nicarbazin in this species. In preliminary range-finding studies it was established that doses of nicarbazin of 600mg/kg bw per day and above resulted in high maternal and fetal mortality. In the main study, four groups of pregnant CD/CRJ rats (24 or 25 per dose group) were given equimolar mixtures of DNC and HDP by gavage as a 1% carboxymethyl cellulose suspension. This provided nicabazin equivalent doses of 0, 70, 200 or 600mg/kg bw per day, from gestation day (GD) 7 to GD 17. Seven of the 25 high-dose rats died (28%) during the treatment period and decreases in food intake and rate of body weight gain were noted from GD 8. Water consumption was increased throughout the treatment period. At 600mg/kg bw, decreases in fetal body weight and ossification were observed, suggesting delayed fetal development, with many abnormalities, particularly hydronephrosis, hyperplasia and bent ribs. No teratogenicity was observed. Based on reduced food intake and body weight gain, and increased mortality at 600mg/kg bw per day, the NOAEL for maternal toxicity was 200mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 200mg/kg bw per day based on reduced fetal body weight and delayed ossification, suggesting retarded fetal development, possibly secondary to maternal toxicity. In 1998, this study was considered by the Committee to establish the ADI (JECFA, 1998).

A well conducted and reported developmental toxicity study in rabbits was available, which was conducted to fulfil the requirements of OECD Guideline 414 and in accordance with GLP compliance (Jardine & Craige, 2005, 2006).

In a pilot study (Jardine & Craige, 2005), mated female New Zealand White rabbits were randomized into four treatment groups each containing six animals. These animals were dosed orally by gavage once daily over GDs 6–28 inclusive, where day 0 was the day of mating. Dose levels were 0, 100, 200 or 400mg/kg bw per day, administered in 0.5% w/v carboxymethylcellulose, delivered

at 2 mL/kg bw. Unacceptable signs of toxicity resulted in cessation of dosing and the premature termination of all animals in the 400mg/kg bw per day group. Treatment with 200mg/kg bw per day resulted in reduced food consumption and weight loss; 3/6 animals were terminated prematurely on day 19, and an increase in late embryonic deaths was noted in two of these rabbits. Five out of six animals had reduced faecal output and findings at necropsy included prominent lobulation of the liver and reddened/thickened glandular mucosa of the stomach. There were no significant findings following administration of 100mg/kg bw per day.

In the main study by Jardine & Craige, (2006), dose levels of 0, 30, 60 or 120mg/kg bw per day for nicarbazin were given by gavage to rabbits (24 per group) during GDs 6–28. There were no significant effects on maternal mortality, clinical signs, body weight, food consumption or pregnancy outcome. At necropsy of the dams the only effect observed was prominent liver lobulation in two of 24 animals dosed at 120mg/kg bw per day; this finding had also been noted in the preliminary toxicity study at 200 and 400mg/kg bw per day and therefore was considered to be associated with nicarbazin treatment. There were no effects on fetal weights, or mortality at any dose level. Occasional increased incidences of incomplete ossification of the skeletal system, and visceral/soft tissue abnormalities, such as central corneal opacity on eyes, common carotid arteries arising separately from the aortic arch and retro-oesophageal right subclavian artery were noted in all groups. However these showed no relationship to dose, and the report states that the abnormalities were within the historical control data ranges for the test laboratory (but data was shown only for common carotid arteries arising separately from the aortic arch). The abnormalities are therefore considered incidental to treatment.

The Committee identified the NOAEL for maternal toxicity of nicarbazin as 60mg/kg bw per day based on the occurrence of prominent liver lobulation in two of 24 animals at the LOAEL of 120mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 120mg/kg bw per day, the highest dose tested.

In a study compliant with OECD TG 414 to evaluate developmental toxicity, pregnant rabbits were treated daily by oral gavage with nicarbazin at doses of 0, 60, 120 or 240mg/kg bw per day, from GD 6 to GD 28. The NOAEL for maternal toxicity was 240mg/kg bw per day, the highest dose tested. The NOAEL for embryo/fetal toxicity was 120mg/kg bw per day, based on changes in skeletal ossification indicative of developmental retardation at 240mg/kg bw per day (EFSA, 2017). Only a brief summary of this study was available, so it was not possible to assess the data in any detail.

In an oral developmental toxicity study based on OECD TG 414, four groups of 24 time-mated female New Zealand White rabbits (Hra:(NZW) SPF) were treated by gavage with the a 3:1 mixture of DNC and

HDP in 0.5% methylcellulose delivered at 0.5 mL/kg bw. Doses were 0 + 0, 22.5 + 7.5, 45 + 15 or 90 + 30mg/kg bw per day, administered once daily on GDs 7–28 (equivalent to doses of nicarbazin at 0, 30, 60 and 120mg/kg bw per day). All animals were observed twice daily for mortality and morbidity. Clinical observations, body weights, and food consumption were recorded. On GD 29 animals were terminated and the uterus, placenta and ovaries examined in all animals. The number of fetuses, early and late resorptions, total implantations and corpora lutea were recorded. Gravid uterine weights were measured. The fetuses were weighed, sexed and examined for external, visceral and skeletal malformations and developmental variations. Treatment with the mixture had no effect on any of the parameters assessed, in either the dams or the fetuses. The NOAEL for both maternal and embryo/fetal toxicity was 90 + 30 mg/kg bw per day DNC + HDP, the highest dose tested.. (EFSA, 2021). The full study report was not available, so it was not possible to assess the data in detail.

2.2.6 Special studies

No special studies were reported.

2.3 Microbiological effects

The impact of nicarbazin residues on the human intestinal microbiome was evaluated through a decision-tree approach adopted by the sixty-sixth meeting of the Committee (JECFA, 2006), which complies with VICH GL36(R) (VICH, 2013). The guideline discusses test systems that can be used to address this toxicological end-point of concern, considering the complexity of the human intestinal microbiome.

This approach can be used to determine if a microbiological acceptable daily intake (mADI) would be necessary based on an evaluation of whether drug residues reaching the human colon are microbiologically active. It entails answering three questions to determine the need for establishing a mADI. First, determine if the drug residue, and/or its metabolites, are microbiologically active against representatives of the human intestinal microbiota. Second, whether the drug residues enter the human colon. Third, if the residues entering the human colon remain microbiologically active. If the answer to any of these questions is "no," then there is no need to calculate a mADI, and the assessment does not need to be completed. However, if a mADI needs to be calculated, two end-points of concern for human health are considered for the assessment: disruption of the colonization barrier of the human intestinal microbiome, and increases in populations of resistant bacteria in the human intestinal microbiome.

Four studies were submitted for consideration, to determine if nicarbazin and/or its metabolites are microbiologically active against a representative

group of microorganisms. A non-GLP study by Bentley, Cross & Ryden, (1983), focused on testing bacteria isolated from chickens receiving a dose of narasin and nicarbazin. This study was not considered for the evaluation of the impact of nicarbazin residues on the human intestinal microbiome because, samples were not tested against nicarbazin alone.

Three studies evaluated the microbiological activity of nicarbazin and its moieties, DNC and HDP, to assess possible antibacterial activity (or the lack such) against more than100 isolates from various bacterial groups.

A non-GLP study by Elliott & Hulman (1981), focussed on testing minimum inhibitory concentrations (MICs) of various compounds, one of which was nicarbazin. Test organisms were two strains of each of *Staphylococcus aureus, Salmonella typhimurium* and *Clostridium perfringens,* and three strains of *Klebsiella pneumoniae* and *Escherichia coli.* This study showed that MICs for all 12 isolates tested against nicarbazin were greater than 128 µg/mL, while DNC and HDP were not tested (Table 5). Results indicated that nicarbazin, one of the non-antibiotic anticoccidials tested, had limited antibacterial activity against the tested micro-organisms.

Another study (Lanckriet et al., 2010) described a study in which MICs of different antibiotics and anticoccidials, one of which was nicarbazin, were determined against 51 strains of *Clostridium perfringens* isolated from broiler chickens. Results showed that nicarbazin did not inhibit in vitro growth of the tested strains up to a concentration of 128 µg/mL (Table 5). Nicarbazin's components DNC and HDP were not tested in this study.

A GLP study (Pridmore, 2001) determined the MIC of nicarbazin, DNC and HDP against a panel of 45 bacterial strains, five from each of the following groups: *Salmonella, Staphylococcus, Enterococcus, Escherichia coli, Proteus, Lactobacillus, Campylobacter, Clostridium* and *Bacteroides*. Results showed that nicarbazin and DNC had no antimicrobial activity against any of the bacterial strains tested (MIC greater than 256 µg/mL). The HDP moiety had no measurable activity against 44 of the 45 bacteria tested with an MIC greater than 256 µg/mL, but towards one strain of *Campylobacter jejuni* HDP alone had an MIC of 256 µg/mL (Table 5).

Table 5
Susceptibility data of nicarbazin, DNC and HDP against over 100 bacterial isolates tested

Reference	Micro-organisms tested	Strains	Nicarbazin (µg/mL)	DNC (µg/mL)	HDP (µg/mL)
Elliot & Hulman, 1981	*Clostridium perfringens*	2			
	Escherichia coli	3			
	Klebsiella pneumoniae	3	> 128	NT	NT
	Salmonella typhimurium	2			
	Staphylococcus aureus	2			
Lanckriet et al., 2010	*Clostridium perfringens*	51	≥ 128	NT	NT
Pridmore, 2001	*Bacteroides fragilis group* (anaerobic)	2			
	Bacteroides vulgatus (anaerobic)	3			
	Campylobacter jejuni * (microaerophilic)	3			
	Campylobacter coli (microaerophilic)	2			
	Clostridium spp. (anaerobic)	1			
	Clostridium bifermentans (anaerobic)	1			
	Clostridium sordellii (anaerobic)	1			
	Clostridium perfringens (anaerobic)	2			
	Enterococcus faecium	3			
	Enterococcus faecalis	2	> 256	> 256	> 256
	Escherichia coli	5			
	Lactobacillus sp. (anaerobic)	5			
	Proteus mirabilis	5			
	Salmonella anatum	1			
	Salmonella Heidelberg	1			
	Salmonella Tennessee	1			
	Salmonella mbandaka	1			
	Salmonella agona	1			
	Staphylococcus aureus	3			
	Staphylococcus sp. (coagulase negative)	2			

NT: not tested
* a single *Campylobacter* strain had an MIC of 256 µg/mL for HDP

2.4 Observations in humans

No data were submitted.

3. Comments

3.1 Biochemical data

Rats received single oral doses of 1, 5, or 10 mg/kg bw bw nicarbazin. Low concentrations of DNC were detected at six hours, but not at 18 hours. By contrast HDP was found at considerably higher concentrations, which increased between six hours and 18 hours. Qualitatively similar findings were obtained in rats given

oral doses for eight days. In urine collected at five hours, concentrations of HDP were an order of magnitude higher than those of DNC (Kuna, 1955).

Male rats received single oral doses by gavage of nicarbazin at 0, 50, 150 or 450 mg/kg bw, DNC at 0, 150, 450 or 900 mg/kg bw, or a DNC + HDP mixture at 0 + 0, 35 + 15, 106 + 44 or 319 + 131 mg/kg bw. Maximum concentration (C_{max}) in plasma for DNC occurred at around two hours in all treated groups. The half-life of DNC was around 12 hours (6–16 hours) and was independent of dose and the form in which it was administrated. The bioavailability of DNC when given alone was less than 2.5% relative to that when given in the form of nicarbazin. When given as a mixture with HDP, the bioavailability of DNC was less than 3.3% relative to that when given in the form of nicarbazin (Lloyd, 2009a).

In a study summarized by EFSA in 2003, [*DNC*-^{14}C] nicarbazin was administered to rats in their diet at about 100 mg/kg feed (1 mg/rat per day) for five days. DNC and metabolite M1 (DNC with one nitro group reduced and acetylated) were the major constituents found in the faeces. M1 and M3 (DNC with both nitro groups reduced and acetylated) were the major constituents found in the urine (EFSA, 2003).

Studies on the physicochemical properties of DNC alone and in complex with HDP (Rogers, 1983) indicated that the presence of an intermolecular bonded moiety such as HDP, as in nicarbazin, was necessary for the effective absorption of DNC.

3.2 Toxicological data

The acute oral toxicity of nicarbazin in rodents was low, the LD_{50} values being greater than 25 000 mg/kg bw in mice and greater than 10 000 mg/kg bw in rats. The individual components of nicarbazin also displayed low acute toxicity; the oral LD_{50} in mice being 4000 mg/kg bw for HDP and greater than 18 000 for DNC.

Only summaries of the short-term studies on nicarbazin were available, and these reports were inadequate for detailed evaluation as they contained minimal detail of the protocols used, limited data on toxicological findings, and were often in the form of progress reports. There was evidence of kidney damage associated with crystalline deposits in the collecting tubules in rats at oral doses of 500 mg/kg bw per day and above. In dogs, bile duct proliferation was the principal finding following an oral dose of 1600 mg/kg bw per day (Kuna,1955).

More recently, 13-week studies of toxicity in rats have been conducted to more closely examine the toxicity of nicarbazin and DNC. These studies were conducted in accordance with current OECD guidelines and complied with GLP. Nicarbazin was administered orally to rats for 13 weeks via the diet, to provide dosages of approximately 0 200, 600 or 1000 mg/kg bw per day (Lloyd, 2009a). There were significant changes in clinical chemistry and urinalysis parameters

indicative of renal toxicity at 200 mg/kg bw per day or greater. A NOAEL could not be identified. Another 13-week toxicity study was conducted in order to identify a NOAEL, and this examined the toxicity of DNC alone. The top dose level was chosen to be equivalent to the dose of DNC that would result from 1000 mg/kg bw per day of nicarbazin. DNC was administered to rats by oral gavage at 0 106, 284 or 709 mg/kg bw per day for 91 days (Lloyd, 2009a). There were no effects on any of the parameters evaluated including histopathological changes in the kidneys. The NOAEL for DNC was 709 mg/kg bw per day (the molar equivalent of 1000 mg/kg bw per day of nicarbazin), the highest dose tested.

In an OECD guideline study reported in summary by EFSA in 2017 (EFSA, 2017), rats were fed for 13 weeks a diet containing nicarbazin to provide a dose of 100 mg/kg bw per day, or an equimolar mixture of DNC and HDP to provide a dose of 71 + 29 mg/kg bw per day. A range of histopathological effects were seen in the kidneys of nicarbazin-treated animals, with associated changes in clinical chemistry and urinalysis. By contrast, for a mixture of DNC + HDP the only dose tested, 71 + 29 mg/kg bw per day, was the NOAEL for the mixture.

In an OECD guideline study reported in summary by EFSA in 2021, rats received a diet for 13 weeks containing nicarbazin [sic], at dose levels providing 0 + 0, 50 + 17, 150 + 50 or 300 + 100 mg/kg bw per day (DNC + HDP). The NOAEL was 300 + 100 mg/kg bw per day DNC + HDP, the highest dose tested (EFSA, 2021).

In an OECD guideline study reported in summary by EFSA in 2021, dogs received nicarbazin [sic] by gavage for 90 days, at dose levels providing 0 + 0, 60 + 20, 180 + 60 or 600 + 200 mg/kg bw per day (DNC + HDP). The NOAEL was 600 + 200 mg/kg bw per day DNC + HDP, the highest dose tested (EFSA, 2021).

In an OECD guideline study rats were fed for 52 weeks a control diet or a DNC + HDP mixture at 20 + 8, 50 + 20.5 or 154 + 63 mg/kg bw per day. The NOAEL was 20 + 8 mg/kg bw per day on the basis of the occurrence of crystals in the urine and associated histopathological changes in the kidney, including tubular basophilia, interstitial chronic inflammation, mononuclear inflammatory infiltrate, tubular dilation, cysts, intraductal inflammatory cells, hyaluronic acid casts, and papillary oedema, at 50 + 20.5 mg/kg bw per day. As summarized in the EFSA report, the effects were described as "rather slight". The full study report was not available, so it was not possible to assess the data in detail (EFSA, 2017).

In a GLP study based on OECD TG 452, rats were fed a diet containing 3:1 mixtures of DNC + HDP at 0 + 0, 52.5 + 17.5, 150 + 50 or 300 + 100 mg/kg bw per day for up to 52 weeks. The NOAEL was 52.5 + 17.5 mg/kg bw per day DNC + HDP based on chronic inflammation in the kidneys which correlated macroscopically with rough surface and/or tan discolouration in animals exposed to 150 + 50 mg/kg bw per day or more of DNC + HDP (EFSA, 2021).

Rats were fed diets containing a mixture of DNC and HDP (purity unspecified) for two years at concentrations calculated to give doses of 0, 50, 150 or 300 mg/kg bw per day of DNC and 0, 17, 50 or 100 mg/kg bw per day of HDP. The administration of the DNC and HDP mixture did not affect the incidence of tumours and there were no signs of any dose-related gross or histopathologic changes, so the highest dose tested was identified as the NOAEL, which was 300 mg/kg bw per day for DNC, and 100 mg/kg bw per day for HDP (Vogin, 1969a).

The Committee concluded that DNC is not carcinogenic in rats.

Dogs were fed diets containing a mixture of DNC and HDP (purity unspecified) in a ratio of 3:1 for six days per week for two years. The actual intakes were 0, 60, 180 or 600 mg/kg bw per day of DNC, and 0, 20, 60 or 200 mg/kg bw per day of HDP. Changes in alanine transaminase (ALT) were seen in some dogs at the highest dose and one dog in this group showed slight bile duct proliferation. Although the relationship between the hepatic findings and treatment was unclear, JECFA 1998 concluded that the conservative NOEL in this study was 240 mg/kg bw day (DNC + HDP) (Vogin, 1969b). Correcting for duration of exposure on six days per week, the NOAEL was 154 mg/kg bw per day for DNC and 51 mg/kg bw per day for HDP, equivalent to 200 mg/kg bw per day of nicarbazin.

The genotoxic potential of nicarbazin was investigated in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was observed, including from in vitro mammalian gene mutation assays, other than a weak positive response, a two-fold increase in revertant colonies at 1000 µg/plate, in *Salmonella typhimurium* TA98 with and without metabolic activation.

Nicarbazin can contain *p*-nitroaniline (PNA) and methyl-(4-nitrophenyl) carbamate (M4NPC) as impurities (EFSA, 2017, 2018) In addition, when chicken meat is subjected to heat treatment, PNA can be expected as a product of DNC breakdown (Bacila et al., 2019). Negative or weakly positive results from the Ames test have been reported for PNA in strain TA98 (Josephy et al., 2016). In 2019 JECFA reviewed 4-chloroaniline (PCA), a close structural analogue of PNA, and concluded that PCA does not exhibit DNA-reactive genotoxicity in vivo. In 2017 EFSA concluded that no safety concern would arise from the impurity PNA if the maximum content in nicarbazin of 0.1% were respected (EFSA, 2017). The impurity M4NPC was also considered safe for the consumer provided a maximum concentration of 0.4% in nicarbazin is not exceeded.

The Committee concluded that nicarbazin is unlikely to be genotoxic in vivo.

As nicarbazin (and DNC) are unlikely to be genotoxic in vivo, any carcinogenic effect would be secondary to prolonged, preneoplastic damage, the only indication of which in repeat-dose studies was renal toxicity due to crystal formation. As this is not observed in long-term studies with DNC, which is the residue of concern, and the mixture of DNC and HDP was not carcinogenic in rats, the Committee concluded that nicarbazin was unlikely to pose a carcinogenic risk to humans from its use as a veterinary drug.

In a three-generation study of reproductive toxicity, rats were fed diets containing a mixture of DNC + HDP, at doses of 0 + 0, 50 + 17, 150 + 50 or 300 + 100 mg/kg bw per day. The NOAELs for parental, reproductive and offspring toxicity of the DNC + HDP mixture were 300 + 100 mg/kg bw per day, the highest dose tested (Kirschner & Vogin, 1970).

In a two-generation reproductive toxicity study, based on OECD guidelines, mixtures of DNC and HDP were administered to rats in their feed to provide DNC + HDP doses of 0 + 0, 100 + 33, 300 + 100 or 580 + 193 mg/kg bw per day. Detailed data were not submitted by the sponsor. A parental NOAEL could not be identified as renal crystal deposits and associated histopathological effects in the kidney were observed at 100 + 33 mg/kg bw per day and greater. The NOAEL for reproductive and offspring toxicity was 580 mg/kg bw per day for DNC and 193 mg/kg bw per day for HDP, the highest dose tested (EFSA, 2017).

In a two-generation reproductive toxicity study based upon OECD TG 416, rats were administered a 3:1 mixture of DNC and HDP in the diet at 0 + 0, 52.5 + 17.5, 150 + 50 or 300 + 100 mg/kg bw per day. The NOAELs for parental, reproductive and offspring toxicity were all 300 + 100 mg/kg bw per day DNC + HDP, the highest dose tested (EFSA, 2021).

In a developmental toxicity study, pregnant rats were given nicarbazin by gavage at doses of 0, 70, 200 or 600 mg/kg bw per day on GD 7–17. No teratogenic effects were observed. The NOAEL for maternal toxicity was 200 mg/kg bw per day on the basis of reduced food intake and body weight gain, and increased mortality at 600 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 200 mg/kg bw per day based on reduced fetal body weight and delayed ossification, suggesting retarded fetal development possibly secondary to maternal toxicity. This study was considered by the forty-eighth meeting of JECFA in 1998 in establishing the ADI (JECFA, 1998).

In a GLP developmental toxicity study based upon OECD TG 414, time-mated rats were administered either vehicle or a 3:1 mixture of DNC and HDP orally by gavage from GD 6–20. Dosage levels were 0 + 0, 52.5 + 17.5, 150 + 50 or 450 + 150 mg/kg bw per day DNC + HDP. The NOAELs for maternal and embryo/fetal toxicity were both 450 + 150 mg/kg bw per day DNC + HDP, the highest dose tested (EFSA, 2021).

In an GLP-compliant, OECD guideline study of developmental toxicity in rabbits, nicarbazin was administered by gavage at dose levels of 0, 30, 60 or 120 mg/kg bw per day from GD 6–28 (Jardine & Craige, 2006). The NOAEL for maternal toxicity was 60 mg/kg bw per day based on the occurrence of prominent liver lobulation in two of 24 animals at the LOAEL of 120 mg/kg bw per day. A similar finding had been noted in the preliminary study where two of six animals were similarly affected at both 200 and 400 mg/kg bw per day, and therefore an association with treatment could not be discounted. The NOAEL for embryo/fetal toxicity was 120 mg/kg bw per day, the highest dose tested.

In an OECD TG 414 study of development toxicity, pregnant rabbits were treated daily by oral gavage with nicarbazin at doses of 0, 60, 120 and 240 mg/kg bw per day, from GD 6 to 28. The NOAEL for maternal toxicity was 240 mg/kg bw per day, the highest dose tested. The NOAEL for embryo/fetal toxicity was 120 mg/kg bw per day, based on changes in skeletal ossification indicative of developmental retardation at 240 mg/kg bw per day (EFSA, 2017).

In an oral developmental toxicity study based upon OECD TG 414, time-mated rabbits received a mixture of DNC and HDP by gavage from GD 7–28, at doses of 0 + 0, 22.5 + 7.5, 45 + 15 or 90 + 30 mg/kg bw per day (DNC + HDP) The NOAELs for maternal and embryo/fetal toxicity were both 90 + 30 mg/kg bw per day DNC + HDP, the highest doses tested (EFSA, 2021).

Table 6
Studies relevant to risk assessment

Species/study type (route of administration)	Doses	Critical end-point	NOAEL (mg/kg bw per day)	LOAEL (mg/kg bw per day)
Rat				
91-day study of toxicity (gavage)	**DNC**; 0, 106, 284 or 709 mg/kg per day	–	709 for DNC	
Two-year study of toxicity/ carcinogenicity (dietary)	**DNC + HDP**; 0 + 0, 50 + 17, 150 + 50 or 300 + 100 mg/kg bw per day	– No increase in tumour incidences	300 + 100	
Three-generation study (dietary)	**DNC + HDP**; 0 + 0, 50 + 17, 150 + 50 or 300 + 100 mg/kg bw per day	**Reproductive toxicity** –	300 + 100[a]	–
		Parental toxicity –	300 + 100[a]	–
		Offspring toxicity –	300 + 100[a]	–
Developmental toxicity study (oral gavage)	**Nicarbazin**; 0, 70, 200, or 600 mg/kg bw per day	**Maternal toxicity** Mortality, decreased food intake and body weight gain	200	600
		Embryo/fetal toxicity reduced fetal body weight and delayed ossification	200	600

Species/study type (route of administration)	Doses	Critical end-point	NOAEL (mg/kg bw per day)	LOAEL (mg/kg bw per day)
Rabbit				
Developmental toxicity study (oral gavage)	**Nicarbazin**; 0, 30, 60 or 120 mg/kg bw per day	**Maternal toxicity:** Prominent liver lobulation	60*	120
		Developmental toxicity:	120[a]	–
Dog				
Two-year toxicity study (dietary)	**DNC + HDP**; 0 + 0, 60 + 20, 180 + 60 or 600 + 200 mg/kg bw per day, six days per week	Elevated serum alanine transaminase and single incidence of mild bile duct proliferation	DNC:154 HDP:51 (correcting for duration of exposure on six days per week)	514 171

* pivotal study for the ADI (Jardine & Craige, 2006);

a the highest dose tested; LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level; GD: gestation day;

3.3 Microbiological data

The impact of nicarbazin residues on the human intestinal microbiome was evaluated through a decision-tree approach adopted by the sixty-sixth meeting of the Committee, which complies with VICH GL36(R) (VICH, 2013). This entails answering three questions to determine the need for establishing a mADI. Determine first if the drug residue, and/or its metabolites, are microbiologically active against representatives of the human intestinal microbiota. Second, determine if the drug residues enter the human colon, and third, whether the residues entering the human colon remain microbiologically active. If the answer to any of these questions is "no", then there is no need to calculate a mADI and the assessment does not need to be completed. However, if a mADI needs to be calculated, two end-points of concern for human health are considered for the assessment: disruption of the colonization barrier of the human intestinal microbiome and increases in populations of resistant bacteria in the human intestinal microbiome.

A non-GLP study (Elliot & Hulman, 1981) determined nicarbazin minimum inhibitory concentrations (MICs) against two strains of each of the following species: *Staphylococcus aureus, Salmonella typhimurium,* and *Clostridium perfringens*. Similarly three strains of the species: *Klebsiella pneumoniae, Escherichia coli* were examined. MIC results for all 12 isolates tested were greater than 128 µg/mL.

A publication from 2010 (Lanckriet et al., 2010) described nicarbazin MICs against 51 strains of *Clostridium perfringens* isolated from broiler chickens. Results showed that nicarbazin did not inhibit in vitro growth of the tested strains up to a concentration of 128 µg/mL.

A GLP study by Pridmore (2001) determined MICs for nicarbazin, DNC and HDP against a panel of 45 bacterial strains, five from each of the

following groups, some of which are representative of the human intestinal microbiome: *Salmonella, Staphylococcus, Enterococcus, Escherichia coli, Proteus, Lactobacillus, Campylobacter, Clostridium* and *Bacteroides*. Nicarbazin and DNC had no antimicrobial activity against any bacterial strains tested (MICs greater than 256 µg/mL). HDP had no measurable activity against 44 of the 45 bacterial strains tested, with MICs greater than 256 µg/mL, while one strain of *Campylobacter jejuni* had an MIC of 256 µg/mL.

Three microbiological studies therefore showed that nicarbazin exhibits no, or almost no, antimicrobial activity, with high MIC values (equal to or greater than 128 µg/mL) against all 108 isolates tested, which included anaerobic and aerobic micro-organisms.

Further, a search of literature available in the public domain did not produce any result indicating antimicrobial activity of nicarbazin against bacteria representative of the human intestinal microbiome. It was concluded that the answer to the first step of the assessment (which asks whether the drug residues and/or their metabolites, are microbiologically active against representatives of the human intestinal microbiota) was negative, therefore there was no need to determine a mADI. The same conclusion applies to determination of a mARfD, for which there is also no need.

4. Evaluation

The Committee established a ADI for nicarbazin (as DNC) of 0–0.9 mg/kg bw on the basis of a NOAEL of 60 mg/kg bw per day (equivalent to 42.5 mg/kg bw per day of DNC) due to prominent liver lobulation, observed in a study of developmental toxicity in the rabbit, applying a safety factor of 50. DNC is the toxic component of nicarbazin, and its absorption alone or in a mixture with HDP is substantially less (less than 5%) than when released from ingested nicarbazin. As DNC is the residue of concern and there is no nicarbazin in products from treated animals, the Committee concluded that despite limitations in the database, a reduction in the default safety factor of 100 used to account for interspecies and intraspecies variability, would be justified. The Committee was unable to quantify just how much of a reduction would be appropriate, but concluded that 50 could certainly be supported, and would still result in a conservative evaluation.

The Committee concluded that it was not necessary to establish an ARfD for nicarbazin (or DNC) in view of their low acute oral toxicity, the absence of developmental toxicity or of any other toxicological effects that would be likely to be elicited by a single dose.

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Appendix 1

Scope of JECFA literature search

The Committee conducted a comprehensive review of the scientific literature from the following publicly accessible databases: Web of Science, PubMed, Google Scholar, CAB Abstracts. The following searches were conducted in each of these databases:

Nicarbazin
Nicarbazin AND toxicity
Nicarbazin AND metabolite
Nicarbazin AND genotoxicity
Nicarbazin AND carcinogen
Nicarbazin AND case study
Nicarbazin AND poisoning

ANNEX 1

Reports and other documents resulting from previous meetings of the Joint FAO/WHO Expert Committee on Food Additives

1. General principles governing the use of food additives (First report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 15, 1957; WHO Technical Report Series, No. 129, 1957 (out of print).
2. Procedures for the testing of intentional food additives to establish their safety for use (Second report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 17, 1958; WHO Technical Report Series, No. 144, 1958 (out of print).
3. Specifications for identity and purity of food additives (antimicrobial preservatives and antioxidants) (Third report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as Specifications for identity and purity of food additives, Vol. I. Antimicrobial preservatives and antioxidants, Rome, Food and Agriculture Organization of the United Nations, 1962 (out of print).
4. Specifications for identity and purity of food additives (food colours) (Fourth report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as Specifications for identity and purity of food additives, Vol. II. Food colours, Rome, Food and Agriculture Organization of the United Nations, 1963 (out of print).
5. Evaluation of the carcinogenic hazards of food additives (Fifth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 29, 1961; WHO Technical Report Series, No. 220, 1961 (out of print).
6. Evaluation of the toxicity of a number of antimicrobials and antioxidants (Sixth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 31, 1962; WHO Technical Report Series, No. 228, 1962 (out of print).
7. Specifications for the identity and purity of food additives and their toxicological evaluation: emulsifiers, stabilizers, bleaching and maturing agents (Seventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 35, 1964; WHO Technical Report Series, No. 281, 1964 (out of print).
8. Specifications for the identity and purity of food additives and their toxicological evaluation: food colours and some antimicrobials and antioxidants (Eighth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 38, 1965; WHO Technical Report Series, No. 309, 1965 (out of print).
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ANNEX 2

Abbreviations used in the monographs

6-CNA	6-chloronicotinic acid
6-HNA	6-hydroxynicotinic acid
AChE	acetylcholinesterase
AD	administered dose
ADI	acceptable daily intake
ALP	alkaline phosphatase
ALT	alanine transaminase
ANOSIM	analysis of similarity
ARfD	acute reference dose
AST	aspartate transaminase
AUC,	area under the concentration–time curve
$AUC_{0-\infty}$	total AUC
BMD	benchmark dose
BuChE	butyrylcholinesterase
BUN	blood urea nitrogen
bw	body weight
ca	circa
Cal-EPA	California Environmental Protection Agency
CAT	catalase
CCRVDF	Codex Committee on Residues of veterinary Drugs in Foods
CL/F	oral clearance
C_{max}	maximum concentration
CMC	carboxymethyl cellulose
CNS	central nervous system
CYP	cytochrome P450
DNC	4,4'-dinitrocarbanilide
DTH	delayed-type hypersensitivity
EFSA	European Food Safety Authority
EMA	European Medicines Agency
FAO	Food and Agriculture Organization of the United Nations
FITC-dextran	fluorescein isothiocyanate-dextran
FOB	functional observational battery
FSH	follicle-stimulating hormone

GD	gestation day
GFAP	glial fibrillary acidic protein
GI	gastrointestinal
GLDH	glutamate dehydrogenase
GLP	good laboratory practice
GP	glutathione peroxidase
GSH	glutathione
HA	haemagglutinating antibody
HBGV	health-based guidance values
Hb	haemoglobin
HDP	2-hydroxy-4,6-dimethylpyrimidine
HPLC	high-performance liquid chromatography
Ht	haematocrit
IL-1β	interleukin 1 beta
IUPAC	International Union of Pure and Applied Chemistry
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
KLH	keyhole limpet hamocyanin
LC-MS/MS	liquid chromatography with tandem mass spectrometry
LC_{50}	median lethal concentration
LD	lactation day
LC_{50}	median lethal dose
LH	luteinizing hormone
LOAEC	lowest-observed-adverse-effect level
LPO	lipid peroxidation
LPS	lipopolysaccheride
mADI	microbiological acceptable daily intake
mARfD	microbiological acute reference dose
MIC	minimum inhibitory concentration
MOA	mode of action
MRL	maximum residue limit
M2 mAChR	M2 muscarinic acetylcholine receptors
nAChR	nicotinic acetylcholine receptors
NCE	normochromic erythrocyte
NOAEC	no-observed-adverse-effect concentration
NOAEL	no-observed-adverse-effect level
OECD	Organisation for Economic Co-operation and Development
OTU	operational taxonomic unit

PCA	4-chloroaniline (*p*-chloroaniline)
PCE	polychromatic erythrocyte
PCR	polymerase chain reaction
PK	pharmacokinetics
PNA	4-nitroaniline (*p*-nitroaniline)
PND	postnatal day
PCoA	principal co-ordinate analysis
qPCR	quantitative real-time polymerase chain reaction
RBC	red blood cell
ROS	reactive oxygen species
SCE	sister chromatid exchange
SD	standard deviation
SOD	superoxide dismutase
sRBC	sheep red blood cell
$t_{½}$	half-life
T3	triiodothyronine
T4	thyroxine
TBA	total bile acid
T_{max}	time to reach maximum concentration (Cmax)
TNF-α	tumour necrosis factor-α
TSH	thyroid-stimulating hormone
UDS	unscheduled DNA synthesis
U.S. EPA	United States Environmental Protection Agency
UHPLC	ultra-high performance liquid chromatography
WHO	World Health Organization
w/v	weight for volume
w/w	weight for weight

ANNEX 3

Participants in the ninety-fourth meeting of the Joint FAO/WHO Expert Committee on Food Additives

Virtual meeting, 16–27 May 2022

World Health Organization (WHO) members

Professor (Emeritus) Alan R. Boobis, National Heart and Lung Institute, Imperial College London, London, United Kingdom (*Chairperson*)

Professor Silvana Lima Górniak, Department of Pathology, School of Veterinary Medicine and Animal Sciences, University of São Paulo, São Paulo, Brazil

Food and Agriculture Organization of the United Nations (FAO) members

Dr Alan Chicoine, Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada (*Vice-Chairperson*)

Mr Peter Cressey, Senior Scientist, Institute of Environmental Science and Research Limited, Christchurch Science Centre, Christchurch, New Zealand

Dr Holly Erdely, Residue Chemistry Team, Division of Human Food Safety, FDA Center for Veterinary Medicine, Rockville, United States of America (*FAO Rapporteur*)

Dr Rainer Reuss, Food Standards Australia New Zealand, Kingston ACT, Australia

Professor Susanne Rath, University of Campinas, Department of Analytical Chemistry, São Paulo, Brazil

FAO experts

Dr Anke Finnah, German Federal Office of Consumer Protection and Food Safety, Berlin, Germany

Mr Samuel Fletcher, United Kingdom Veterinary Medicines Directorate, Surrey, United Kingdom

Professor Fernando Ramos, University of Coimbra, Faculty of Pharmacy, Coimbra, Portugal

Professor Jae-Han Shim, Distinguished Research Emeritus Professor, Chonnam National University, Gwangju, Republic of Korea

WHO experts

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FAO Resource contributors (not present at meeting)

Professor Benjamin U. Ebeshi, Department of Pharmaceutical and Medicinal Chemistry, Niger Delta University, Bayelsa State, Nigeria

Professor Jianzhong Shen, Dean of College of Veterinary Medicine, China Agricultural University, Beijing, China

Secretariat

Dr Vittorio Fattori, Food Systems and Food Safety Division, Food and Agriculture Organization of the United Nations (*FAO Secretariat*)

Ms Ngai Yin Ho, Department of Nutrition and Food Safety (NFS), World Health Organization (*WHO Consultant*)

Dr Markus Lipp, Food Systems and Food Safety Division, Food and Agriculture Organization of the United Nations (*FAO Secretariat*)

Dr Keya Mukherjee, Food Systems and Food Safety Division, Food and Agriculture Organization of the United Nations (*FAO Secretariat*)

Mr Soren Madsen, Department of Nutrition and Food Safety (NFS), World Health Organization (*WHO Secretariat*)

Dr Russell Parry, Shrewsbury, United Kingdom (*WHO Editor*)

Dr Moez Sanaa, Department of Nutrition and Food Safety (NFS), World Health Organization (*WHO Secretariat*)

ANNEX 4

Recommendations on the substances on the agenda

Imidacloprid (parasiticide)

Acceptable daily intake	In view of the absence of a study to assess the impact of imidacloprid on representative human intestinal microbiota, it was not possible to determine a mADI, thus the Committee was unable to establish an ADI for imidacloprid. The Committee established a toxicological acceptable daily intake (tADI) of 0–0.05 mg/kg bw on the basis of a NOAEL of 5.25 mg/kg bw per day for decreased body weight gain in the extended one-generation reproduction study, with the application of a safety factor of 100 to allow for interspecies and intraspecies differences.
Acute reference dose	In view of the absence of a study to assess the impact of imidacloprid on representative human intestinal microbiota, it was not possible to determine a mARfD, thus the Committee was unable to establish an ARfD for imidacloprid. The Committee established a toxicological acute reference dose (tARfD) of 0.09 mg/kg bw based on a $BMDL_{05}$ of 9 mg/kg bw reported by Cal EPA for an acute neurotoxicity study in rats and a safety factor of 100 to allow for interspecies and intraspecies differences. This value was supported by a NOAEL of 7.5 mg/kg bw per day for tremors in a 90-day toxicity study in dogs occurring during the first week of treatment, although it is not known whether tremors occurred after the first dose.
Estimated chronic dietary exposure	While estimates of dietary exposure were derived, there are no HBGVs with which to compare them. Based on incurred residues in Atlantic salmon (fillet) and a withdrawal period of 98 degree-days:

	The GECDE for adults and the elderly is 1.0 µg/kg bw per day. The GECDE for children and adolescents is 2.7 µg/kg bw per day. The GECDE for infants and toddlers is 0.9 µg/kg bw per day. Based on incurred residues in fish meat and a withdrawal period of 98 degree-days: The GECDE for adults and the elderly is 1.8 µg/kg bw per day. The GECDE for children and adolescents is 3.8 µg/kg bw per day. The GECDE for infants and toddlers is 1.2 µg/kg bw per day.
Estimated acute dietary exposure	Acute dietary exposures were assessed at 98 degree-days post dose. The adjusted (MR:TRR = 0.7) 95/95 UTL concentrations used were 859 µg/kg. No ARfD was available. Based on consumption of Atlantic salmon: The GEADE for adults is 6.2 µg/kg bw per day. The GEADE for children is 6.6 µg/kg bw per day. Based on consumption of all fin fish: The GEADE for adults is 34.1 µg/kg bw per day. The GEADE for children is 23.8 µg/kg bw per day.
Residue definition	The marker residue for imidacloprid in fillets of salmonids is the parent molecule, imidacloprid.
Maximum residue limits	As the Committee could not establish an ADI or an ARfD, no MRLs could be recommended for imidacloprid.

Ivermectin (broad-spectrum antiparasitic agent)

Acceptable daily intake	The Committee established an ADI of 0–10 µg/kg bw at the eighty-first meeting.

Acute reference dose	The Committee established an ARfD of 200 µg/kg bw at the eighty-first meeting.
Residue definition	The marker residue in sheep, pigs and goats is ivermectin B_{1a} (H_2B_{1a}, or 22,23-dihydroavermectin B_{1a}).
Estimated chronic	The GECDE for adults and the elderly is 0.72 µg/kg bw per day, which represents 7.2% of the upper bound of the ADI of 10 µg/kg bw. The GECDE for children and adolescents is 0.93 µg/kg bw per day, which represents 9.3% of the upper bound of the ADI of 10 µg/kg bw. The GECDE for infants and toddlers is 0.48 µg/kg bw per day, which represents 4.8% of the upper bound of the ADI of 10 µg/kg bw.
Estimated acute dietary exposure	The GEADE for cattle muscle, applicable to children and the general population, is 69 µg/kg bw, which represents 35% of the ARfD of 200 µg/kg bw. The GEADE for sheep muscle, applicable to children and the general population, is 73 µg/kg bw, which represents 37% of the ARfD of 200 µg/kg bw. The GEADE for pig muscle, applicable to children and the general population, is 30 µg/kg bw, which represents 15% of the ARfD of 200 µg/kg bw.

Recommended maximum residue limits (MRLs) for ivermectin

Species	Muscle (µg/kg)	Liver (µg/kg)	Kidney (µg/kg)	Fat (µg/kg)
Pigs	15	30	20	50
Sheep and goats	30	60	20	100

Nicarbazin (coccidiostat)

Toxicological effects	The NOAEL was 60 mg/kg bw per day (equivalent to 42.5 mg/kg bw per day of DNC) due to an increased incidence of prominent liver lobulation, observed in a study of developmental toxicity in the rabbit.

Toxicological ADI	The tADI for nicarbazin was established at 0–0.9 mg/kg bw (DNC).
Microbiological effects	Nicarbazin and/or its metabolites show no antimicrobial activity towards representative bacteria of the human intestinal microbiota.
Microbiological ADI	The Committee concluded that it was not necessary to establish a mADI for nicarbazin.
Acceptable daily intake	The ADI for nicarbazin was established at 0–0.9mg/kg bw based on toxicological effects.
Uncertainty factor	When considering nicarbazin it is DNC that is the toxic component, and its absorption alone or in a mixture with HDP is substantially less (< 5%) than when released from ingested nicarbazin. As DNC is the residue of concern and there is no nicarbazin in products from treated animals, the Committee concluded that despite limitations in the database, a reduction in the default safety factor of 100 used to account for interspecies and intraspecies variability, would be justified. The Committee was unable to quantify just how much of a reduction would be appropriate, but concluded that 50 could certainly be supported, and would still result in a conservative evaluation.
Acute reference dose	The Committee concluded that it was not necessary to establish an ARfD for nicarbazin.
Residue definition	The marker residue in chickens is DNC.
Estimated dietary exposure	Based on incurred DNC residues in chicken muscle, offal, and skin with fat, at 24 hours withdrawal time and 125 mg/kg feed: The GECDE for adults and the elderly is 120 µg/kg bw per day, which represents 13% of the upper bound of the ADI of 900 µg/kg bw.

The GECDE for children and adolescents is 160 µg/kg bw per day, which represents 18% of the upper bound of the ADI of 900 µg/kg bw.

The GECDE for infants and toddlers is 210 µg/kg bw per day, which represents 23% of the upper bound of the ADI of 900 µg/kg bw.

Based on incurred DNC residues in chicken muscle, offal, and skin with fat, at zero days withdrawal time and 50 mg/kg feed:

The GECDE for adults and the elderly is 95 µg/kg bw per day, which represents 11% of the upper bound of the ADI of 900 µg/kg bw.

The GECDE for children and adolescents is 120 µg/kg bw per day, which represents 14% of the upper bound of the ADI of 900 µg/kg bw.

The GECDE for infants and toddlers is 160 µg/kg bw per day, which represents 18% of the upper bound of the ADI of 900 µg/kg bw.

Recommended maximum residue limits (MRLs) for nicarbazin

Species	Liver (µg/kg)	Kidney (µg/kg)	Muscle (µg/kg)	Skin with fat (µg/kg)
Chicken	15 000	8000	4000	4000

Selamectin (broad-spectrum parasiticide)

Acceptable daily intake	The Committee withdrew the previous ADI and established an ADI of 0–0.05 mg/kg bw, based on a NOAEL of 5 mg/kg bw per day for increased liver and uterus/cervix weights at 15 mg/kg bw per day in a one-year rat study, with application of a safety factor of 100 to account for interspecies and intraspecies variability. Although the NOAEL for effects seen in a 13-week dietary neurotoxicity/toxicity study in rats, assessed by the Committee at its last meeting was 1 mg/kg bw per day, the LOAEL, at 15 mg/kg bw per day, and the effects observed were the same as those on which the ADI is based. The Committee concluded that the ADI established at the present meeting would be sufficiently protective of these findings.
Acute reference dose	The Committee concluded that the ARfD of 0.4 mg/kg bw established at the eighty-eighth meeting was still appropriate.
Residue definition	The marker residue in Atlantic salmon fillet is selamectin.
Estimated dietary exposure	Dietary exposure was assessed for some possible scenarios, but no GVP has been established.
Maximum residue limits	Specific MRLs could not be recommended at this time due to a lack of an established GVP.

This volume contains monographs prepared at the ninety-fourth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met in a virtual format, from 16–27 May 2022.

The toxicological monographs in this volume summarize data on the veterinary drug residues that were evaluated toxicologically by the Committee: imidacloprid and nicarbazin. Annexed to the report is a summary of the Committee's recommendations on these and other drugs (ivermectin and selamectin) discussed at the ninety-fourth meeting, including acceptable daily intakes (ADIs), acute reference doses (ARfDs) and proposed maximum residue limits (MRLs).

This volume and others in the WHO Food Additives Series contain infrmation that is useful to those who produce and use food additives and veterinary drugs, and those involved with controlling contaminants in food, government and food regulatory officers, industrial testing laboratories, toxicological laboratories and universities.

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