Toxicological evaluation of certain veterinary drug residues in food

Food and Agriculture Organization of the United Nations

World Health Organization

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CONTENTS

PREFACE

The monographs contained in this volume were prepared at the eighty-first meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met at FAO headquarters in Rome, Italy, on 17–26 November 2015. These monographs summarize the data on the safety of residues in food of selected veterinary drugs reviewed by the Committee.

The eight-first report of JECFA has been published by WHO as WHO Technical Report No. 997. 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 M. Sheffer, Ottawa, Canada. 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Many unpublished proprietary reports are submitted to the Committee by various producers of the veterinary drugs under review and in many cases represent the only data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 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 World Health Organization 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 Food Safety and Zoonoses, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland.

RESIDUES OF VETERINARY DRUGS

Diflubenzuron

First draft prepared by
Tong Zhou[1] and Arturo Anadón[2]

[1] Office of New Animal Drug Evaluation, Center for Veterinary Medicine, Food and Drug Administration, Department of Health and Human Services, Rockville, Maryland, United States of America (USA)

[2] Department of Toxicology and Pharmacology, Faculty of Veterinary Medicine, Universidad Complutense de Madrid, Madrid, Spain

1. Explanation

Diflubenzuron (International Union of Pure and Applied Chemistry name: 1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea, Chemical Abstracts Service no. 35367-38-5; Fig. 1) is an acyl urea derivative (halogenated benzoylphenylurea). Diflubenzuron is approved for use as a veterinary drug in Norway and Chile in the treatment of sea lice (*Lepeophtheirus salmonis* and *Caligus rogercresseyi*) infestations in Atlantic salmon (*Salmo salar*) as an oral dosage of 3–6 mg/kg body weight (bw) in feed for 14 consecutive days, with a withdrawal period in the range of 105–300 degree-days. It is also used as an insecticide/acaricide in agriculture and forestry against larvae of *Lepidoptera*, *Coleoptera*, *Diptera* and *Hymenoptera* (USEPA, 1997) and as a vector control agent in drinking-water sources and drinking-water storage containers (WHO, 2008).

Fig. 1
Structure of diflubenzuron
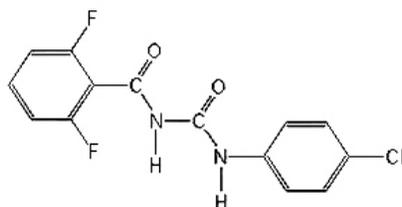
The mechanism of action of diflubenzuron is to inhibit the formation of new chitin in the insect cuticle during the moulting process by inducing both chitinase and phenoloxidase.

Diflubenzuron has not previously been evaluated by the Joint FAO/ WHO Expert Committee on Food Additives (JECFA). The Committee evaluated diflubenzuron at the current meeting at the request of the Twenty-second Session of the Codex Committee on Residues of Veterinary Drugs in Foods (FAO/WHO,

2015). The Committee was asked to establish an acceptable daily intake (ADI) and recommend maximum residue limits (MRLs) for diflubenzuron in salmon muscle and skin in natural proportion.

The Committee noted that the toxicity of diflubenzuron had been previously evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1981, 1985 and 2001 (FAO/WHO, 1982, 1986, 2002) and by a number of other scientific or regulatory bodies, such as the WHO Task Group on Environmental Health Criteria for Diflubenzuron, which prepared Environmental Health Criteria 184 (IPCS, 1996). In 2001, JMPR established an ADI of 0–0.02 mg/kg bw for diflubenzuron, based on the no-observed-adverse-effect level (NOAEL) of 2 mg/kg bw per day for haematological effects observed in 2-year toxicity studies in rats and a 52-week toxicity study in dogs (FAO/WHO, 2002).

The metabolism of diflubenzuron is known to lead to the formation of 4-chloroaniline (*p*-chloroaniline [PCA]; Fig. 2) in some species, but not the rat, and it is not known whether PCA is formed in salmon or humans. PCA might also occur as an impurity in the product formulation or as a degradation product generated during food processing. PCA is considered by many scientific and regulatory bodies (e.g. the International Agency for Research on Cancer; IARC, 1993) as genotoxic and carcinogenic. The Committee therefore evaluated the toxicity of PCA, focusing particularly on its genotoxicity and carcinogenicity as well as on its possible carcinogenic mode of action, based on studies retrieved from a search of published literature.

Fig. 2
Structure of *p*-chloroaniline (PCA)
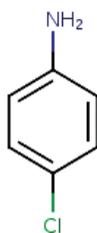
Because the sponsor did not provide any toxicological data on diflubenzuron, the Committee relied mainly on the summary evaluation prepared by JMPR in 2001. The Committee considered JMPR's summary evaluation of studies on the short-term and long-term toxicity, reproductive and developmental toxicity, and genotoxicity and carcinogenicity of diflubenzuron.

In addition, the Committee considered information obtained from literature searches on diflubenzuron and PCA. A literature search was conducted in June 2015 on the following search terms using PubMed, Embase and Web

of Sciences: diflubenzuron and toxicity or toxicology, diflubenzuron and toxicokinetics. In total, 215 unique references were retrieved. Of these, five were cited in the current monograph. The Committee also considered information obtained from literature searches conducted in October 2015 using PubMed and the broad search term 4-chloroaniline or *p*-chloroaniline. Four of the 323 references retrieved were cited in the current monograph.

The original studies provided to the 2001 JMPR were performed over a period of approximately 40 years, and all the studies were considered adequate for their intended purpose unless otherwise specified in the JMPR monograph. Some of the critical studies did not comply with good laboratory practice (GLP) regulations, as the data were generated before the implementation of GLP regulations. Overall, however, the present Committee considered that the database was adequate to assess the risk of diflubenzuron.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

(a) Oral administration

(i) Mice

When mice (strain not specified) were given a single oral dose of diflubenzuron at 12, 64, 200 or 920 mg/kg bw, excretion was almost complete within 48 hours. The cumulative percentage of the dose excreted in the urine decreased from 15% at the lowest dose to about 2% at the highest dose, showing that the relationship between urinary excretion and dose in mice is similar to that in rats (see below) (de Lange & Post, 1978; GLP status not specified).

(ii) Rats

Radiolabelled diflubenzuron, with the ^{14}C label uniformly distributed in the anilino moiety, was administered by oral gavage to Wistar rats as a single dose of 4, 16, 48, 128 or 1000 mg/kg bw. Urine was collected every 24 hours up to 120 or 144 hours. The intestinal absorption of diflubenzuron in rats decreased with increasing dose. The cumulative excretion in urine as a percentage of the dose decreased from 28% at the lowest dose to only 1% at the highest dose, while the recovery remained constant (90%) (de Lange, Fronik & Post, 1977; Willems et al., 1980; GLP status not specified).

In female Wistar rats with cannulated bile ducts orally administered [^{14}C]diflubenzuron at a dose of 4, 16, 128 or 900 mg/kg bw, bile was collected at

6, 24, 48 and 72 hours, and urine and faeces were collected at 24-hour intervals for 72 hours. The sum of the excretion in urine and bile decreased from 42% of the dose at the lowest dose to about 4% at the highest dose. In Wistar rats that had received radiolabelled diflubenzuron with the ^{14}C in the carbamoyl group of the benzoyl moiety, 1% of the radiolabel was found in expired air. Seventy-two hours after administration of a single oral dose of 5 mg/kg bw of double-labelled diflubenzuron (^{14}C in the anilino moiety and ^{3}H in the benzoyl moiety), 1.3% of the ^{14}C and 3.5% of the ^{3}H were retained in the carcass (de Lange, Fronik & Post, 1977; Willems et al., 1980; GLP status not specified).

An extensive GLP-compliant investigation of the absorption, distribution and excretion of diflubenzuron was reported. Groups of Sprague-Dawley rats received [^{14}C]diflubenzuron uniformly labelled in both phenyl rings (specific activity 37 or 722 kBq/mg) by gavage in 1% gum tragacanth. Details of the dosing and sampling are given in Table 1. Samples were retained for subsequent determination of metabolites (see section 2.1.2 below). Radiolabel was determined by liquid scintillation counting (LSC) with correction against an external standard after appropriate processing. The results (Table 2) showed that at a dose of 5 mg/kg bw, about 30% of the administered dose was excreted in urine and bile; absorption was less complete at 100 mg/kg bw. In animals with bile duct cannulae, reduced excretion was seen in urine (about 7%), indicating some degree of enterohepatic recirculation. $^{14}CO_2$ in exhaled air represented less than 0.1% of the administered dose. The peak concentrations of radiolabel in blood (C_{max}) were achieved at 4 hours (T_{max}) after administration. The highest tissue concentrations at 4 hours were seen in liver and fat. More radiolabel was found in erythrocytes relative to plasma with increasing duration after dosing. Autoradiography showed a wide distribution of radiolabel, the highest concentrations being found in the

Table 1

Design of study of absorption, distribution and excretion of [^{14}C]diflubenzuron in rats

No. of animals	Dosing schedule	Routine samples[a]	Terminal samples
5/sex	1 × 5 mg/kg bw	Urine, faeces, air	Fluids, tissues at 168 h
5/sex	14 × 5 mg/kg bw per day (^{12}C)	–	–
	1 × 5 mg/kg bw (^{14}C)	Urine, faeces, air	Fluids, tissues at 168 h
5/sex	1 × 100 mg/kg bw	Urine, faeces, air	Fluids, tissues at 168 h
3/sex	1 × 5 mg/kg bw	Blood	–
3/sex per time	1 × 5 mg/kg bw	–	Fluids, tissues at 4, 14, 48 and 72 h
3/sex	1 × 5 mg/kg bw	Bile, urine, faeces	Gastrointestinal tract, carcass at 24 h
1/sex per time	1 × 5 mg/kg bw	–	Autoradiography[b]

bw: body weight

[a] Urine and faecal samples were taken at 8 and 24 hours, then every 24 hours until 168 hours. Air samples were taken at 8 and 24 hours. Tail vein blood was taken at 0.25, 0.5, 1, 2, 4, 8, 16, 24 and 48 hours. Bile samples were taken hourly for 24 hours.

[b] At times corresponding to plasma C_{max}, $C_{max/2}$ and 48 hours.

Source: Dunsire, Cameron & Spiers (1990)

Table 2
Absorption, distribution and excretion of [^{14}C]diflubenzuron in rats (mean values)

	1 × 5 mg/kg bw		14 + 1 × 5 mg/kg bw		1 × 100 mg/kg bw	
	Males	**Females**	**Males**	**Females**	**Males**	**Females**
Urine (%)						
0–8 h	8	9	4	4	1	1
0–24 h	20	20	14	12	3	2
Faeces (%)						
0–8 h[a]	16	11	<1	11	66	35
0–24 h	73	75	64	72	95	93
Total recovery (%)	97	99	97	99	99	99
Time to blood C_{max} (h)	4	4	ND	ND	ND	ND
Time to blood $C_{max/2}$ (h)	14	14	ND	ND	ND	ND
Plasma concentration (ng eq/mL)						
C_{max}	695	752	ND	ND	ND	ND
$C_{max/2}$	187	260	ND	ND	ND	ND
48 h	13	20	ND	ND	ND	ND
168 h (7 days)	3	3	4	5	0	10
Liver concentration (ng eq/mL)						
C_{max}	2 090	2 440	ND	ND	ND	ND
$C_{max/2}$	693	1 170	ND	ND	ND	ND
48 h	336	526	ND	ND	ND	ND
168 h (7 days)	187	151	153	152	330	370
Erythrocyte concentration (μg eq/mL)						
C_{max}	548	449	ND	ND	ND	ND
$C_{max/2}$	566	318	ND	ND	ND	ND
48 h[a]	360	398	ND	ND	ND	ND
168 h (7 days)	169	251	157	152	590	780
Bile (%)						
0–8 h	3	3	ND	ND	ND	ND
0–24 h	19	15	ND	ND	ND	ND

bw: body weight; C_{max}: maximum concentration; eq: equivalents; ND: not determined
[a] Great inter-animal variation.
Source: Dunsire, Cameron & Spiers (1990)

gastrointestinal tract. Excretion was relatively rapid, with more than 90% of the doses of 5 and 100 mg/kg bw being excreted within 24 hours. Animals given repeated doses showed evidence of delayed absorption and excretion, but no change in the pattern of excretion or distribution of radiolabelled residues was seen at 7 days. The highest concentrations of residues of radiolabel 7 days after administration were found in erythrocytes and liver, but the total radiolabelled residues represented less than 1% of the administered dose. There were no notable differences between males and females (Dunsire, Cameron & Spiers, 1990).

(iii) Cats

Diflubenzuron labelled with ^{14}C and ^{3}H was administered orally to cats (strain not specified) at a dose of about 7 mg/kg bw on day 10 of a 15-day dosing regimen with unlabelled diflubenzuron. Within 72 hours of administration, 9% of the oral dose had been excreted in the urine, and 77% of the ^{14}C and 71% of the ^{3}H had been excreted in the faeces (Hawkins, Jackson & Roberts, 1980).

(b) Dermal application

(i) Rats

In a GLP-compliant study, the dermal absorption of [U-^{14}C-anilino]diflubenzuron (purity 97.2%; specific activity 666 kBq/mg) was investigated in groups of four male Sprague-Dawley rats (one control per time point). A suspension of diflubenzuron (100 µL) in 0.25% gum tragacanth was applied to a 10 cm^2 clipped area at a concentration of 0.005 or 0.05 mg/cm^2 and kept under semi-occluded conditions. The rats were killed at 1, 4 or 10 hours after application, and the skin site was washed 5 times with soap solution. Samples of excreta, cage wash, dermal application site and carcass were analysed by LSC after appropriate processing. More than 96% of the applied radiolabel was recovered in all groups, more than 80% being removed in the first wash. The radiolabelled residues at the dermal application site represented 4.5–6% of the applied dose. The mean absorption was less than 0.5% in all groups, with a range of 0.03–1.2%. There was no evidence of increased absorption with longer exposure (Andre, 1996).

(ii) Rabbits

The dermal absorption of [U-^{14}C-anilino]diflubenzuron (purity >99%; specific activity 11.2 µg/g) was investigated in a group of eight male New Zealand rabbits. A 10% suspension of diflubenzuron in 1% gum tragacanth was applied at 150 mg/kg bw to shaved areas of the skin and occluded for 6 hours, when the application site was washed. Excretion of radiolabel in urine and faeces was determined over 48 hours. The concentration of radiolabel in faeces was at the limit of quantification (LOQ) (<1% of the applied dose), and about 0.1% of the applied dose was recovered in urine. Diflubenzuron is thus poorly absorbed through rabbit skin (de Lange, 1979; GLP status not specified).

2.1.2 Biotransformation

(a) Rats

The basic metabolic pathways of diflubenzuron were established in studies in rats. The main pathway involves hydroxylation of the phenyl moieties of the intact compound. About 80% of the metabolites in rat urine were identified as

2,6-difluoro-3-hydroxybenzuron, 4-chloro-2-hydroxydiflubenzuron and 4-chloro-3-hydroxydiflubenzuron. Approximately 20% underwent scission at the ureido bridge, and most was excreted as 2,6-difluorobenzoic acid (de Lange, Fronk & Post, 1977; Willems et al., 1980).

In the study described above in section 2.1.1, in which Wistar rats were administered radiolabelled diflubenzuron as a single dose of 5 mg/kg bw by oral gavage, there was no indication of the presence of either PCA or 4-chlorophenylurea (CPU), a metabolite that may be reduced to PCA, in the urine or bile of the rats (Willems et al., 1980).

In a GLP-compliant study, the metabolites produced by Sprague-Dawley rats after administration of [^{14}C]diflubenzuron labelled in both phenyl rings were identified and characterized in samples from the study of Dunsire, Cameron & Spiers (1990) (see section 2.1.1 above). Pooled (0–24 hours) samples of urine, faeces or bile were prepared from males and females orally dosed once at 5 or 100 mg/kg bw or 15 times at 5 mg/kg bw per day. After extraction, the metabolites were determined by high-performance liquid chromatographic (HPLC) separation, LSC and comparison with standards. The presence of conjugates was determined by the use of a glucuronidase–arylsulfatase preparation. A specific investigation for the presence of PCA was performed, as routine methods failed to separate this compound from CPU. The metabolic profiles were similar for males and females. Biliary samples were difficult to analyse, as 75–80% of the radiolabel was present in a five-component peak. Enzyme hydrolysis of bile increased the proportion of 2-hydroxydiflubenzuron from about 5% to 19%, at the expense of the major multicomponent peak. Diflubenzuron represented about 7% of the biliary radiolabel. Initial investigations of faecal samples gave variable recovery rates, with diflubenzuron representing more than 93% of the recovered radiolabel. A second method involving enzyme hydrolysis and dilution with control urine was used to investigate samples from animals given the high single dose and repeated doses. Various compounds were identified, the main peaks being diflubenzuron (65–75% of recovered radiolabel), 2-hydroxydiflubenzuron (7–10%), CPU (5–6%) and 2,6-difluorobenzamide (2–4%). Investigations of urine samples gave reliable recoveries (85–105%), and various metabolites were identified, although there was an incomplete separation. The results showed that absorbed diflubenzuron was extensively metabolized. Enzyme treatment of the urine samples indicated conjugation of both 2′-hydroxydiflubenzuron and diflubenzuron. The only notable difference between the groups was the higher concentration of unconjugated 2′-hydroxydiflubenzuron and the presence of peak B in the urine of animals given repeated doses. Specific investigations for PCA did not reveal its presence in bile or urine using a method with an LOQ of 7.5 ng/mL (Cameron, Henderson & McGuire, 1990).

The production of PCA and CPU was investigated in four male Fischer 344 rats given a single dose of [U-^{14}C-anilino]diflubenzuron (specific activity 164 Bq/mg; radiochemical purity 99.9%) at 104 mg/kg bw in 1% gum tragacanth by oral gavage. Urine and faecal samples were taken after 20, 48, 72 and 96 hours. Pooled samples (0–20 hours) were analysed by HPLC, liquid chromatography with mass spectrometry (LC-MS) and liquid chromatography with tandem mass spectrometry (LC-MS/MS). Faecal samples were extracted initially with acetonitrile, then sequentially with hexane, acetone and water. Most of the administered dose was excreted within 20 hours, with 68% in faeces and 2% in urine. The only compound detected in faeces was diflubenzuron. Three metabolites were identified in significant amounts in urine: 4-chloroaniline-2-sulfate (45% of the total urinary radiolabelled residues), *N*-(4-chlorophenyl)-oxamic acid (13%) and 4-chloroacetanilide-2-sulfate (2%). Diflubenzuron, PCA, CPU and their *N*-hydroxy derivatives were not identified in urine. 2-Hydroxydiflubenzuron was present at less than 1% of the total radiolabelled residues (Wang & Gay, 1999; GLP status not specified).

There is no clear reason for the marked differences between the findings of Cameron, Henderson & McGuire (1990) and Wang & Gay (1999). The overall conclusion from these studies is that the primary steps in the metabolism of diflubenzuron are hydrolysis of the anilino ring, cleavage of the ureido bridge and conjugation (mainly to sulfate). Both Willem et al. (1980) and Wang & Gay (1999) indicated that PCA and CPU might not be formed directly in rats. It is possible that hydroxylation of the anilino ring occurs before cleavage of the ureido bridge.

JMPR (FAO/WHO, 2002) proposed a metabolic pathway for diflubenzuron in rats, as presented in Fig. 3.

(b) **Other species**

PCA was not detected (limits of detection [LODs] not given) in urine or faeces of sheep or cows following administration of a single oral dose of diflubenzuron at 10 mg/kg bw (Ivie, 1978). However, when diflubenzuron was given as a single oral dose of 5 mg/kg bw, PCA was detected in small quantities in the urine of swine (1.03% of the dose; Opdycke, Miller & Menzer, 1982a) and chicken excreta (0.44% of the dose; Opdycke, Miller & Menzer, 1982b).

Following administration of an oral dose of diflubenzuron, CPU was detected in small quantities in the urine of swine (0.82% of a 5 mg/kg bw dose; Opdycke, Miller & Menzer, 1982a), in the urine of cows (0.6% of a 10 mg/kg bw dose; Ivie, 1978) and in chicken excreta (3.14% of a 5 mg/kg bw dose; Opdycke, Miller & Menzer, 1982b).

Fig. 3
Proposed metabolic pathway for diflubenzuron in rats

Bold arrows: major pathways.
Light arrows: minor pathways.

2.1.3 Effects on enzymes and other biochemical parameters

The effects of diflubenzuron on the concentrations of methaemoglobin and sulfhaemoglobin and the incidence of Heinz bodies were investigated in mice and rats dosed once with a 25% weight per weight (w/w) formulation of diflubenzuron. Groups of 15 male Swiss mice (Keet, 1977a) or male Wistar rats (Keet, 1977b) received the formulation at 0 (co-formulants only) or 10 000 mg/kg bw (2500 mg/kg bw as diflubenzuron) in 1% gum tragacanth by oral gavage. Samples of blood were taken 4 and 24 hours after dosing. In mice, there were no effects on body weight, sulfhaemoglobin concentration or incidence of Heinz bodies. The methaemoglobin concentrations were increased ($P < 0.003$) at 4 hours, but not at 24 hours. The increase at 4 hours was relatively small (2.2% versus 2.6%), but the ranges (1.3–2.5% versus 1.5–3.3%) indicated a treatment-related effect. A similar pattern was found in rats, with no effects on body weight, sulfhaemoglobin concentration or incidence of Heinz bodies. The methaemoglobin concentrations were increased ($P < 0.01$) at 24 hours, but not at 4 hours. The increase at 24 hours was relatively small (1.5% versus 1.9%), but there was a small overlap in the ranges (1.2–1.8% versus 1.6–2.1%), showing a clear treatment-related effect. A single high dose of diflubenzuron (2500 mg/kg bw) can thus increase methaemoglobin concentrations (Keet, 1997a,b).

2.2 Toxicological studies

2.2.1 Acute toxicity

(a) Lethal doses

The results of acute toxicity studies show that diflubenzuron (purity 99.6%) has very low acute toxicity when given by the oral, inhalation or dermal route. The median lethal dose (LD_{50}) in mice and rats given diflubenzuron in 1% tragacanth by oral gavage was greater than 4600 mg/kg bw (van Eldik, 1973). In rats treated dermally for 24 hours, the LD_{50} was greater than 10 000 mg/kg bw (Koopman, 1977). In rats treated by whole-body inhalation of a preparation with a mass median aerodynamic diameter of less than 5 μm, the median lethal concentration (LC_{50}) was greater than 2.9 mg/L of air (Berczy, Cobb & Cherry, 1973). No clinical signs were seen in these studies, although the level of detail in the study reports was inadequate to permit an independent confirmation. No haematological examinations were performed.

(b) Dermal irritation

In a non-GLP-compliant study, diflubenzuron (purity unknown) was reported not to be irritating to the intact or abraded skin of rabbits (strain and sex not

specified), although the level of detail in the report was inadequate to permit an independent confirmation (Taylor, 1973).

(c) Ocular irritation

In a non-GLP-compliant study, diflubenzuron (purity 99.6%) was slightly irritating to the eyes of New Zealand White rabbits (sex not specified) after instillation of 40 mg (0.1 mL). The findings were similar when the eyes were rinsed 5 minutes or 24 hours after administration (Davies & Liggett, 1973).

(d) Dermal sensitization

In a GLP-compliant study, the skin sensitizing potential of diflubenzuron (purity 95.6%) was investigated in groups of 10 CRL HA BR guinea-pigs exposed in a maximization test. For induction, 10% diflubenzuron in maize oil with Freund's complete adjuvant was injected, or 30% diflubenzuron in a petroleum jelly product was applied topically. The animals were challenged by application of 10% or 30% diflubenzuron in the petroleum jelly product. Mild responses were seen after induction by either route. After challenge, mild reactions were seen in one control and two test animals. Diflubenzuron was not a skin sensitizer in this study (Prinsen, 1992).

2.2.2 Short-term studies of toxicity

(a) Oral administration

(i) Rats

In a GLP-compliant study, groups of 40 Sprague-Dawley rats of each sex (90 of each sex for controls) received diets containing diflubenzuron (two lots; purity 96% and 97.2%) at a concentration of 0, 160, 400, 2000, 10 000 or 50 000 mg/kg feed (equivalent to 0, 8, 20, 100, 500 and 2500 mg/kg bw per day, respectively) for 13 weeks. About half of the animals were killed at week 7, and the remainder at 13 weeks. Routine clinical examinations were performed. Samples were taken from 10 animals of each sex per group for haematology, clinical chemistry and urine analysis in weeks 7 and 13. All animals were examined grossly, and extensive histological investigations were performed on controls and animals at the highest dose, with more limited examination (but including liver, spleen and bone marrow) of other groups.

There were no treatment-related effects on mortality rate, clinical signs or feed consumption. Body weight gain was reduced by about 20% in females receiving 50 000 mg/kg feed and in males receiving 2000 mg/kg feed and above, but with no clear dose–response relationship. Haematology at week 7 showed a range of dose-related alterations in erythrocyte parameters (erythrocyte counts, haemoglobin, reticulocytes, methaemoglobin and sulfhaemoglobin)

in animals of each sex receiving dietary concentrations of 400 mg/kg feed and above, with minimal effects at 160 mg/kg feed; there were small increases in methaemoglobin concentration in males (statistically insignificant) and females (statistically significant) at 160 mg/kg feed. The haematological findings were reproduced at week 13. Alterations in a number of clinical chemistry parameters were seen in animals at the two highest doses, but the findings were sporadic, generally occurred in only a few animals in a group and were considered to be not biologically significant. The absolute and relative weights of the spleen were increased in males and females at 400 mg/kg feed and above and in males receiving 160 mg/kg feed for 7 weeks. At week 13, the absolute weight of the liver was increased by more than 10% in females at the highest dose, and the relative liver weights were increased by more than 10% in males at the highest dose and in females at dietary concentrations of 400 mg/kg feed and above. Pathological lesions related to treatment were chronic hepatitis, haemosiderosis and congestion of the spleen, and erythroid hyperplasia of the bone marrow in all treated groups and haemosiderosis of the liver at dietary concentrations of 400 mg/kg feed and above. The lesions increased in severity with increasing dose and duration of dosing.

From this study, a NOAEL could not be identified, as there were small but statistically significant increases in methaemoglobin concentration, with associated findings in the spleen (increased spleen weight, spleen haemosiderosis and congestion) and bone marrow (erythroid hyperplasia), at the lowest dose, 160 mg/kg feed (equivalent to 8 mg/kg bw per day) (Burdock et al., 1980; Goodman, 1980).

(ii) Dogs

In a non-GLP-compliant study, purebred Beagle dogs (three of each sex per group), aged 16–23 weeks, received diets (400 g/day) containing diflubenzuron (purity unspecified) at a concentration of 0, 10, 20, 40 or 160 mg/kg feed for 13 weeks. The achieved intakes were 0, 0.4, 0.8, 1.6 and 6.4 mg/kg bw per day, respectively. Blood samples for haematology were taken before dosing and at weeks 2, 4, 6 and 12. Clinical chemistry (limited), urine analysis and ophthalmological investigations were performed before dosing and at weeks 6 and 12. All animals were examined grossly at sacrifice, and an extensive range of tissues was examined microscopically. The results were presented for the two sexes combined.

There were no deaths or adverse clinical signs. Body weights and feed and water consumption were not affected by treatment. The body weights fluctuated considerably during the study, which was expected, given the spread of ages, and there was no indication of an association with treatment. The results of urine analysis, ophthalmology and gross pathological and histopathological

examinations were similar in all groups. Increases in aspartate aminotransferase activity were seen in animals at the highest dose in weeks 6 and 12, but the finding was of dubious significance, as it was not reproduced when the samples were retested. At week 2, haematological end-points were similar in all groups. At week 4, the haemoglobin concentration of animals at 160 mg/kg feed was reduced by about 10%. At week 6, both the haemoglobin concentration and erythrocyte count were reduced and the methaemoglobin and free haemoglobin concentrations were increased in animals at 160 mg/kg feed. At week 12, haematological end-points were similar in all groups, although there was a clear increase in the myeloid:erythroid ratio in bone marrow in dogs at 160 mg/kg feed (1.2 versus 0.7 in controls). There was an indication of an increase in spleen weight (by about 40%) at 160 mg/kg feed, but intergroup variation was high, and the ranges in treated and control groups showed considerable overlap.

The NOAEL was 40 mg/kg feed (equal to 1.6 mg/kg bw per day), on the basis of alterations in haematological end-points and bone marrow at 160 mg/kg bw (equal to 6.4 mg/kg bw per day) (Chesterman et al., 1974).

In a non-GLP-compliant study, groups of six male and six female Beagle dogs (treated groups: six of each sex; controls: 12 of each sex) received gelatine capsules containing diflubenzuron (purity 97.6%) at a dose of 0, 2, 10, 50 or 250 mg/kg bw per day for 52 weeks. The animals underwent routine clinical investigations. Blood samples for haematology and clinical chemistry were taken before dosing and at weeks 4, 7, 13, 26 and 51. Urine was analysed before dosing and at weeks 7, 13, 26 and 51. Ophthalmoscopy was performed before dosing and at weeks 26 and 51. All animals were examined grossly and microscopically.

One female at the highest dose died from liver failure, and one female at 50 mg/kg bw per day died from pneumonia, but neither death was clearly related to treatment. No consistent differences were found between control and test animals with respect to clinical signs, feed consumption, body weight, water consumption or urinary parameters. A range of effects related to impaired erythrocytes was seen at 50 and 250 mg/kg bw per day from week 13 onwards. At doses of 50 mg/kg bw per day and above, signs of haemolytic anaemia, destruction of erythrocytes and compensatory regeneration of erythrocytes were observed. Increases in methaemoglobin and sulfhaemoglobin concentrations were evident at doses of 10 mg/kg bw per day and above. The severity of the haematological alterations appeared to increase with duration of dosing, stabilizing at week 26 and with some evidence of adaptation at week 52. Increased sulfhaemoglobin concentrations seen in animals at the lowest dose in week 26 were considered not to be adverse, as they were not statistically significant and were not seen at week 52. Other findings included increased platelet numbers at doses of 10 mg/kg bw per day and above in females and 50 mg/kg bw per day and above in males; increased lactate

dehydrogenase activity from week 7 in animals of each sex receiving 250 mg/kg bw per day; and increases in liver (10%) and spleen weights (35%) at doses of 50 mg/kg bw per day and above. The only notable histopathological findings were in the liver, comprising increased pigmentation of Kupffer cells and macrophages at 10 mg/kg bw per day and above. The increased incidence of pigmentation of Kupffer cells observed in males at the lowest dose (2 mg/kg bw per day) was considered not to be biologically significant, as it was graded '"minimal", and the frequency was within the normal range of background findings.

The NOAEL was 2 mg/kg bw per day, based on significant effects on methaemoglobin and sulfhaemoglobin concentrations and platelet counts and increased incidences of hepatic pigmentation at 10 mg/kg bw per day (Greenough et al., 1985).

(b) Dermal application

In a GLP-compliant study, Crl:CD BR (VAF/Plus) rats (10 of each sex per group) received diflubenzuron (purity 96.7%) in 0.25% aqueous gum tragacanth applied dermally to shaved, unabraded sites under semi-occlusive dressings at a dose of 0, 20, 500 or 1000 mg/kg bw per day, 6 hours/day, for 21 days. The application sites represented about 10% of the body area at the middle and high doses and 1% at the low dose. Routine clinical investigations were performed. Blood samples for haematology and clinical chemistry were taken before sacrifice. All animals were examined grossly, and liver, kidney and skin from controls and animals at the high dose were examined histologically.

One female at the middle dose and one at the high dose died on day 9. As these were isolated findings and there were no subsequent deaths, the relationship to exposure to diflubenzuron was uncertain. Body weight, feed consumption, clinical signs, clinical chemistry parameters and gross and microscopic appearance were unaffected. There were no signs of irritation at the dermal application site. Reductions in erythrocyte parameters were seen in females at 500 mg/kg bw per day and in animals of each sex at 1000 mg/kg bw per day, together with increased incidences and severity of polychromasia, hyperchromasia and anisocytosis in animals of each sex at doses of 500 mg/kg bw per day and above. Statistically significant increases in leukocyte counts were seen in males at 500 mg/kg bw per day and above, and a non-significant increase in leukocyte counts was seen in females at the high dose. The findings were linked to increased numbers of segmented neutrophils and lymphocytes, but the association with diflubenzuron was unclear.

The NOAEL was 20 mg/kg bw per day, based on haematological effects at 500 mg/kg bw per day (Goldenthal, 1996).

(c) **Exposure by inhalation**

In a GLP-compliant study, Sprague-Dawley rats (10 of each sex per group) were exposed for 6 hours/day, 5 days/week, for 4 weeks, by nose only, to atmospheres of diflubenzuron (purity 96.5%) that were generated from a jet mill and contained particles with a mass median aerodynamic diameter of about 2 mm and a gravimetric concentration of 0.2 (control), 12, 34 or 110 mg/m³. The concentrations varied during the study by ±10%, but homogeneity within the chamber was confirmed. The animals were observed routinely during and after exposure with a basic neurofunctional assessment. Samples for urine analysis, haematology and clinical chemistry parameters were taken before sacrifice. All animals were examined grossly. A wide range of tissues from animals in the control and high-concentration groups as well as kidney, liver and lungs from all groups were examined microscopically.

One animal died during the terminal bleeding. Body weight, feed consumption, clinical signs, urine analysis, and gross and microscopic appearance were unaffected. A reduction in "grid count" was evident in the neurofunctional assessment of male and female rats exposed to 110 mg/m³. Statistically significant ($P < 0.05$) decreases in some erythrocyte parameters were seen at this concentration, with evidence of a concentration-related effect. Slight, but statistically significant, increases in bilirubin concentrations were seen in both male and female rats at the highest level of exposure.

The no-observed-adverse-effect concentration (NOAEC) was 34 mg/m³ (approximately 10 mg/kg bw per day), based on effects on behaviour, haematological end-points and bilirubin concentration at 110 mg/m³ (Newton, 1999).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) **Mice**

In a GLP-compliant study, HC/CFLP mice (controls: 104 mice of each sex; treated: 52 mice of each sex per group) were administered diflubenzuron (purity 97.6%) in the diet at a concentration of 0, 16, 80, 400, 2000 or 10 000 mg/kg feed (equal to 0, 1.2, 6.4, 32, 160 and 840 mg/kg bw per day for males and 0, 1.4, 7.3, 35, 190 and 960 mg/kg bw per day for females, respectively) for 91 weeks. Groups of 24 controls and 12 mice in each treated group were sacrificed at weeks 27, 52 and 78. Detailed observations, feed consumption and body weight determinations were performed weekly. Haematology (weeks 26, 52, 78 and 91), clinical chemistry (weeks 24, 50, 76 and 89), urine analysis (weeks 25, 51, 77 and 90) and water consumption determination were performed periodically. All animals were examined grossly, and a wide range of tissues from all animals was examined microscopically.

The mortality rates were similar in all groups, with survival to week 78 being more than 50% except for females receiving dietary concentrations of 80

or 2000 mg/kg feed. Clinical signs (blue-grey coloration of the extremities) were noted at week 1 in animals receiving dietary concentrations of 2000 mg/kg feed and higher and subsequently in animals in all groups receiving dietary concentrations of 80 mg/kg feed and higher. Body weights and feed consumption were similar in all groups. Water consumption was increased at some time points for females receiving dietary concentrations of 2000 mg/kg feed and higher, with an associated increase in urine volume. Urine analysis did not indicate any impairment of renal function. Statistically significant, dose-related changes were seen in a number of haematological parameters from week 26 onwards in animals receiving dietary concentrations of 80 mg/kg feed and higher. Statistically significant increases in the concentrations of sulfhaemoglobin were seen at 16 mg/kg feed in animals of both sexes at week 52 and in females at week 78. A dose-related increase in the incidence of Heinz bodies was seen consistently in mice at dietary concentrations of 400 mg/kg feed and higher and sporadically in males at 80 mg/kg feed. Increased serum alkaline phosphatase and aspartate aminotransferase activities were seen in males and females at the highest dietary concentration at week 26 and in males at this concentration subsequently. Gross pathological examination showed increased absolute liver and spleen weights (Table 3) and cyanotic skin as the only consistent findings. Histopathological examination confirmed that the liver and spleen were the primary target organs for non-neoplastic effects (Table 3). No increase in tumour incidence was associated with administration of diflubenzuron. The increased sulfhaemoglobin concentrations at 16 mg/kg feed are of questionable biological significance, as they occurred in only a proportion of animals in the group and the frequency was within the range of fluctuating control values seen during the study.

The NOAEL was 16 mg/kg feed (equal to 1.2 mg/kg bw per day), on the basis of increased methaemoglobin and sulfhaemoglobin concentrations and incidences of cyanosis at 80 mg/kg feed (equal to 6.4 mg/kg bw per day). The NOAEL for carcinogenicity was 10 000 mg/kg feed (equal to 840 mg/kg bw per day), the highest concentration tested (Colley et al., 1984).

(b) Rats

In a non-GLP-compliant study, groups of CD rats (60 of each sex per group) were fed diets containing diflubenzuron (purity unspecified) at a concentration of 0, 10, 20, 40 or 160 mg/kg feed (equivalent to 0, 0.5, 1, 2 and 8 mg/kg bw per day, respectively) for 2 years. The achieved dietary concentrations and homogeneity were not confirmed. All animals were investigated for clinical signs, body weight, feed consumption, and gross and microscopic pathology of a limited range of tissues. Samples from controls and animals at the highest concentration were taken at 3, 6, 12, 18 and 24 months for haematology (10 of each sex), limited

Table 3
Findings in mice given diflubenzuron in the diet for up to 91 weeks

Finding	Sex	0 mg/kg feed	16 mg/kg feed	80 mg/kg feed	400 mg/kg feed	2 000 mg/kg feed	10 000 mg/kg feed
Week 26							
Leukocyte count (10^3/mm^3)	Male	6.5	7.7	8.7	8.1	7.2	11*
	Female	6.5	5.3	6.4	6.7	11*	8.3*
Erythrocyte count (10^6/mm^3)	Male	7.5	7.8	7.4	7.6	7.1	7.0
	Female	8.7	9.1	8.4	8.9	8.3	8.1
Haemoglobin (g/dL)	Male	14	15	14	14	14	15
	Female	16	16	16	16	17*	17*
Mean cell haemoglobin (pg)	Male	19	19	19	19	20*	22*
	Female	18	17	18	18	20*	21*
Methaemoglobin (%)	Male	1.6	1.9	2.2*	2.9*	4.9*	5.9*
	Female	1.6	1.8	2.3*	3.4*	5.3*	5.9*
Platelet count (10^3/mm^3)	Male	890	930	900	1 200*	1 200*	1 200*
	Female	1 100	1 100	1 100	1 400*	1 400*	1 400
Absolute liver weight (g)	Male	2.6	2.7	2.5	2.6	3.0*	3.0*
	Female	2.5	2.3	2.5	2.4	2.6	2.8
Absolute spleen weight (g)	Male	0.13	0.16	0.14	0.16	0.22*	0.29*
	Female	0.15	0.14	0.16	0.22*	0.29*	0.41*
Week 78							
Leukocyte count (10^3/mm^3)	Male	10	10	13	14	9.5	13
	Female	7.1	5.7	5.9	5.5	9.4	4.9
Erythrocyte count (10^6/mm^3)	Male	7.3	7.1	6.8	6.9	6.9	6.6
	Female	7.9	8.1	6.9	7.2	7.2	6.3*
Haemoglobin (g/dL)	Male	14	15	12	14	15	16*
	Female	15	15	12	14	14	15
Mean cell haemoglobin (pg)	Male	21	21	18	21	22	24*
	Female	19	18	18	20	20	23*
Methaemoglobin (%)	Male	0.66	0.87	1.4*	2.3*	3.2*	4.6*
	Female	1.3	1.4	1.9*	3.6*	5.2*	6.0*
Sulfhaemoglobin (%)	Male	0.1	0.2	0.4*	4.0*	3.8*	6.4*
	Female	0.1	0.6*	0.6*	3.0*	3.5*	3.7*
Platelet count (10^3/mm^3)	Male	1 000	1 000	1 100	1 200	1 500*	1 600*
	Female	590	600	700	930	1 300	1 500
Absolute liver weight (g)	Male	3.4	3.0	3.9	3.5	3.6	3.5
	Female	2.9	2.9	3.4	3.2	3.9	4.3*
Absolute spleen weight (g)	Male	0.22	0.48	0.97	0.52	0.22	0.33
	Female	0.27	0.22	0.38	0.50	0.55	0.46
Week 91							
Total number of tissues/sex per group		104	52	52	52	52	52
Hepatocyte enlargement	Male	36	12	11	23	35*	31*
	Female	15	4	5	11	15*	29*

Finding	Sex	0 mg/kg feed	16 mg/kg feed	80 mg/kg feed	400 mg/kg feed	2 000 mg/kg feed	10 000 mg/kg feed
Hepatocyte vacuolation	Male	22	17	9	12	18	26*
	Female	33	17	17	17	12	26
Pigmented Kupffer cells	Male	0	0	1	1	3	15*
	Female	1	0	1	0	±4	11*
Splenic extramedullary	Male	27	7	15	21	19	22*
haematopoiesis	Female	37	18	19	19	25	25
Splenic siderocytes	Male	5	1	2	16*	32*	30*
	Female	15	6	13	30*	31*	43*

*: $P < 0.05$
Source: Colley et al. (1984)

clinical chemistry (five of each sex) and urine analysis (five of each sex). Ophthalmological investigations were performed on controls and animals at the highest concentration at 3, 6, 12, 18 and 24 months. Haematological investigations were performed on animals at 40 mg/kg feed (10 of each sex) at 12, 18 and 24 months.

The survival rate was unaffected by treatment but was low, with less than 30% of animals surviving in all groups at termination. There were no treatment-related effects on urine analysis, clinical chemistry, ophthalmology, feed consumption or body weight gain. Statistically significant ($P < 0.05$) increases in methaemoglobin concentrations were seen at 12 and 18 months in males and females receiving 160 mg/kg feed, but not in those receiving 40 mg/kg feed. Sulfhaemoglobin concentration was unaffected by treatment, but the free haemoglobin concentration was reduced in groups at the highest concentration at 3 and 12 months. No increase in the incidence of tumours was seen in treated animals; however, the poor survival and limited range of tissues examined severely limited the power of this study to detect a tumorigenic compound.

The NOAEL was 40 mg/kg feed (equivalent to 2 mg/kg bw per day), on the basis of haematological changes (increases in methaemoglobin concentrations) at 160 mg/kg feed (equivalent to 8 mg/kg bw per day). The NOAEL for carcinogenicity was 160 mg/kg feed (equivalent to 8 mg/kg bw per day), the highest concentration tested (Hunter et al., 1976; Colley & Offer, 1977).

In a GLP-compliant study, Sprague-Dawley rats (controls: 100 of each sex; treated: 50 of each sex per group) were fed diets containing diflubenzuron (purity 97.6%) at a concentration of 0, 160, 620, 2500 or 10 000 mg/kg feed (equal to 0, 7.1, 28, 112 and 472 mg/kg bw per day for males and 0, 9.3, 37, 128 and 612 mg/kg bw per day for females, respectively) for 2 years. All animals were

investigated for clinical signs, body weight, feed consumption, and gross and microscopic pathology of a wide range of tissues. Haematology (10 of each sex per group) was performed at 12 and 24 months. No clinical chemistry, urine analysis or ophthalmological examinations were performed; because these parameters were not affected in rats at 160 mg/kg feed in the previous non-GLP-compliant study, the highest dose tested in the non-GLP-compliant study and the lowest dose tested in this GLP-compliant study, this was considered not to be a significant omission.

The survival rate was similar in all groups and was greater than 50% at termination. Feed consumption was similar in all groups, but body weight gain was reduced by more than 10% in females at 2500 and 10 000 mg/kg feed from week 4, achieving statistical significance at week 26. A range of erythrocyte parameters was altered in both males and females at 620 mg/kg feed and above at 12 and 24 months, although there was no marked progression with duration of dosing (Table 4). The methaemoglobin and sulfhaemoglobin concentrations were increased significantly in males at the lowest concentration at 12 months, but there was no clear dose–response relationship, and the biological significance of the finding is debatable. Increased numbers of reticulocytes and the extent of bone marrow hyperplasia indicated that the rats responded to the effects on erythrocytes. The only gross finding was enlarged spleens, and this was confirmed by the data on organ weights. The main treatment-related histopathological findings were pigmented (golden brown) macrophages in the spleen and liver and erythroid hyperplasia of the bone marrow. The background incidence of pigmented macrophages in the spleen was high, however, and there was no clear dose–response relationship. Evidence of irritation in the stomach was seen in males receiving diflubenzuron at dietary concentrations greater than 2500 mg/kg feed that survived to termination. The overall incidence of tumours was low, with no evidence of atypical findings or any dose–response relationship.

No NOAEL for toxicity could be identified, as increased methaemoglobin and sulfhaemoglobin concentrations were noted at 160 mg/kg feed (equal to 7.1 mg/kg bw per day), the lowest dose tested, and were consistent with the pattern of toxicity of diflubenzuron seen at other doses. The NOAEL for carcinogenicity was 10 000 mg/kg feed (equal to 472 mg/kg bw per day), the highest dietary concentration tested (Burdock et al., 1984).

2.2.4 Genotoxicity

Table 5 summarizes the eight in vitro and four in vivo studies performed to examine the genotoxic potential of diflubenzuron. JMPR (FAO/WHO, 2002) reviewed all of the genotoxicity studies except for the two recent in vivo studies by de Barros et al. (2013). JMPR indicated that the overall quality of the studies was acceptable, positive controls giving positive results and the concentration ranges

Table 4
Findings in rats given diflubenzuron in the diet for up to 104 weeks

Finding	Sex	0 mg/kg feed	160 mg/kg feed	620 mg/kg feed	2 500 mg/ kg feed	10 000 mg/ kg feed
Week 52						
Erythrocyte count (10^6/mm^3)	Male	8.7	8.3	7.6*	8.0	7.4*
	Female	8.1	7.8	7.6	7.1*	6.5*
Haemoglobin (g/dL)	Male	17	16	15*	16	15*
	Female	15	15	14	14*	13*
Erythrocyte volume fraction (%)	Male	48	48	44	46	43*
	Female	46	46	45	43	41*
Reticulocyte count (%)	Male	0.9	0.8	1.6	1.7*	2.1*
	Female	1.0	1.1	1.8	3.1*	5.0*
Methaemoglobin (%)	Male	0.2	2.0*	1.5	2.4*	2.5*
	Female	0.6	0.9	1.4	1.9*	2.2*
Sulfhaemoglobin (%)	Male	0.1	1.3*	0.9*	0.7	1.1*
	Female	0.1	0.2	1.0*	0.1	0.4
Week 104						
Methaemoglobin (%)	Male	0.8	1.1	1.3	1.8*	1.8*
	Female	0.7	1.3	1.6*	2.1*	2.3*
Sulfhaemoglobin (%)	Male	0.2	0.2	0.6	0.8	0.4
	Female	0.2	0.3	0.6	1.1*	1.1*
Myeloid:erythroid cell ratio	Male	2.5	1.6	1.8	1.5*	1.5
	Female	1.9	1.1*	1.3	1.3	1.1*
Absolute spleen weight (g)	Male	1.0	1.0	1.3	1.4*	1.5*
	Female	0.7	0.9	0.9	1.1*	1.2*
Sternum, marrow hyperplasia (%)	Male	16	10	8	60*	72*
	Female	4	6	2	6	2
Sternum, erythroid hyperplasia (%)	Male	4	6	26*	38*	30*
	Female	1	2	12*	22*	26*
Liver, pigmented macrophages (%)	Male	22	20	50*	82*	72*
	Female	26	38	74*	80*	86*
Spleen, pigmented macrophages (%)	Male	66	84	86	88	74
	Female	80	90	100	94	100
Stomach, acanthosis or hyperkeratosis (%)	Male	1	0	3	11*	24*
Stomach, gastritis (%)	Male	1	0	0	7*	21*

*: $P < 0.05$
Source: Burdock et al. (1984)

of diflubenzuron being sufficient to induce signs of toxicity or precipitation. JMPR concluded that diflubenzuron was not genotoxic in vitro (in the presence or absence of metabolic activation) or in vivo (FAO/WHO, 2002).

Table 5
Results of genotoxicity assays with diflubenzuron

Test system	Test object	Concentration/dose	Results	References
In vitro				
Reverse mutation (Ames)	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538	8, 40, 200, 1 000 µg/plate	Negative	Koorn (1990)
Reverse mutation (Ames)	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538; *Saccharomyces cerevisiae* D4	0.1, 1, 10, 500 µg/plate	Negative	Brusick & Weir (1977a)
Reverse mutation (Ames)	*S. typhimurium* TA98, TA100, TA1537, TA1538	10, 100, 1 000 µg/plate	Negative	MacGregor et al. (1979)
Forward mutation	L5178Y mouse lymphoma	1, 5, 19, 38, 75, 150, 300 µg/mL	Negative	MacGregor et al. (1979)
Cell transformation	BALBc/3T3 cells	20, 40, 80, 160, 310 µg/mL	Negative	Brusick & Weir (1977b)
Unscheduled DNA synthesis	WI-38 human cells	50, 100, 500, 1 000 µg/mL	Negative	Brusick & Weir (1977c)
Unscheduled DNA synthesis	Wistar rat hepatocytes (primary culture)	1, 3, 10, 33, 100, 333 µg/mL	Negative	Enninga (1990)
Chromosomal aberrations	Chinese hamster ovary cells	100, 150, 200, 250 µg/mL	Negative	Taalman & Hoorn (1986)
In vivo				
Dominant lethal mutation	Albino (CR) mice	1 000, 2 000 mg/kg bw (in corn oil, intraperitoneally)	Negative	Arnold, Kennedy & Keplinger (1974)[a]
Micronucleus induction	Bone marrow (Swiss mice)	15, 150, 1 500 mg/kg bw (in corn oil, by oral gavage)	Negative	MacGregor et al. (1979)
Micronucleus induction	Peripheral blood (Swiss mice)	0.3, 1, 3 mg/kg bw	Positive (clasto-genic)	de Barros et al. (2013)
Comet formation	Peripheral blood (Swiss mice)	0.3, 1, 3 mg/kg bw	Positive	de Barros et al. (2013)

bw: body weight; DNA: deoxyribonucleic acid
[a] Not validated.

The two more recent in vivo genotoxicity studies were identified from a literature search (de Barros et al., 2013; GLP status not specified). The genotoxic potential of diflubenzuron in vivo was examined. Fifty adult male Swiss mice (60 days old, weighing 30–40 g) were divided into five groups (10 per group). Three groups received diflubenzuron (purity 98%) at a single dose of 0.3, 1 or 3 mg/kg bw by oral gavage. The negative control group received the vehicle, corn oil. The positive control group was treated with cyclophosphamide dissolved in saline at a dose of 100 mg/kg bw.

Peripheral blood was collected from each animal for the comet assay and micronucleus test. The comet assay was conducted on blood collected 24 hours after treatment. A total damage score was determined by multiplying the number of cells assigned to each class of damage by the numeric value of the class and

summing all resulting values. The micronucleus test was conducted on blood collected from the tail of animals at 24, 48 and 72 hours after treatment. Two thousand cells per animal (two slides per animal per treatment) were examined. Micronucleated cells were counted using a fluorescence microscope with a 100× magnification, and the frequency of micronuclei was determined.

Diflubenzuron increased the incidence of comet formation at all doses tested. A significant increase in the frequency of micronuclei was observed at the sampled time points at all doses tested when compared with the negative (vehicle) control. The results indicated that diflubenzuron exerts genotoxic effects in a dose-dependent manner. It was noted that the highest dose (3 mg/kg bw) showed a significantly higher frequency of micronuclei than the positive control group at all time points measured.

2.2.5 Reproductive and developmental toxicity

(a) Multigeneration reproductive toxicity

In a GLP-compliant two-generation reproductive toxicity study conducted according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 416, groups of 32 male and 32 female Crl:CD(SD)BR (VAF/Plus) rats (28 of each sex in the F_1 generation) received diets containing diflubenzuron (purity 97.1%) at a concentration of 0, 500, 5000 or 50 000 mg/kg feed (equal to 0, 42, 430 and 4300 mg/kg bw per day for males and 0, 36, 360 and 3800 mg/kg bw per day for females, respectively) for 70 days before mating (1:1). The animals were examined routinely for clinical signs and body weight changes. Litters were culled, when possible, to four of each sex on day 4. Blood samples for haematological examination were taken from F_0 and F_1 parents before sacrifice. Haematological end-points and the spleen were not investigated in young animals. All pups and parental animals were examined grossly, whereas histopathological examinations were limited to the reproductive organs, liver and spleen of parental animals. Routine investigations of reproductive performance along with evaluations of developmental end-points, including surface righting reflex, startle reflex, air righting reflex, vaginal opening and preputial separation, were conducted.

Two deaths occurred, a male at the high dose and a female at the low dose, neither of which appeared to be related to treatment. The only clinical sign associated with treatment was pale faeces at the high dose. Animals at this dose had reduced feed consumption at week 1, but not subsequently. Water consumption was increased in all treated groups, but this was considered to be secondary to the high concentrations of diflubenzuron in the diet and not biologically significant. Reproductive parameters and sperm quality and quantity were not impaired by exposure to diflubenzuron. Pup weights were reduced in a dose-related manner

in the F_0 generation, but not in the F_1 generation, and the reduction achieved statistical significance ($P < 0.05$) between day 4 and weaning in animals at 50 000 mg/kg feed. The body weight gain of treated animals after weaning was similar to or greater than that of controls. Attainment of the air righting reflex and startle response was enhanced slightly in diflubenzuron-treated F_0 litters, but unchanged in F_1 animals. Statistically significant ($P < 0.01$) alterations in erythrocyte parameters and increases in lymphocyte counts and platelet numbers were seen in all parental groups. The spleen was the primary target organ, with increases in weight, congestion and haemosiderosis at all doses and an increase in the incidence of congested red pulp in F_0 animals at the middle and high doses. Increased incidences of centrilobular hepatocyte hypertrophy were seen in both generations at concentrations at 5000 mg/kg feed and above, and the prevalence of brown pigmentation of Kupffer cells was increased in all treated groups.

The only effect on reproductive outcome and pup development was a reduction in pup body weight gain during lactation, with statistically significant decreases in F_1 pup weights on days 4, 8 and 21 of lactation. The NOAEL for reproductive effects was 50 000 mg/kg feed (equal to 3800 mg/kg bw per day), the highest dose tested. The NOAEL for toxicity in offspring was 5000 mg/kg feed (equal to 360 mg/kg bw per day), on the basis of reductions in pup body weight at 50 000 mg/kg feed (equal to 3800 mg/kg bw per day) in the F_0 generation. No NOAEL could be identified for toxicity in parental animals, as haematological effects consistent with those seen in other studies with diflubenzuron were observed at all doses tested (Brooker, 1995).

(b) **Developmental toxicity**

(i) Rats

In a GLP-compliant study, diflubenzuron (purity 97.6%) was administered in 1% tragacanth solution by oral gavage to timed-mated female Crl:CD(SD)BR rats (24 per group) on days 6–15 of gestation at 0 or 1000 mg/kg bw per day (limit dose). The dams were killed on day 20 of gestation and examined grossly, and the uterine contents were removed. About 50% of the fetuses were examined by dissection, followed by staining with alizarin red S for skeletal investigations; the remainder were examined by sectioning and dissection.

There were no deaths or evidence of clinical signs in the dams. Maternal body weights and reproductive parameters were unaffected by treatment. The fetal weights and sex ratio were similar in control and treated groups. Two major malformations were seen in the offspring of treated dams: a conjoined twin and a pup with a short body, malrotated hindlimbs and a horseshoe kidney. Conjoined twins are generally considered to be a preimplantation event and, as such, would have occurred before the first dose of diflubenzuron. The abnormalities in the

other pup had been seen in previous studies in the laboratory, and an isolated incidence was considered not to be related to treatment. The incidences of bilobed thoracic centra and retarded ossification of the third and sixth sternebrae were higher in the treated group than in concurrent controls, but were within the range of incidences in contemporary controls in other studies.

The NOAELs for maternal toxicity and embryo/fetal toxicity were the limit dose of 1000 mg/kg bw per day (Kavanagh, 1988a).

(ii) Rabbits

In a GLP-compliant study, groups of 16 timed-mated female New Zealand White rabbits received diflubenzuron (purity 97.6%) by oral gavage in 1% tragacanth at 0 or a limit dose of 1000 mg/kg bw per day on days 7–19 of gestation. The does were killed on day 28 of gestation and examined grossly, and the uterine contents were removed. Fetuses were examined by dissection, followed by staining with alizarin red S for skeletal investigations.

One control rabbit died, and one treated animal was killed because it developed hindlimb paralysis. The latter effect was considered unlikely to be related to treatment, as it was an isolated finding, and no lesions were found in nerves or muscles in other studies with diflubenzuron. Maternal clinical signs, body weights and reproductive parameters were not affected by treatment. Fetal weights and sex ratios were similar in control and treated groups. The overall incidences of malformations and variations were lower in treated animals than in controls, with no significant increase for any individual finding.

The NOAELs for maternal toxicity and embryo/fetal toxicity were the limit dose of 1000 mg/kg bw per day (Kavanagh, 1988b).

2.2.6 Special studies

(a) Metabolite: 4-chlorophenylurea (CPU)

(i) Metabolism

The metabolism of CPU was studied in four male Fischer 344 rats that received [U-^{14}C-phenyl]CPU (specific activity 70.3 GBq/mol; radiochemical purity 97%) in 1% gum tragacanth by gavage at 198 mg/kg bw. Urine and faeces were collected at 20, 48, 72, 96, 120 and 144 hours (termination). The total radiolabel in urine, faeces and carcass was determined by LSC after appropriate processing. Pooled faecal and urine samples (0–20 and 20–48 hours) were examined by HPLC, thin-layer chromatography (TLC), LC-MS and gas chromatography with mass spectrometry (GC-MS) and comparison with standards. Metabolites in faeces were identified after sequential extractions with acetonitrile, hexane, acetone and water. Urine samples were also investigated after treatment with glucuronidase or sulfatase.

Over 90% of the administered dose was excreted in urine. Four faecal metabolites were identified, all of which were also found in urine. The major faecal metabolite was 4-hydroxyphenylurea (about 3% of the administered dose), with unchanged CPU present at 0.6%. Thirteen distinct peaks were obtained from urine samples, the major components being 4-chlorophenylurea-2-sulfate (25% of the administered dose), 4-hydroxyphenylurea (18%), 4-chlorophenylurea-2-glucuronide (17%), 4-chlorophenylurea-3-sulfate (8%) and phenylurea-4-sulfate (7%). PCA was not detected in urine at an LOD of 0.02 μg/mL. The metabolic profile of CPU showed that it undergoes extensive ring hydroxylation and conjugation with either sulfate or glucuronide (Gay, Wang & Long, 1999; GLP status not specified).

(ii) Acute toxicity

The acute toxicity of CPU was investigated in male Fischer 344 rats as part of a study of metabolism. The rats were dosed once with CPU (purity 99.3%) at 190, 220 or 230 mg/kg bw in 1% gum tragacanth. No deaths were seen at the middle and high doses, but pronounced clinical signs of central nervous system depression (lethargy, ataxia, loss of righting reflex) were seen within 30 minutes of dosing. The rat given 190 mg/kg bw showed mild signs of central nervous system depression within 90 minutes of dosing. Chromodacryorrhoea lasting 36–48 hours was seen in all animals (Gay, Wang & Long, 1999).

(b) **Metabolite: *p*-chloroaniline (PCA)**

(i) General toxicity

Repeated exposure to PCA leads to cyanosis and methaemoglobinaemia, followed by effects in blood, liver, spleen and kidneys, as evidenced by changes in haematological parameters, splenomegaly and haemosiderosis (from moderate to heavy) in spleen, liver and kidney, partially accompanied by extramedullary haematopoiesis. The lowest-observed-adverse-effect levels (LOAELs) for a significant increase in methaemoglobin levels in rats and mice were 5 and 7.5 mg/kg bw per day, respectively, for a 13-week oral gavage administration of PCA. The LOAEL for a 103-week oral gavage study in rats (with administration 5 days/week) was 2 mg/kg bw per day, based on a significant increase in methaemoglobin levels and fibrotic changes of the spleen in male rats; hyperplasia of bone marrow was observed in female rats at and above 6 mg/kg bw per day (IPCS, 2003). This information demonstrated that PCA exhibits toxicity end-points similar to those of diflubenzuron, but is more potent than diflubenzuron.

(ii) Carcinogenicity

Mice

Groups of B6C3F1 mice (50 of each sex per group), 6 weeks of age, were fed a diet containing PCA (technical grade, purity unspecified, melting point 68–71 °C) at a concentration of 2500 or 5000 mg/kg feed (equivalent to 375 and 750 mg/kg bw per day, respectively) for 78 weeks, followed by a 13-week observation period. A group of 20 male and 20 female controls received the diet alone. Body weights were recorded once a week for the first 6 weeks, every 2 weeks for the next 12 weeks and monthly thereafter. All animals were inspected twice daily. Feed consumption was measured monthly for 20% of the animals in each group.

The numbers of surviving animals at 91 weeks were 18/20 (control), 44/50 (low dose) and 44/50 (high dose) for males; and 20/20 (control), 41/50 (low dose) and 39/50 (high dose) for females. Decreased body weight gain was observed in both treated males and females relative to that of controls. Non-neoplastic proliferative and chronic inflammatory lesions were found in the spleens of treated animals. Haemangiosarcomas occurred in all organs and tissues (e.g. subcutaneous tissue, spleen, liver, kidney) in 2/20 control, 9/50 low-dose and 14/50 high-dose males; one haemangioma was observed in males in the low-dose group. The increased incidence of total vascular tumours was significant ($P < 0.025$, Cochran-Armitage trend test). Among female mice, haemangiosarcomas occurred at all of the sites in 0/18 control, 3/49 low-dose and 7/42 high-dose animals; one haemangioma was observed in females in the high-dose group. The Cochran-Armitage trend test indicated a significant positive association between incidence of combined vascular tumours (haemangiosarcomas or haemangiomas) and doses in females ($P = 0.012$) but not in males ($P > 0.05$); although not significant with the Fisher exact tests for both sexes, the increased incidence of combined vascular tumours in treated animals of both sexes also exceeded the historical control incidence for combined haemangiosarcomas or haemangiomas (about 3% for either sex). It was concluded that there was insufficient evidence to conclude that PCA was carcinogenic in mice under the conditions of this bioassay (USNCI, 1979).

Groups of B6C3F1 mice (50 of each sex per group), 7 weeks old, were administered PCA (purity 99.1%; lot no. 127) by oral gavage in aqueous hydrochloric acid (molar equivalents) at 3, 10 or 30 mg/kg bw per day, 5 days/week, for 103 weeks. Controls received deionized water at a volume of 5 mL/kg bw. All animals were observed twice per day. Clinical signs were recorded once per month. Individual body weights were recorded once every week for the 13 weeks of the study and once every month thereafter. Necropsy was performed on all animals. Histopathological examinations on a standard set of tissues were performed on all vehicle control and high-dose animals, all animals dying before the end of the study

and all grossly visible lesions in all dose groups. Liver and spleen (the target tissues) from the lower-dose groups (3 and 10 mg/kg bw per day) were also examined.

Mean body weights of dosed animals were generally within 5% of those of controls throughout the study. No test article–related clinical signs were observed. Survival after week 103 was 43/50 (controls), 36/50 (low dose), 29/50 (middle dose; $P = 0.005$) and 35/50 (high dose) for males; and 39/50 (controls), 42/50 (low dose), 44/50 (middle dose) and 41/50 (high dose) for females. Incidences of proliferation of haematopoietic cells in the liver were increased in dosed females. Multifocal renal tubular pigmentation (haemosiderin) was observed in high-dose females, but not in any dosed males. Hepatocellular adenomas were observed in male mice with a significant negative trend, and hepatocellular carcinomas occurred with a significantly positive trend. The incidences of hepatocellular carcinomas in mid- and high-dose males and the incidences of hepatocellular adenomas or carcinomas (combined) in low-, mid- and high-dose males were significantly greater than those in vehicle controls; hepatocellular carcinomas metastasized to the lung (see Table 6). No significant increase in the incidence of such tumours occurred in females. The incidence of haemangiosarcomas in high-dose males was marginally increased relative to that in the controls; nearly all incidences of haemangiosarcomas occurred in the liver or spleen. The incidences of malignant lymphomas in low- and high-dose males and in mid- and high-dose females were significantly lower than those in controls. It was concluded that there was some evidence of carcinogenicity in male mice and no evidence in female mice (USNTP, 1989).

Rats

Groups of Fischer 344 rats (50 of each sex per group), 6 weeks of age, were fed diets containing PCA (technical grade; purity unspecified) at 250 or 500 mg/kg feed (equivalent to 12.5 and 25 mg/kg bw per day, respectively) for 78 weeks, followed by a 24-week observation period. Control groups (20 of each sex) received the diet alone. At the end of the 24-week observation period, the surviving animals were sacrificed. Body weights were recorded once a week for the first 6 weeks, every 2 weeks for the next 12 weeks and monthly thereafter. All animals were inspected twice daily. Feed consumption was measured monthly for 20% of the animals in each group. Gross and comprehensive histological examinations were performed on all animals.

There was no difference in body weight gain in the treated animals compared with the controls. Survival at week 102 was 18/20 (controls), 46/50 (low dose) and 38/50 (high dose) for males; and 18/20 (controls), 49/50 (low dose) and 45/50 (high dose) for females. Non-neoplastic proliferative and chronic inflammatory lesions were found in the spleens of treated rats. Mesenchymal tumours (fibroma, fibrosarcoma, haemangiosarcoma, osteosarcoma and sarcoma

Table 6
Incidences of significant findings in mice treated with PCA hydrochloride

	Sex	Incidence of finding[a]			
		Control	Low dose	Middle dose	High dose
Neoplastic lesions (tumours)					
Hepatocellular adenomas	M	9/50 (18%)*	15/49 (31%)	10/50 (20%)	4/50 (8%)
Hepatocellular carcinoma	M	3/50 (6%)**	7/49 (14%)	11/50 (22%)*	17/50 (34%)**
Metastatic to lung		*1/50*	*1/49*	*2/50*	*9/50*
Hepatocellular adenomas or carcinomas (combined)	M	11/50 (22%)	21/49 (43%)*	20/50 (40%)*	21/50 (42%)*
Haemangiosarcomas, liver	M	2/50 (4%)	2/50 (4%)	1/50 (2%)	6/50 (12%)
Haemangiosarcomas, spleen	M	3/50 (6%)	2/50 (4%)	0/50 (0%)	5/50 (10%)
Haemangiosarcomas, all sites	M	4/50 (8%)*	4/49 (8%)	1/50 (2%)	10/50 (20%)
Malignant lymphomas	M	10/50 (20%)	3/49 (6%)*	9/50 (18%)	3/50 (6%)*
	F	19/50 (38%)	12/50 (24%)	5/50 (10%)**	10/50 (20%)*
Non-neoplastic lesions					
Proliferation of haematopoietic	M	7/50 (14%)	4/49 (8%)	7/50 (14%)	5/50 (10%)
cells, liver	F	15/50 (30%)	29/50 (58%)	24/50 (48%)	31/50 (62%)
Multifocal renal tubular pigmentation	F	0/50 (0%)	0/7 (0%)	0/4 (0%)	38/49 (78%)

F: female; M: male; *: $P < 0.05$; **: $P < 0.001$ (logistic regression test for neoplastic lesions)
[a] Number of animals with the specific lesions/total number of animals examined, or percentage.
Source: USNTP (1989)

not otherwise specified) in the spleen or splenic capsule occurred in 0/20 control, 0/49 low-dose and 10/49 high-dose male rats ($P = 0.001$, Cochran-Armitage trend test) and in 0/18 control, 2/49 low-dose and 5/42 high-dose female rats. The small number of fibromas and sarcomas in the spleens of male rats was considered suggestive of carcinogenicity because of the rarity of these tumours in the spleens of control rats. However, there was insufficient evidence to conclude that PCA was carcinogenic in rats (USNCI, 1979).

In a GLP-compliant study, groups of F344/N rats (49 or 50 of each sex per group), 8 weeks old, were administered PCA (purity 99.1%; lot no. 127) by oral gavage in aqueous hydrochloric acid (molar equivalents) at 2, 6 or 18 mg/kg bw, 5 days/week, for 103 weeks. Controls (50 of each sex) received deionized water at a volume of 5 mL/kg bw. All animals were observed twice per day. Clinical signs were recorded once per month. Individual body weights were recorded once every week for the first 13 weeks of the study and once every month thereafter. Blood samples for haematological parameters and methaemoglobin determination were collected from 15 randomly selected rats from each group at 6, 12, 18 and 24

months. Rats were dosed for 2 consecutive days before blood collection, except at 24 months, when collection was made 11–14 days after administration of the last dose. Animals found moribund or those surviving to the end of the study were humanely sacrificed. Necropsy was performed on all animals. Histopathological examinations were performed on a standard set of tissues from all vehicle control and high-dose animals, all animals dying before the end of the study and all grossly visible lesions in all dose groups. The following tissues in the low- and mid-dose groups were examined: adrenal glands, bone, bone marrow, kidneys, liver, spleen and testes.

The mean body weights of high-dose females were 4–6% lower than those of controls after week 70. The mean body weights of dosed males were generally within 5% of those of controls throughout the study. Mid- and high-dose males and high-dose females had blue extremities, which was indicative of cyanosis. Survival at week 103 in low-dose and mid-dose males was significantly higher than that in the control group: 18/50, 32/50 ($P = 0.007$), 32/50 ($P = 0.005$) and 21/50 for the control, low, middle and high doses, respectively; as was that of low- and high-dose females: 27/50, 39/50 ($P = 0.011$), 36/50 and 37/50 ($P = 0.043$) for the control, low, middle and high doses, respectively. There were changes in haematological parameters at various time points measured, such as decreases in haemoglobin concentration, erythrocyte count, haematocrit value, mean corpuscular volume, nucleated erythrocytes and mean corpuscular haemoglobin. Compound-related non-neoplastic lesions included bone marrow hyperplasia, hepatic haemosiderosis and splenic fibrosis. The incidences of proliferative mesenchymal lesions and fatty metaplasia of the spleen were increased in dosed animals: the incidences of splenic fibrosis were increased in dosed males and females; the incidence of uncommon primary sarcomas of the spleen in high-dose males (Table 7), many of which metastasized to one or more sites, was significantly higher than that in the controls; and cellular infiltration of lipocytes (fatty metaplasia) was observed in high-dose animals (males: 0/49, 0/50, 0/50, 24/50; females: 0/50, 0/50, 0/50, 11/50, for the control, low, middle and high doses). An increased incidence of medullary hyperplasia of the adrenal gland was noted in high-dose females. The incidences of adrenal phaeochromocytomas or malignant phaeochromocytomas combined were significantly higher in the high-dose males. The incidence of mononuclear cell leukaemias was decreased in all treated groups: 21/49, 3/50, 2/50 and 3/50 for males and 10/50, 2/50, 1/50 and 1/50 for females in the control, low-dose, mid-dose and high-dose groups, respectively. Incidences of femoral hyperplasia were increased in high-dose males and mid- and high-dose females (males: 26/49, 36/50, 35/49, 46/50; females: 11/50, 12/48, 21/50, 37/47); and incidences of femoral reticular cell hyperplasia were increased in mid- and high-dose females (males: 0/49, 0/50, 3/49, 0/50; females: 1/50, 2/48, 7/50, 7/47). Incidences of haemosiderin pigmentation were

Table 7

Incidences of findings in the spleen and adrenal gland in rats treated with PCA

Findings	Incidence of finding			
	Control	Low dose	Middle dose	High dose
Spleen, males				
Fibrosis	3/49 (6%)	11/50 (22%)	12/40 (24%)	41/50 (82%)
Fibroma	0/49 (0%)	0/50 (0%)	0/50 (0%)	2/50 (4%)
Fibrosarcoma	0/49 (0%)	1/50 (2%)	2/50 (4%)	17/50 (34%)**
Osteosarcoma	0/49 (0%)	0/50 (0%)	1/50 (2%)	19/50 (38%)**
Haemangiosarcoma	0/49 (0%)	0/50 (0%)	0/50 (0%)	4/50 (8%)
Fibrosarcoma, osteosarcoma or haemangiosarcoma, combined	0/49 (0%)	1/50 (2%)	3/50 (6%)	38/50 (76%)**
Spleen, females				
Fibrosis	1/50 (2%)	2/50 (4%)	3/50 (6%)	42/50 (84%)
Fibrosarcoma	0/50 (0%)	0/50 (0%)	1/50 (2%)	0/50 (0%)
Osteosarcoma	0/50 (0%)	0/50 (0%)	0/50 (0%)	1/50 (2%)
Adrenal gland, males				
Medullary hyperplasia	16/49 (31%)	21/48 (44%)	15/48 (31%)	17/49 (35%)
Phaeochromocytoma	13/49 (27%)	14/48 (29%)	14/48 (29%)	25/49 (51%)*
Malignant phaeochromocytoma	1/49 (2%)	0/48 (0%)	1/48 (2%)	1/49 (2%)
Phaeochromocytoma or malignant phaeochromocytoma, combined	13/49 (27%)	14/48 (29%)	15/48 (31%)	26/49 (53%)*
Adrenal gland, females				
Medullary hyperplasia	4/50 (8%)	4/50 (8%)	7/50 (14%)	24/50 (48%)
Phaeochromocytoma	2/50 (4%)	3/50 (6%)	1/50 (2%)	6/50 (12%)

*: $P < 0.05$; **: $P < 0.001$ (logistic regression test)
Source: USNTP (1989)

increased in high-dose males (males: 1/49, 0/50, 0/49, 26/49; females: 0/50, 0/50, 0/50, 1/50). It was concluded that there was clear evidence of carcinogenicity in male rats and equivocal evidence in female rats (USNTP, 1989).

(iii) Genotoxicity

The in vitro genotoxicity of PCA was examined in a variety of test systems in the 1989 studies by the United States National Toxicology Program (NTP) (results are summarized in Table 8), which indicated that PCA is clearly genotoxic in vitro. The WHO Concise International Chemical Assessment Document 48 on PCA (IPCS, 2003) provided a table summarizing the results of numerous in vitro genotoxicity studies, including the 1989 NTP studies. Inconsistent and conflicting results have been reported; nonetheless, weak mutagenic activity was repeatedly shown in the presence of metabolic activation (S9).

Table 8
Results of USNTP (1989) genotoxicity assays with PCA

Test system	Test object	Concentration/dose	Results
In vitro			
Reverse mutation (Ames)[a]	*Salmonella typhimurium* TA97, TA98, TA100, TA1535	33–1 600 µg/plate	Positive (+S9, TA98)
	S. typhimurium TA98, TA100	33–2 000 µg/plate	Positive (+S9, TA98) Weakly positive (+S9, TA100)
	S. typhimurium TA98, TA100, TA1535, TA1537	10–3 300 µg/plate	Negative (±S9, all strains)
Forward mutation[b]	L5178Y mouse lymphoma cells	50–600 µg/mL (−S9)	Positive
		9.375–200 µg/mL (+S9)	Positive
		31.3–1 000 µg/mL (−S9)	Positive
		7.8–200 µg/mL (+S9)	Positive
Chromosomal aberrations[b]	Chinese hamster ovary cells	400–600 µg/mL (−S9)	Negative
		800–1 000 µg/mL (+S9)	Positive
		30–600 µg/mL (−S9)	Negative
		1.6–800 µg/mL (+S9)	Negative
		100–550 µg/mL (−S9)	Weakly positive
		100–600 µg/mL (+S9)	Negative
Sister chromatid exchange[b]	Chinese hamster ovary cells	16.7–500 µg/mL (−S9)	Positive
		900–1 200 µg/mL (+S9)	Positive
		0.5–500 µg/mL (−S9)	Equivocal
		50–500 µg/mL (−S9)	Weakly negative
		50–500 µg/mL (−S9)	Negative
		1.6–1 600 µg/mL (+S9)	Negative
		100–600 µg/mL (+S9)	Equivocal

S9: 9000 × *g* supernatant fraction from rat liver homogenate
[a] Parallel tests in three laboratories.
[b] Parallel tests in two laboratories.
Source: USNTP (1989)

There are a few in vivo genotoxicity studies that are available in the public literature. In a wing somatic mutation and recombination test, *Drosophila melanogaster* was exposed to PCA at 7.84 mmol/L by 6-hour feeding. PCA was genotoxic in both repair-proficient and repair-defective larvae of the mei-9 cross, which indicates a potential to induce point mutation, chromosome breakages and mitotic recombinations (Graf, Hall & van Schaik, 1990).

In a comet assay, Sprague-Dawley rats (five per group) were treated with PCA orally by gavage at a dose level of 37.5, 75 or 150 mg/kg bw per day at 10 mL/kg bw on three occasions, the second dose being administered approximately 24 hours after the first dose and the third dose being administered approximately 21 hours after the second dose, 3 hours before sampling. A number of clinical

observations, including a blue skin colour, dark eyes, red staining of eyes, underactive behaviour and coat piloerection, were observed at 75 and 150 mg/kg bw per day. Both eyelids partially closed and hunched posture were also observed at 150 mg/kg bw per day. Statistically significant increases in the mean and median per cent tail intensity were observed in the liver and stomach of animals in all treated groups. It was concluded that PCA was clearly positive for genotoxicity in the liver and stomach of rats when administered orally by gavage (Barfield & Burlinson, 2015).

EFSA (2012) reviewed three in vivo genotoxicity studies (repeat micronucleus test in mice, rat liver unscheduled DNA synthesis study, rat comet assay) that were submitted by its notifier and concluded that, based on the weight of evidence, PCA is an in vivo genotoxic agent.

(c) Microbiological effects

Considering the chemical structure and mode of action of diflubenzuron, the Committee did not anticipate any adverse effects of diflubenzuron residues on human gastrointestinal microbiota.

2.3 Observations in humans

No reports of adverse effects or poisoning incidents associated with diflubenzuron were found, and no adverse effects have been reported during field use (Dykstra, 2001).

Diflubenzuron has been produced in a plant in the Netherlands since 1973, where employees involved in its production and formulation underwent regular medical examinations, including haematological analyses. Although no actual exposures have been described, no disturbances in the health status of these employees were observed that could be linked to exposure to diflubenzuron (Dykstra, 2001).

3. Comments

3.1 Biochemical data

Diflubenzuron is rapidly absorbed to a moderate extent from the gastrointestinal tract. In a single-dose oral study with ^{14}C-labelled diflubenzuron in rats, about 30% of the administered dose was absorbed at 5 mg/kg bw, and less was absorbed at 100 mg/kg bw (Dunsire, Cameron & Spiers, 1990). Once absorbed, diflubenzuron is extensively metabolized and rapidly excreted, mostly in the urine, although some enterohepatic circulation occurs. In the radiolabel study, more than 90% of

the administered dose (5 and 100 mg/kg bw) was excreted within 24 hours. When mice were given a single oral dose of diflubenzuron at 12, 64, 200 or 920 mg/kg bw, excretion was almost complete within 48 hours (de Lange & Post, 1978).

The primary metabolic pathways are hydroxylation of the aniline ring, cleavage of the ureido bridge and conjugation, mainly with sulfate. In rats, about 80% of the metabolites were identified as involving hydroxylation of the phenyl moieties of diflubenzuron, and approximately 20% underwent scission at the ureido bridge (de Lange et al., 1975; Willems et al., 1980).

PCA was not detected in bile or urine using a method with an LOQ of 7.5 ng/mL in rats (Cameron, Henderson & McGuire, 1990). A radiolabel study with rats given a single dose of [U-^{14}C-anilino]diflubenzuron at 104 mg/kg bw also did not identify PCA in urine (Wang & Gay, 1999). PCA was not detected (LODs not given) in urine or faeces of sheep or cow following administration of a single oral dose of diflubenzuron at 10 mg/kg bw (Ivie, 1978) or in rat urine following administration of a single oral dose of diflubenzuron at 5 mg/kg bw (Willems et al., 1980). However, when diflubenzuron was given as a single oral dose of 5 mg/kg bw, PCA was detected in small quantities in swine urine (1.03% of the dose; Opdycke, Miller & Menzer, 1982a) and chicken excreta (0.44% of the dose; Opdycke, Miller & Menzer, 1982b).

When diflubenzuron was given as a single oral dose, CPU, a metabolite that may be reduced to PCA, was detected in small quantities in the urine of swine (0.82% of a 5 mg/kg bw dose; Opdycke, Miller & Menzer, 1982a), in the urine of cows (0.6% of a 10 mg/kg bw dose; Ivie, 1978) and in chicken excreta (3.14% of a 5 mg/kg bw dose; Opdycke, Miller & Menzer, 1982b).

3.2 Toxicological data

Critical studies relevant to the risk assessment are summarized in Table 9.

Diflubenzuron was of low acute toxicity when given to mice and rats by the oral, inhalation or dermal route. The oral LD$_{50}$ was greater than 4600 mg/kg bw in mice and rats (van Eldik, 1973), the dermal LD$_{50}$ was greater than 10 000 mg/kg bw in rats (Koopman, 1977) and the inhalation LC$_{50}$ was greater than 2.9 mg/L in rats (Berczy, Cobb & Cherry, 1973).

Diflubenzuron was not irritating to the skin of rabbits (Taylor, 1973) and was slightly irritating to the eyes of rabbits (Davies & Liggett, 1973). Diflubenzuron was not a skin sensitizer in a study in guinea-pigs (Prinsen, 1992).

The primary target for toxicity is the erythrocytes, with secondary effects on liver and spleen. Dose-related methaemoglobinaemia has been consistently demonstrated in both sexes of various species (mice, rats and dogs) after short-term or long-term oral exposure to diflubenzuron.

Table 9
Studies relevant to 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)
Diflubenzuron				
Mouse				
Ninety-one-week toxicity and carcinogenicity study (diet)	Males: 0, 1.2, 6.4, 32, 160, 840 Females: 0, 1.4, 7.3, 35, 190, 960	Toxicity: Increased methaemoglobin concentration	1.2	6.4
		Carcinogenicity: None	840[a]	–
Rat				
Two-year toxicity and carcinogenicity studies[b] (diet)	Study 1: 0, 0.5, 1, 2, 8 Study 2: Males: 0, 7.1, 28, 112, 472 Females: 0, 9.3, 37, 128, 612	Toxicity: Increased methaemoglobin and sulfhaemoglobin concentrations	2[c]	7.1[d]
		Carcinogenicity: None	472[a]	–
Two-generation reproductive toxicity study (diet)	Males: 0, 42, 430, 4 300 Females: 0, 36, 360, 3 800	Reproductive toxicity: None	3 800[a]	–
		Parental toxicity: Changes in haematological parameters	–	36[e]
		Offspring toxicity: Decreased F_1 pup weights	360	3 800
Developmental toxicity study (gavage)	0, 1 000	Maternal toxicity: None	1 000[f]	–
		Embryo/fetal toxicity: None	1 000[f]	–
Rabbit				
Developmental toxicity study (gavage)	0, 1 000	Maternal toxicity: None	1 000[f]	–
		Embryo/fetal toxicity: None	1 000[f]	–
Dog				
One-year toxicity study (capsule)	0, 2, 10, 50, 250	Increased methaemoglobin and sulfhaemoglobin concentrations, platelet counts and hepatic pigmentation	2	10
4-Chloroaniline (PCA)				
Mouse				
Two-year carcinogenicity study (gavage)	0, 3, 10, 30	Increased incidence of hepatocellular adenomas and carcinomas in male mice	–	3[e]
Rat				
Two-year carcinogenicity study (gavage)	0, 2, 6, 18	Increased incidence of spleen sarcoma in male rats	6	18

[a] Highest dose tested.
[b] Two or more studies combined.
[c] Overall NOAEL.
[d] Overall LOAEL.
[e] Lowest dose tested.
[f] Limit dose.

In a 13-week study, rats were fed diets containing diflubenzuron at a concentration of 0, 160, 400, 2000, 10 000 or 50 000 mg/kg feed (equivalent to 0, 8, 20, 100, 500 and 2500 mg/kg bw per day, respectively). A range of dose-related changes in erythrocyte parameters (erythrocyte counts, haemoglobin, reticulocytes, methaemoglobin and sulfhaemoglobin) were noted in both sexes at 400 mg/kg feed and above, with minimal effects at 160 mg/kg feed. The absolute and relative weights of the spleen were increased in males at 160 mg/kg feed and above and in females at 400 mg/kg feed and above for 7 weeks. Pathological findings included chronic hepatitis, haemosiderosis and congestion of the spleen, and erythroid hyperplasia of the bone marrow in all treated groups; and haemosiderosis of the liver at 400 mg/kg feed and above. A NOAEL could not be identified, because there were small, but statistically significant, increases in methaemoglobin concentration and associated changes in the spleen (increased spleen weight, spleen haemosiderosis and spleen congestion) and bone marrow (erythroid hyperplasia) at the lowest dose tested (160 mg/kg feed, equivalent to 8 mg/kg bw per day) (Burdock et al., 1980; Goodman, 1980).

In a 13-week non-GLP-compliant study in dogs, animals received diets containing diflubenzuron at a concentration of 0, 10, 20, 40 or 160 mg/kg feed (equal to 0, 0.4, 0.8, 1.6 and 6.4 mg/kg bw per day, respectively). At week 6, haemoglobin concentration and erythrocyte count were reduced and methaemoglobin and free haemoglobin concentrations were increased at 160 mg/kg feed. There was an increase in the myeloid:erythroid ratio in bone marrow at 160 mg/kg feed at week 12. A NOAEL of 40 mg/kg feed (equal to 1.6 mg/kg bw per day) was identified, based on changes in haematological end-points and bone marrow at 160 mg/kg feed (equal to 6.4 mg/kg bw per day) (Chesterman et al., 1974).

In a 52-week non-GLP-compliant study, dogs received gelatine capsules containing diflubenzuron at a dose of 0, 2, 10, 50 or 250 mg/kg bw per day. A range of effects related to impaired erythrocytes was seen at the two highest doses from week 13 onwards. Increases in methaemoglobin and sulfhaemoglobin concentrations and in platelet counts were seen at 10 mg/kg bw per day and above. The only histopathological findings were in the liver (increased pigmentation of Kupffer cells and macrophages) at 10 mg/kg bw per day and above. A NOAEL of 2 mg/kg bw per day was identified, based on effects on methaemoglobin and sulfhaemoglobin concentrations, platelet counts and hepatic pigmentation at 10 mg/kg bw per day (Greenough et al., 1985).

In a chronic toxicity and carcinogenicity study, diflubenzuron was given to mice in the diet at a concentration of 0, 16, 80, 400, 2000 or 10 000 mg/kg feed (equal to 0, 1.2, 6.4, 32, 160 and 840 mg/kg bw per day for males and 0, 1.4, 7.3, 35, 190 and 960 mg/kg bw per day for females, respectively) for 91 weeks. Significant, dose-related changes were seen in a number of haematological

parameters from week 26 onwards (methaemoglobin and sulfhaemoglobin at 80 mg/kg feed and above; haemoglobin at 2000 mg/kg feed and above; leukocyte and erythrocyte counts at 10 000 mg/kg feed). On week 26, absolute spleen weights were significantly increased at 2000 mg/kg feed and above. Increased incidences of splenic siderocytes at 400 mg/kg feed and above and of pigmented Kupffer cells at 10 000 mg/kg feed were noted. A NOAEL of 16 mg/kg feed (equal to 1.2 mg/kg bw per day) was identified, based on methaemoglobin formation at 80 mg/kg feed (equal to 6.4 mg/kg bw per day). There was no evidence of carcinogenicity in this study (Colley et al., 1984).

In a non-GLP-compliant chronic toxicity and carcinogenicity study in rats, animals received diflubenzuron in the diet at a concentration of 0, 10, 20, 40 or 160 mg/kg feed (equivalent to 0, 0.5, 1, 2 and 8 mg/kg bw per day, respectively) for 2 years. The achieved dietary concentrations and homogeneity of diflubenzuron in the feed were not confirmed. The NOAEL was 40 mg/kg feed (equivalent to 2 mg/kg bw per day), based on increases in methaemoglobin concentration and reduced free haemoglobin concentration at 160 mg/kg feed (equivalent to 8 mg/kg bw per day). There was no increase in the incidence of tumours in treated animals. However, the poor survival (<30% in all groups at termination) and limited range of tissues examined limited the power of this study to detect any carcinogenicity of diflubenzuron (Hunter et al., 1976; Colley & Offer, 1977).

In a GLP-compliant combined 2-year toxicity and carcinogenicity study, rats received diflubenzuron in the diet at a concentration of 0, 160, 620, 2500 or 10 000 mg/kg feed (equal to 0, 7.1, 28, 112 and 472 mg/kg bw per day for males and 0, 9.3, 37, 128 and 612 mg/kg bw per day for females, respectively). Erythrocyte parameters (e.g. methaemoglobin and sulfhaemoglobin concentrations) were altered, with no marked progression with duration and dosing. The main treatment-related histopathological findings were pigmented macrophages in the spleen and liver and erythroid hyperplasia of the bone marrow at 620 mg/kg feed and above. A NOAEL for toxicity could not be identified, owing to increases in methaemoglobin and sulfhaemoglobin concentrations noted at 160 mg/kg feed (equal to 7.1 mg/kg bw per day), the lowest dose tested. The overall incidences of tumours were low, with no treatment- or dose-related findings (Burdock et al., 1984).

The overall NOAEL for toxicity in the 2-year studies in rats was 2 mg/kg bw per day, and the overall LOAEL was 7.1 mg/kg bw per day.

The Committee concluded that diflubenzuron is not carcinogenic in mice or rats.

The genotoxicity of diflubenzuron was evaluated in an adequate range of assays, both in vitro and in vivo. No evidence of genotoxicity was found, other than the studies by de Barros et al. (2013), in which positive findings in micronucleus induction and comet formation in the peripheral blood (the target of toxicity) were reported in mice given diflubenzuron at a dose of 0.3, 1 or 3

mg/kg bw. The genotoxicity potency reported in this study was inconsistent with what was reported in other studies and has not been replicated. The Committee concluded that diflubenzuron is not genotoxic based on the weight of evidence of genotoxicity information available.

In view of the lack of genotoxicity and the absence of carcinogenicity in mice and rats, the Committee concluded that diflubenzuron is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation reproductive toxicity study, rats received diets containing diflubenzuron at a concentration of 0, 500, 5000 or 50 000 mg/kg feed (equal to 0, 42, 430 and 4300 mg/kg bw per day for males and 0, 36, 360 and 3800 mg/kg bw per day for females, respectively). Haematological parameters and the spleen were not examined in young animals. Reproductive parameters were not affected. Pup weights were reduced in a dose-related manner in the F_1 generation, but not in the F_2 generation. Alterations in erythrocyte parameters and increases in lymphocyte counts and platelet numbers were seen in all parental groups. The spleen was the primary target organ, showing increases in weight, congestion and haemosiderosis at all doses and an increase in the incidence of congested red pulp in F_0 animals at the middle and high doses. Effects on liver included increased incidences of centrilobular hepatocyte hypertrophy at the middle and high doses and brown pigmentation of Kupffer cells in all treated groups. The NOAEL for reproductive effects was 50 000 mg/kg feed (equal to 3800 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 5000 mg/kg feed (equal to 360 mg/kg bw per day), based on reductions in pup body weight at 50 000 mg/kg feed (equal to 3800 mg/kg bw per day) in the F_1 generation. A NOAEL for parental toxicity could not be identified because of the haematological effects observed at all doses tested (Brooker, 1995).

In a developmental toxicity study, rats were dosed orally by gavage with diflubenzuron at 0 or 1000 mg/kg bw per day (the limit dose) from days 6 to 15 of gestation. The dams were killed on day 20 of gestation. No treatment-related effects on the dams or fetuses were noted. The NOAEL for both maternal and embryo/fetal toxicity was 1000 mg/kg bw per day, the only dose tested (Kavanagh, 1988a).

In another developmental toxicity study, rabbits were dosed orally by gavage with diflubenzuron at 0 or 1000 mg/kg bw per day (the limit dose) from days 7 to 19 of gestation. The does were killed on day 28 of gestation. No treatment-related effects on the does or fetuses were noted. There was no evidence of developmental toxicity in rabbits. The NOAEL for both maternal and embryo/fetal toxicity was 1000 mg/kg bw per day, the only dose tested (Kavanagh, 1988b).

3.3 Toxicological data on PCA (a metabolite of diflubenzuron)

Repeated exposure to PCA leads to cyanosis and methaemoglobinaemia, followed by effects in blood, liver, spleen and kidneys, as evidenced by changes in haematological parameters, splenomegaly and haemosiderosis (from moderate to heavy) in spleen, liver and kidney, partially accompanied by extramedullary haematopoiesis. The LOAELs for a significant increase in methaemoglobin levels in rats and mice were 5 and 7.5 mg/kg bw per day, respectively, for a 13-week oral gavage administration of PCA. The LOAEL for a 103-week oral gavage study in rats (with administration 5 days/week) was 2 mg/kg bw per day, based on a significant increase in methaemoglobin levels and fibrotic changes of the spleen in male rats; hyperplasia of bone marrow was observed in female rats at and above 6 mg/kg bw per day (IPCS, 2003). This information demonstrated that PCA exhibits toxicity end-points similar to those of diflubenzuron, but is more potent than diflubenzuron.

PCA was tested for carcinogenicity in mice and rats by administration in the diet and by oral gavage.

In a dietary carcinogenicity study in mice, animals received PCA at a concentration of 0, 2500 or 5000 mg/kg feed (equivalent to 0, 375 and 750 mg/kg bw per day, respectively) for 78 weeks, followed by a 13-week observation period. Decreased body weight gain was observed in treated animals. Non-neoplastic proliferative and chronic inflammatory lesions were found in the spleens of treated mice. There was an increased incidence of haemangiosarcomas in the spleen, liver, kidney and subcutaneous tissue (combined) for both sexes. It was concluded that there was insufficient evidence to conclude that PCA was carcinogenic in mice (USNCI, 1979).

In a second carcinogenicity study in mice, animals were administered PCA by oral gavage in aqueous hydrochloric acid at 0, 3, 10 or 30 mg/kg bw per day, 5 days/week, for 103 weeks. Incidences of proliferation of haematopoietic cells in the liver were increased in dosed females. Multifocal renal tubular pigmentation (haemosiderin) was observed in high-dose females. There were increases in the incidences of hepatocellular carcinomas in males dosed at 10 and 30 mg/kg bw per day (3/50, 7/49, 11/50, 17/50), incidences of combined hepatocellular adenomas and carcinomas in all treated males (11/50, 21/49, 20/50, 21/50) and incidences of haemangiosarcomas of the liver and spleen (combined) in males at 30 mg/kg bw per day (4/50, 4/49, 1/50, 10/50). It was concluded that there was some evidence of carcinogenicity in male mice and no evidence in female mice (USNTP, 1989).

In a dietary carcinogenicity study in rats, animals received PCA at a concentration of 0, 250 or 500 mg/kg feed (equivalent to 0, 12.5 and 25 mg/kg bw per day, respectively) for 78 weeks, followed by a 24-week observation period. Mesenchymal tumours (fibroma, fibrosarcoma, haemangiosarcoma,

osteosarcoma and sarcoma not otherwise specified) in the spleen were observed in males at the high dose and in females at both doses; no tumours were found in the controls. It was concluded that there was insufficient evidence to conclude that PCA was carcinogenic in rats (USNCI, 1979).

In a second carcinogenicity study in rats, animals were administered PCA by oral gavage in aqueous hydrochloric acid at 0, 2, 6 or 18 mg/kg bw per day, 5 days/week, for 103 weeks. Changes in haematological parameters (e.g. decreases in haemoglobin concentration, erythrocyte count and haematocrit) were noted at various time points. Non-neoplastic findings included bone marrow hyperplasia, hepatic haemosiderosis and splenic fibrosis. The incidence of uncommon sarcomas of the spleen in high-dose male rats was significantly higher than that in the vehicle controls (fibrosarcomas, osteosarcomas or haemangiosarcomas, combined: 0/49, 1/50, 3/50, 38/50); some of these tumours metastasized to one or more sites. One mid-dose female developed fibrosarcoma, and one high-dose female developed osteosarcoma; the controls showed zero incidence of either of these tumours. The incidence of adrenal phaeochromocytomas or malignant phaeochromocytomas combined was significantly higher in the high-dose males. There was a non-significant increase in the incidence of phaeochromocytomas in high-dose females (2/50, 3/50, 1/50, 6/50). It was concluded that there was clear evidence of carcinogenicity in male rats and equivocal evidence in female rats (USNTP, 1989).

The oral gavage carcinogenicity study is considered to be more appropriate than the dietary admixture feeding study for determining carcinogenicity because (1) PCA is unstable in feed and (2) mice and rats in the feeding studies were dosed for 78 weeks and killed and examined for histopathology following a further 13-week (mice) or 24-week (rats) observation period. Nonetheless, both studies showed some similar effects: splenic toxicity in male and female rats, a treatment-related increase in uncommon splenic sarcomas in male rats and a treatment-related increase in haemangiosarcomas in male mice.

The Committee concluded that PCA is carcinogenic in mice and rats.

PCA has been tested for genotoxicity in various in vitro and in vivo systems. PCA is genotoxic in vitro (USNTP, 1989) and in vivo (Barfield & Burlinson, 2015). The Committee is aware of the existence of additional in vivo genotoxicity studies (EFSA, 2012); however, these were not available to the Committee.

The Committee concluded that PCA is genotoxic.

There is no established mode of action for PCA carcinogenesis; it is not known whether it is mediated through a genotoxic and/or non-genotoxic mechanism. Several hypotheses regarding the mechanism of splenic carcinogenicity have been proposed. However, because PCA is genotoxic and carcinogenic, the Committee could not exclude the possibility that the carcinogenesis of PCA occurs by a genotoxic mode of action.

3.4 Observations in humans

No reports of adverse effects or poisoning incidences associated with diflubenzuron were found (Dykstra, 2001).

3.5 Microbiological data

Considering the chemical structure and mode of action of diflubenzuron, the Committee did not anticipate any adverse effects of diflubenzuron residues on human gastrointestinal microbiota.

4. Evaluation

In the absence of adequate information on exposure to PCA, a genotoxic and carcinogenic metabolite and/or degradate of diflubenzuron, and on whether diflubenzuron can be metabolized to PCA in humans, the present Committee was unable to establish an ADI for diflubenzuron because it was not possible to assure itself that there would be an adequate margin of safety from its use as a veterinary drug. The Committee also noted that it was not possible to calculate a margin of exposure for PCA in the absence of adequate information on exposure to PCA.

4.1 Additional information that would assist in the further evaluation of the compound

- A comparative metabolism study of diflubenzuron in humans and rats (e.g. in hepatocytes)
- Information on PCA exposure associated with the consumption of treated fish
- Information on the amount of PCA present (if any) as an impurity in the product formulation
- Information on the amount of PCA generated during food processing.

4.2 Recommendation

The Committee recommended that JMPR consider the re-evaluation of diflubenzuron at a future meeting and that the WHO Pesticide Evaluation Scheme (WHOPES) and the WHO *Guidelines for Drinking-water Quality* (GDWQ) Chemical Working Group reconsider their recommendations for the use of diflubenzuron as a vector control agent in drinking-water.

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Ivermectin (addendum)

First draft prepared by
Chris Schyvens[1] and João Palermo-Neto[2]

[1] Scientific Assessment and Chemical Review Program, Australian Pesticide and
  Veterinary Medicines Authority, Canberra, Australia
[2] Faculty of Veterinary Medicine, University of São Paulo, São Paulo, Brazil

1. Explanation

Ivermectin (Chemical Abstracts Service no. 70288-86-7)[1] is a macrocyclic lactone that is a member of the avermectin series and is widely used as a broad-spectrum antiparasitic endectocide against nematode and arthropod parasites in food-producing animals. In human medicine, ivermectin is used to treat onchocerciasis, lymphatic filariasis, strongiloidiasis and scabies (Gonazález Canga et al., 2007). Ivermectin consists of two homologous compounds, 22,23-dihydroavermectin B_{1a} (H_2B_{1a} or ivermectin B_{1a}) and 22,23-dihydroavermectin B_{1b} (H_2B_{1b} or ivermectin B_{1b}), in the H_2B_{1a}:H_2B_{1b} ratio of 80:20. Ivermectin is used in cattle, sheep, goats, pigs, horses, reindeer and American bison at doses of 0.1–0.5 mg/kg body weight (bw) given subcutaneously, topically or orally as a single-dose treatment only. Withdrawal periods range from 14 to 122 days where ivermectin is approved for use.

Ivermectin was previously considered by the Committee at its thirty-sixth, fortieth, fifty-eighth, seventy-fifth and seventy-eighth meetings (Annex 1, references *91*, *104*, *157*, *208* and *217*). At its fortieth meeting, the Committee established an acceptable daily intake (ADI) of 0–1 µg/kg bw based on developmental toxicity of ivermectin in CF-1 mice and recommended maximum residue limits (MRLs) of 40 µg/kg for fat, 100 µg/kg for liver and 10 µg/kg for milk as ivermectin in cattle (using the marker ivermectin B_{1a}). At its seventy-eighth meeting, the Committee recommended an MRL of 4 µg/kg for cattle muscle based on 2 times the limit of quantification of the analytical method.

At its seventy-fifth meeting, the Committee concluded that there was a need to evaluate the toxicological information on ivermectin with a view to identifying a critical effect other than in the CF-1 mouse for the establishment of an ADI. The Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF) at its Twenty-second Session requested that JECFA re-evaluate the ADI and the MRLs in all cattle tissues (FAO/WHO, 2015). CCRVDF noted that the draft MRL for ivermectin in bovine muscle recommended at the seventy-eighth meeting was in some cases ≥2.5 times lower than the MRL established in other countries where ivermectin was used. This did not reflect good veterinary practice. Furthermore, JECFA had not recommended an MRL for bovine kidney.

The Committee considered data from a safety, tolerability and pharmacokinetics study in humans and information on various repeated-dose ivermectin treatment regimens in patients, which were provided by a sponsor. The Committee also considered previous evaluations by JECFA on ivermectin

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[1] International Union of Pure and Applied Chemistry name: (1′*R*,2*R*,4′*S*,10′*E*,14′*E*,16′*E*,21′*R*)-6-(butan-2-yl)-21′,24′-dihydroxy-12′-{[(2*R*,4*S*,6*S*)-5-{[(2*S*,4*S*,6*S*)-5-hydroxy-4-methoxy-6-methyloxan-2-yl]-oxy}-4-methoxy-6-methyloxan-2-yl]oxy}-5,11′,13′,22′-tetramethyl-3′,7′,19′-trioxaspiro[oxane-2,6′-tetracyclo[15.6.1.1;{4,8}.0;{20,24}]pentacosane]-10′,14′,16′,22′-tetraen-2′-one.

in various animal species and the pharmacokinetics of ivermectin in dogs in particular, so that a more appropriate animal model could be used to establish an ADI. In light of the possibility for acute exposure to high concentrations of ivermectin from the injection site, the Committee also considered the acute toxicity of ivermectin with a view to establishing an acute reference dose (ARfD).

The critical animal studies were not performed to good laboratory practice (GLP) because the data were generated prior to the implementation of GLP. The human study was conducted according to the principles of the Declaration of Helsinki. The Committee considered that the database was adequate for the evaluation.

1.1 CF-1 mouse

The ADI established at the fortieth meeting of JECFA was based upon the developmental toxicity of ivermectin to the CF-1 mouse, as it was the most sensitive species studied. A no-observed-effect level (NOEL) (which would now be referred to as a no-observed-adverse-effect level [NOAEL]) of 0.1 mg/kg bw per day for maternal toxicity in the CF-1 mouse was combined with a safety (uncertainty) factor of 100 to derive an ADI for ivermectin of 0–1 µg/kg bw. However, subsequent consideration by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and JECFA regarding the unique sensitivity of the CF-1 mouse to avermectins suggested that this mouse model of toxicity was inappropriate for establishing health-based guidance values for avermectins.

Data submitted to the 1994 meeting of JMPR as part of its consideration of abamectin-induced toxicity indicated that the high sensitivity of CF-1 mice to the neurotoxicity of avermectins was associated with P-glycoprotein deficiency in the small intestine and in the capillary endothelial cells of the blood–brain barrier (WHO, 1994). JMPR speculated that the heterogeneity of the response in CF-1 mice may explain the absence of a dose–response relationship for maternal toxicity in the studies of teratogenicity. Data submitted to JMPR in 1998 resolved the issue of the variability seen in earlier studies in CF-1 mice (WHO, 1998). In recent evaluations, JMPR and JECFA have moved to discount the effects observed in the CF-1 mouse in identifying critical effects for the establishment of ADIs for avermectins (FAO/WHO, 2016; Annex 1, reference *208*).

1.2 P-glycoprotein

P-glycoprotein is a 170 kDa transmembrane protein that belongs to the adenosine triphosphate binding cassette (ABC) protein superfamily and is coded for the multiple drug resistance (*MDR1*) gene, also known as the *ABCB1* gene (Hugnet, Lespine & Alvinerie, 2007). Although P-glycoprotein does not have any intrinsic metabolic functions, it is important for the active cellular efflux of a large number

of drugs, including macrocyclic lactones, and toxic compounds. Most of these P-glycoprotein substrates are also substrates of the major drug-metabolizing cytochrome P450 (CYP) 3A4 isotype enzyme. CYP3A4 and P-glycoproteins are expressed at high levels in the villus tip of enterocytes in the gastrointestinal system (Dowling, 2006). In the central nervous system, P-glycoprotein is found in the capillary endothelial cells that form the blood–brain barrier (Hugnet, Lespine & Alvinerie, 2007). P-glycoprotein is also found in the placenta (Ceckova-Novotna, Pavek & Staud, 2006).

In the intestinal tract, substrate drugs may be absorbed passively by the enterocytes; then they enter the systemic circulation, undergo metabolism by CYP3A4 or are extruded by P-glycoprotein back into the intestinal lumen. This extrusion effectively allows the substrate drug further access to CYP3A4 in enterocytes farther down the intestinal tract (Dowling, 2006). Although non-P-glycoprotein substrate drugs may need to pass through the enterocyte only once, P-glycoprotein substrate drugs may continuously cycle between the enterocyte and the intestinal lumen, resulting in repeated access to CYP3A4 or faecal excretion (Dowling, 2006).

Similarly, in the central nervous system, P-glycoprotein substrate drugs, such as avermectins, are transported by P-glycoprotein from the inside to the outside of the endothelial cells back into the lumen of the capillary, thus preventing further diffusion in the central nervous system (Hugnet, Lespine & Alvinerie, 2007).

In the absence of P-glycoprotein, avermectins may be absorbed more freely from the gastrointestinal tract and are capable of diffusing freely and accumulating in the central nervous system, which may lead to drug-induced neurotoxicity. P-glycoprotein-deficient animals such as the CF-1 mouse strain (Lankas, Cartwright & Umbenhauer, 1997), genetically engineered mice (e.g. *mdr1a−/−*) (Schinkel et al., 1996), Murray red cattle and certain dogs of the Collie breed are uniquely sensitive to the adverse effects of ivermectin (Hugnet, Lespine & Alvinerie, 2007). Genetic studies in dogs have documented the *mdr* gene deletion in a number of canine breeds (Australian Shepherds, Collies, English Shepherds, Longhaired Whippets, McNabs, Miniature Australian Shepherds, Old English Sheepdogs, Shetland Sheepdogs, Silken Windhounds and White German Shepherds), with the highest incidence in Collies (30% homozygous and 40% heterozygous). The gene deletion frequency in other herding breeds of Collie lineage is much lower (Dowling, 2006). There are no data to indicate that Beagles carry this deletion.

Importantly, studies in humans have established that P-glycoprotein is expressed at near adult levels in the newborn. Although polymorphisms of P-glycoprotein are known in humans, their impact on P-glycoprotein activity has been considered to be relatively modest (Annex 1, reference *208*).

More than 50 naturally occurring single-nucleotide polymorphisms have been identified in the human *ABCB1* gene (Macdonald & Gledhill, 2007). The vast majority are silent – that is, either they do not occur in the coding regions of the gene or, owing to the inherent redundancy of codon usage, they do not alter the amino acid sequence of the protein. Although there are conflicting reports of the effects of individual *ABCB1* single-nucleotide polymorphisms on P-glycoprotein expression and function in various tissues (Sakaeda, 2005; Kerb, 2006), there is currently no evidence for the existence of mutations in humans that might result in a loss of function analogous to that seen in the CF-1 mouse and Collie dog. Where human blood–brain barrier P-glycoprotein levels have been measured directly, the most common halotypes were found to have equal functionality. As heterozygous P-glycoprotein +/− mice and dogs do not exhibit ivermectin neurotoxicity at clinically relevant doses, it is likely that humans carrying at least one functional copy of the *ABCB1* gene will not be more susceptible to avermectin toxicity at clinically relevant doses or at the low exposure levels resulting from pesticide use. Calculations using allelic frequencies of known haplotypes indicated that homozygosity for any as yet uncharacterized haplotypes with severely reduced blood–brain barrier functionality is likely to be very rare in human populations (Macdonald & Gledhill, 2007). Taken together, this may indicate that individuals with significantly compromised P-glycoprotein functionality analogous to that seen in the CF-1 mouse and Collie dog do not exist or are very rare.

Overall, when the level or functionality of P-glycoprotein is reduced in animals, the animals absorb more avermectins following oral administration, develop higher avermectin levels in blood, accumulate greater amounts of avermectins in the central nervous system and appear to be more sensitive to the adverse health effects caused by these compounds compared with animals with a normal expression of P-glycoprotein (Shoop & Soll, 2002; Annex 1, reference *157*).

1.3 Response to JECFA call for data

In May 2015, JECFA issued a call for all data necessary to review the ADI for ivermectin and recommend MRLs for ivermectin in edible tissues of cattle.

In response to this call for data, the sponsor submitted a meeting abstract on a safety, tolerability and pharmacokinetics study in adults (Guzzo et al., 2002a) and a clinical report on the same study (Lasseter, 2001). The clinical study report was subsequently published as Guzzo et al. (2002b).

2. Biological data

2.1 Biochemical aspects

2.1.1 Human pharmacokinetics

Pharmacokinetics data were obtained from the safety, tolerability and pharmacokinetics study in humans (Lasseter, 2001). Twelve healthy human subjects per dose group were administered oral doses of ivermectin of 30 or 60 mg on days 1, 4 and 7 or single doses of 90 or 120 mg. An additional four healthy human subjects per dose group were administered a placebo. All subjects were fasted prior to dosing. A group of the subjects that received 30 mg were allowed a 1-week washout and then fed prior to administration of a single oral dose of 30 mg ivermectin.

Analysis of the pharmacokinetics data revealed that concentrations of ivermectin in plasma were generally proportional up to a single oral dose of 120 mg in fasted subjects. Dose linearity of the peak concentration in plasma (C_{max}) and the area under the plasma concentration–time curve (AUC) was confirmed after dose normalization. There were no differences in pharmacokinetic variables between men and women. Minimal accumulation was observed with repeated dosing, and the elimination half-life ranged from 18.8 to 20.1 hours for fasted subjects (Table 1). The ivermectin AUC was 2.57-fold greater in fed versus fasted subjects receiving a single dose of 30 mg, but the elimination half-life was shorter, at 15.0 hours.

Table 1

Arithmetic means (and standard deviations) for pharmacokinetic parameters on study day 1 by treatment regimen

Parameter	Ivermectin 30 mg (fed) (n = 11[a]) 0.4 mg/kg bw[c]		Ivermectin 30 mg (fasted) (n = 12[b]) 0.4 mg/kg bw[c]		Ivermectin 60 mg (fasted) (n = 12) 0.8 mg/kg bw[c]		Ivermectin 90 mg (fasted) (n = 12) 1.2 mg/kg bw[c]		Ivermectin 120 mg (fasted) (n = 12) 1.5 mg/kg bw[c]	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
$AUC_{0-\infty}$ (ng·h/mL)	4 564.6	1 892.5	1 724.3	830.5	2 984.0	1 530.1	2 910.2	1 801.0	4 547.7	2 402.9
C_{max} (ng/mL)	260.5	172.1	84.8	42.7	165.2	98.6	158.1	87.6	247.8	158.9
$t_{1/2}$ (h)[d]	15.0	NA	20.1	NA	19.6	NA	18.8	NA	19.1	NA
T_{max} (h)	4.6	0.9	4.3	1.0	3.6	0.9	4.9	1.8	4.2	0.9
Dose-normalized $AUC_{0-\infty}$ (ng·h/mL)	152	–	57	–	50	–	32	–	38	–
Dose-normalized C_{max} (ng/mL)	8.7	–	2.8	–	2.8	–	1.8	–	2.1	–

AUC$_{0-\infty}$: area under the plasma concentration–time curve from time 0 to infinity; bw: body weight; C_{max}: peak plasma concentration; n: number of subjects per treatment regimen; NA: not applicable; SD: standard deviation; $t_{\frac{1}{2}}$: elimination half-life; T_{max}: time to reach C_{max}
[a] One subject discontinued prior to receiving treatment.
[b] As prespecified in the study protocol amendment (Lasseter, 2001), plasma samples from three subjects were not analysed for ivermectin concentration and did not contribute to the pharmacokinetics analysis.
[c] Dose expressed as milligrams per kilogram median body weight.
[d] Harmonic mean.
Source: Lasseter (2001)

2.1.2 Canine pharmacokinetics

No data on the toxicokinetics of ivermectin were available from the pivotal 14-week oral toxicity study in dogs (see section 2.2.1 below). Therefore, a literature search was undertaken to identify any additional scientific articles that contained data on the pharmacokinetics of ivermectin in laboratory animals. The search strategy included the following terms: "ivermectin AND pharmacokinetics AND (mouse OR rat OR dog OR monkey)". The following database was searched on 19 October 2015: PubMed (United States National Library of Medicine, National Institutes of Health). Of the 153 articles retrieved, only six studies in dogs were identified that had relevant pharmacokinetics data (i.e. where ivermectin was given as an oral formulation). The pharmacokinetics data from these studies are tabulated in Table 2.

Table 2

Comparison of pharmacokinetics data following administration of a single oral dose of ivermectin to dogs

Breed (number)	Dose (µg/ kg bw)	$t_{\frac{1}{2}}$ (days)	T_{max} (day)	C_{max} (ng/mL)	Dose-normalized C_{max} (ng/mL)	AUC (ng·d/mL)	Dose-normalized AUC (ng·d/mL)	Reference
Beagle (9)[a]	81.5	4.4	0.22	24.0	0.3	38.9	11.5	Dunn et al. (2011)
Beagle (16)[b]	100	ND	0.18	44.3	0.4	43	6.7	Daurio et al. (1992)
Cross-breed (5)[c]	200	3.3	0.23	116.8	0.6	237	28	Gokbulut et al. (2006)
Beagle (8)[d]	250	3.3	0.17	132.6	0.5	233	22.4	Al-Azzam et al. (2007)
Not defined (12)[e]	300	1.8	0.30	9.64	0.03	27.8	2.2	Gong et al. (2010)
Beagle (10)[f]	300	2.07	0.29	92.70	0.31	141.96	11.4	Walther, Allan & Roepke (2015)

AUC: area under the plasma concentration–time curve; bw: body weight; C_{max}: peak plasma concentration; ND: no data; $t_{\frac{1}{2}}$: elimination half-life; T_{max}: time to reach C_{max}
[a] Sex not defined. Body weight not defined. Fasting status not provided.
[b] Sixteen females. Body weight not defined. Fasting status not provided.
[c] Five females (15–30 kg). Not fasted.
[d] Four of each sex (7.9–16.3 kg) inoculated with *Brugia pahangi*. Fasting status not provided.
[e] Six of each sex (mean body weight ± SD: 9.80 ± 0.48 kg). Fasted.
[f] Five of each sex (mean body weight ± SD: 13.1 ± 1.2 kg). Fasting status not provided.

The data indicate that for a 3-fold increase in ivermectin dose (81.5–250 μg/kg bw), there was approximately a 5.5-fold increase in the ivermectin C_{max} (24.0–132.6 ng/mL) and a 6-fold increase in the plasma ivermectin AUC (38.9–237 ng·d/mL). The studies that used 300 μg/kg bw were not included in this comparison because (1) the study with the shortest elimination half-life for ivermectin in dogs (Gong et al., 2010) was associated with animals that were fasted prior to dosing, whereas in the other studies, the animals were assumed not to have been fasted; and (2) the C_{max} and AUC values obtained by Walther, Allan & Roepke (2015) were inconsistent with the higher values obtained for the 200 and 250 μg/kg bw doses by Gokbulut et al. (2006) and Al-Azzam et al. (2007). Dose normalization of the pharmacokinetics data from the oral dosing studies in dogs for the dose range 81.5–250 μg/kg bw confirmed the non-linear plasma pharmacokinetics of ivermectin in dogs. For this dose range, the elimination half-life ranged from 3.3 to 4.4 days.

2.2 Toxicological studies

The evaluation of the toxicity of ivermectin in laboratory animals has previously been undertaken by JECFA (Annex 1, reference *92*). As the toxicological effects noted in CF-1 mice are no longer considered relevant to humans, the derivation of an appropriate ADI should be based upon appropriate toxicity studies in either humans or other laboratory animals. However, no long-term, repeated-dose toxicity studies in humans or other laboratory animals were submitted to JECFA for consideration.

2.2.1 Short-term studies of toxicity

(a) Rats

A 14-week repeated-dose toxicity study in rats was previously evaluated by JECFA (Annex 1, reference *92*). The findings from this study are reproduced below:

> A fourteen-week toxicity study following <u>in utero</u> exposure was reported. Twenty rat pups of each sex between 3 and 4 weeks of age weighing 49 to 86 grams (males) or 43 to 77 grams (females) were treated at dosage levels of 0.4, 0.8, and 1.6 mg/kg b.w./day. No changes due to treatment occurred at 0.4 mg/kg b.w./day. The following effects could not be excluded as being treatment-related in the two other dosage level groups: spleen-enlargement and reactive bone-marrow hyperplasia, which occurred in 1 animal at 0.8 mg/kg b.w./day and in 3 animals at 1.6 mg/kg b.w./day (Merck & Co., Inc., 1979b).

Based on the findings of spleen enlargement and reactive bone marrow hyperplasia at 0.8 mg/kg bw per day, a NOAEL of 0.4 mg/kg bw per day was identified in this study.

The significance of the toxicological findings from this 14-week study in rats is questionable. Splenic enlargement and bone marrow hyperplasia have not been reported in other species. In particular, there was insufficient information in the description of the study to clearly identify the study design and to interpret the study's findings.

(b) **Dogs**

A 14-week repeated-dose toxicity study with ivermectin in Beagle dogs has previously been evaluated by JECFA (Annex 1, reference *92*). The findings from this study are reproduced below:

> Twenty male and 20 female beagle dogs, 39-43 weeks of age, weighing initially 8.2 - 12.1 kg (males) and 6.2 - 9.2 kg (females) were selected for oral treatment (gastric intubation) in five groups of four males and four females at doses of 0.5, 1.0, 2.0 mg/kg b.w./day. Controls received water or vehicle (sesame oil). At 2.0 mg/kg b.w./day, three males and one female developed tremors, ataxia, anorexia, and dehydration. All of these animals exhibited ptyalism and mydriasis followed by slight tremors, characterized by intermittent or constant shaking of all limbs, which generally increased in severity over 3 to 6 days. These animals were frequently found laterally recumbent and were ataxic when standing. They were sacrificed between weeks 4 and 12. Mydriasis was observed in all dogs at this level (beginning in week 1 and continuing until week 12 when it decreased in incidence). The four dogs sacrificed showed weight losses between 1.0 and 1.6 kg. At 1.0 mg/kg b.w./ day, mydriasis was occasionally seen, particularly in week 3. Weight gain was retarded. At 0.5 mg/kg b.w./day, only slight retardation of weight gain was observed. No significant drug-related changes were observed for the following parameters: ocular abnormalities, electrocardiograms, haematologic parameters, urine-analysis, and pathological changes (Merck & Co., Inc., 1978).

Based on occasional mydriases and retarded weight gain, a NOAEL of 0.5 mg/kg bw per day was identified in this study.

It is noted that the NOAEL from this study was considered the most relevant for establishing an ADI for ivermectin by the European Medicines Agency (EMEA, 2005, 2014) and the United States Food and Drug Administration (USFDA, 2014).

(c) **Rhesus monkeys**

A 16-day repeated-dose toxicity study with ivermectin in immature rhesus monkeys has previously been evaluated by JECFA (Annex 1, reference *92*). The findings from this study are reproduced below:

A 16-day oral toxicity study with ivermectin was conducted to determine its toxicity in immature rhesus monkeys (13 - 21 months old, weighing 2.1 to 3.2 kg (males) and 1.9 to 2.7 kg (females) at initiation). Each of the treatment groups (4 females, 4 males per group) were dosed daily by nasogastric intubation with ivermectin in sesame oil at dose levels of 0.3, 0.6, and 1.2 mg/kg body weight.

These dose levels were chosen to provide an appropriate 6-fold safety margin relative to the human clinical dose, and based on the acute toxicity in rhesus monkeys. All animals were treated for at least 14 days and then sacrificed on days 15, 16 or (one animal) 17. No drug-related effects (physical signs, body weight, ocular lesions, haematology, serum biochemical parameters, or necropsy findings) were noted in any of the treated animals (Merck & Co., Inc., 1986).

A NOAEL of 1.2 mg/kg bw per day, the highest dose tested, was identified in this study.

JMPR (WHO, 1998) noted that:

P-glycoprotein was present on the endothelial surface of capillaries in the cerebellum, cerebellar peduncle, and pons of rhesus monkey foetuses. The staining intensity was comparable in all areas of the brain. P-glycoprotein was also present in the placenta, but none was detected in fetal jejunum. The brain levels of P-glycoprotein in monkey foetuses were comparable to those in the brain of one to two year old rhesus monkeys examined in another study.

This comment from JMPR would suggest that fetal, neonatal and juvenile rhesus monkeys have a functional blood–brain barrier insofar as having adequate levels of P-glycoprotein in their central nervous system. This is supported by the lack of adverse findings in the 16-day toxicity study in rhesus monkeys.

2.2.2 Long-term studies of toxicity and carcinogenicity

JECFA has not evaluated any chronic toxicity or carcinogenicity studies in laboratory animals administered ivermectin. The Committee at its thirty-sixth meeting (Annex 1, reference *91*) concluded that given the structural similarities and comparative toxicological profiles of ivermectin and abamectin, such studies were not required. In a 94-week dietary carcinogenicity study in mice using abamectin doses of 0, 2, 4 and 8 mg/kg bw per day, a NOEL (NOAEL) of 4.0 mg/kg bw per day was identified (Merck & Co., Inc., 1983). Furthermore, in a 105-week dietary carcinogenicity study in rats with abamectin doses of 0, 0.75, 1.5 and 2.0 mg/kg bw per day, a NOEL (NOAEL) of 1.5 mg/kg bw per day was identified (Merck & Co., Inc., 1982).

2.2.3 **Genotoxicity**

JECFA has previously evaluated the genotoxicity of ivermectin and concluded that it was negative in three in vitro assays for genotoxicity, but no test of clastogenicity had been performed (Annex 1, reference *91*).

2.2.4 **Reproductive and developmental toxicity**

A reproductive toxicity study and a series of multigeneration toxicity studies have been carried out in rats. All of these studies have previously been evaluated by JECFA (Annex 1, reference *91*). The findings from these studies are reproduced below:

> Ivermectin was administered orally once daily to three groups of 15 female rats at dose levels of 0.4, 0.8, and 1.6 mg/kg body weight from 15 days prior to mating until 20 days post-partum. Two vehicle control groups received sesame oil in the same dosing regimen as the treated animals.
>
> There was no mortality or clinical evidence of toxicity in the females. Average body weight was significantly increased among females at 0.8 and 1.6 mg/kg b.w./day during the prebreeding period and at all dose levels during gestation.
>
> Ivermectin had no effect on mating, reproductive status, average length of gestation or post implantation survival rate. Statistically significant treatment-related increases in mortality among pups in the 1.6 mg/kg b.w./day group were observed on day 1 and from days 7-14 post-partum. Prior to death, several pups were observed to be hypothermic and to have no externally observable milk in the epigastric region. Throughout the lactation period, average pup weights were slightly higher than controls in the 0.4 mg/kg b.w./day group and significantly higher in the two other dose-level groups.
>
> Development (eye opening, ear opening, incisor eruption, and hair growth) was also slightly accelerated (Merck & Co., Inc., 1979a,b).

The NOAEL for maternal toxicity was 1.6 mg/kg bw per day, the highest dose tested. Based on pup mortality at 1.6 mg/kg bw per day, the NOAEL for offspring toxicity was 0.8 mg/kg bw per day.

* * *

> A series of three multigeneration studies was initiated in rats, the first two of which were halted prior to scheduled termination because neonatal toxicity was apparent at all dose levels tested.
>
> Dose rates of 0.4, 1.2, and 3.6 mg/kg b.w./day were used in the first study. It was necessary, however, to terminate this study before mating of the F_{1b}-generation because

it became apparent from toxic symptoms observed in the F_{1a}-, F_{1b}-, and F_{2a}-generations that a NOEL could not be derived from this study.… (Merck & Co., Inc., 1980).

A second multigeneration study was initiated at a dose of 2.0 mg/kg b.w./day in order to provide clear evidence of toxicity while allowing sufficient surviving offspring to permit continuous dosing throughout the production of two litters in each of three generations. This study was terminated prior to the production of the F_{1b}-litter when it became apparent that there was treatment-related neonatal toxicity present in the above concurrent multigeneration study at dose levels 1.2 and 0.4 mg/kg b.w./day (Merck & Co., Inc., 1981).

In a final multi-generation study the following dose groups were included: 0.05, 0.1, 0.2, and 0.4 mg/kg b.w./day. A vehicle control group received sesame oil daily in the same volume as drug-treated rats. The animals were 28 days old at the onset of the daily treatment and were mated 71 days later. Exposure was continued for the entire life-span.

The F_{1a}-litter was sacrificed on day 21 post-partum. Approximately three weeks later the F_0-rats were mated again to produce the F_{1b}-litter. On day 21 post partum of the F_{1b}-offspring, the F_0-generation was sacrificed. After 71 days of treatment, the F_{1b}-rats were mated to produce the F_{2a}-offspring which were also sacrificed on day 21 post-partum. Approximately three weeks later the F_{1b}-rats were again mated to produce the F_{2b}-offspring. Twenty-one days post partum of this offspring, the F_{1b}-generation was sacrificed. After 71 days of drug treatment, F_{2b}-rats were mated to produce the F_{3a}-offspring which were sacrificed on day 21 postpartum.

Approximately three weeks later the F_{2b}-rats were again mated to produce the F_{3b}-litter. The parents were sacrificed after weaning of the F_{3b}-litter. Twenty males and 20 females from each F_{3b}-offspring group were randomly selected for necropsy at 28 to 43 days of age. There was no treatment-related mortality or physical signs of toxicity among parents or offspring in any dosage group throughout the production of two litters in each of the F_0-, F_1-, and F_2-generations. Ivermectin had no treatment-related effects on the reproductive performance of male or female rats in any dosage group.

Treatment-related effects on body weight gain were limited to a slight but statistically significant decrease during the postweaning period in mean body weight gain among F_{1b}-females in the 0.4 mg/kg b.w./day group and among F_{2b}-males from the 0.2 and 0.4 mg/kg b.w./day groups. External, visceral, and skeletal examination of both the F_{3a}- and F_{3b}-offspring revealed no evidence of teratogenicity. Doses of less than or equal to 0.2 mg/kg b.w./day had no adverse effects on parents or progeny (Merck & Co., Inc., 1980, 1981).

The NOAEL for parental toxicity, reproductive toxicity and offspring toxicity was 0.4 mg/kg bw per day, the highest dose tested.

The significance of the toxicological findings from these reproductive toxicity studies in rats for human toxicology is questionable. The adverse effects observed in these studies appear to be related to in utero and postnatal exposure (via maternal milk) to ivermectin. Ivermectin has been shown to be more toxic to juvenile rats than to young adults, as a result of enhanced sensitivity to ivermectin due to an underdeveloped blood–brain barrier. Significantly higher brain–plasma drug concentration ratios in neonatal rats compared with adult rats have been reported (Lankas & Gordon, 1989), as well as higher drug levels in maternal milk compared with maternal plasma (WHO, 1998).

JMPR (WHO, 1998), in its consideration of the contribution of P-glycoprotein development in rodents to the observed toxicity of abamectin, noted:

> In the immature rat (about six weeks old), P-glycoprotein is present in the brain and in the brush border epithelial cells of the jejunum. In fetal animals (day 20 of gestation), however, minimal P-glycoprotein was detected in the brain, the levels being less than 1% of that in adult animals up to day 14 and then increasing rapidly. No P-glycoprotein was detected in the jejunum of fetal rats or in rats on days 2 or 5 post-partum; P-glycoprotein was detectable by day 8 post-partum, and the levels increased with time thereafter. These data indicate late expression of P-glycoprotein, occurring some 10-15 days post-partum. In non-pregnant adult rats, P-glycoprotein was not observed in the uterus; it was present, however, on the luminal surface of the uterine epithelium in pregnant rats.

In the same evaluation, JMPR (WHO, 1998) also commented on the ivermectin multigeneration study:

> Post-natal toxicity was assessed further in a series of cross-fosterings of newborn pups, with the toxicity being shown to be due to postnatal and not in-utero exposure….These data were interpreted by the JMPR to indicate that the development of the blood-brain barrier in rat offspring is delayed, occurring sometime after parturition. The postnatal toxicity observed in rats may be a function of the accessibility of the target organ to the toxin, owing to the late formation of the blood-brain barrier and to possible mobilization of ivermectin from adult fatty tissues.

Although dogs are not routinely used in developmental and reproductive toxicity studies, an ivermectin dose of 600 µg/kg bw was not found to have negative effects on reproductive status, as measured by the numbers of implantations, resorptions, and live or dead puppies. Furthermore, continuation of treatment after whelping had no effects on the puppies (Pulliam & Preston, 1989). In addition, no adverse effect on reproductive status was observed in dogs treated with 600 µg/kg bw monthly for 8 months and bred to untreated bitches (Daurio et al., 1987).

The hypersensitivity of fetal and neonatal rats to ivermectin (and other avermectins) indicates the limited utility of using a toxicological end-point from the abovementioned subchronic toxicity studies in rats.

Interestingly, P-glycoprotein has been detected in the brain capillaries of human fetuses aborted at 24 weeks, but not at earlier gestational ages. Nonetheless, the levels found were comparable to those in the adult brain. JMPR noted that in human placenta, P-glycoprotein was found in the syncytiotrophoblast microvillus border and in some placental macrophages in the first trimester, but mainly in the placental macrophages at term (WHO, 1998).

2.2.5 Special studies

(a) Microbiological effects

Considering the chemical structure and mode of action, the Committee did not anticipate any adverse effects of ivermectin residues on human gastrointestinal microbiota.

2.3 Observations in humans

2.3.1 Clinical study

The sponsor submitted a human clinical safety, tolerability and pharmacokinetics study and associated abstract. This study has been evaluated, and the key findings are summarized below.

Sixty-eight healthy, non-smoking human subjects (21–45 years of age, weighing 50–90 kg) were assigned to one of four treatment panels to receive either placebo ($n = 4$) or ivermectin ($n = 12$) in the fasted state as follows:

1. 30 mg (three doses over 1 week), followed by a 1-week wash-out after the last dose and then a single 30 mg dose after being fed a "standard high-fat diet", equivalent to 0.4 mg/kg bw based on the median body weight in this group;
2. 60 mg (three doses over 1 week), equivalent to 0.8 mg/kg bw based on the median body weight in this group;
3. 90 mg (single dose), equivalent to 1.2 mg/kg bw based on the median body weight in this group; or
4. 120 mg (single dose), equivalent to 1.5 mg/kg bw based on the median body weight in this group.

Clinical observation for adverse experiences (e.g. any neurological signs of toxicity, such as vomiting, mydriasis or gait disturbance) as well as neurological examinations (mental status, optic, coordination and gait, reflex, sensory and motor examinations), physical examinations, vital signs (blood

pressure, heart rate, respiratory rate and temperature), electrocardiograms and clinical laboratory tests (haematology, blood chemistry and urine analysis) were performed.

Sixty-six subjects completed the study. There were no deaths during the study. There were no reports of ataxia or mydriasis. All clinical adverse observations were considered to be transient and mild. There were no ivermectin-related effects on pupillometry, neurological examinations, vital signs, electrocardiography, physical examination, urine analysis or haematology.

Oral doses of ivermectin of up to 120 mg were well tolerated by human subjects. No adverse effects on human health, in particular upon the neurological system, were identified. The NOAEL for acute oral toxicity of ivermectin was identified as 120 mg (equivalent to 1.5 mg/kg bw based on the median body weight of 77.9 kg), the highest dose tested. These findings were considered to have utility in establishing an ARfD.

The findings from this study (Lasseter, 2001) have been published as Guzzo et al. (2002b).

2.3.2 Clinical experience

Ivermectin has been in use as a human therapeutic for more than 20 years. As noted by JECFA at its fifty-eighth meeting (Annex 1, reference *157*):

> Ivermectin has been administered to several million human patients in Africa and Latin America since its introduction in 1987 as the main treatment for onchocerciasis at a recommended dose level of 150 μg/kg bw administered once every 12 months. The adverse reactions that have been observed in treated patients have been described as allergic or inflammatory responses resulting from killing of microfilariae, referred to as the "Mazotti reaction". No signs of acute central nervous system toxicity have been reported. Ivermectin is now considered safe for use in pregnant women, on the basis of finding of P-glycoprotein in human placentae and in human foetuses by week 28 of gestation and the absence of adverse effects to the fetus when pregnant women were inadvertently treated with ivermectin.

Ivermectin is also used in the treatment of lymphatic filariasis, strongiloidiasis and scabies in several countries. The treatment of scabies may generally require a single oral dose of 200 μg/kg bw, but two or three repeated doses may be required, separated by an interval of 1 or 2 weeks, to be fully effective (Dourmishev, Dourmishev & Schwartz, 2005).

The sponsor identified a number of reported studies in which parasitized patients received up to 13 oral doses of ivermectin (800 μg/kg bw) during the course of treatment. These studies reported that ivermectin was tolerated and

noted no serious adverse health effects. A recent review of the acute toxicity of macrocyclic lactones reported that adverse health effects of ivermectin treatment in patients with onchocerciasis were related not to the dosage of ivermectin, but to the skin microfilarial load (Yang, 2012).

The sponsor has provided information on a safety and tolerability study (Lasseter, 2001) and published studies in humans in which subjects/patients were given multiple doses of ivermectin (Table 3). Although a number of studies outlined in Table 3 indicate that patients may have received up to 13 doses of ivermectin during the course of treatment, there is little to suggest that these patients maintained significant systemic levels of ivermectin, thus limiting their utility in establishing an ADI for chronic toxicity. This is further supported by the pharmacokinetics profile of ivermectin in humans. Data from the safety and tolerability study (Lasseter, 2001) revealed that the half-life of ivermectin in plasma was approximately 18 hours in healthy subjects given a single oral dose of 30–120 mg (equivalent to 333–2000 μg/kg bw) (Table 1), which is similar to other published pharmacokinetics data for ivermectin in humans, with half-lives of 28 hours (Edwards & Breckenridge, 1988) and 37 hours (Baraka et al., 1996).

Similarly, the EMEA (2005), in its review of the Lasseter (2001) study, noted that:

> However, it was considered that the human study could not be used to establish the ADI because [of] the dosing regimen used. As indicated…, the time to reach steady state would be much longer than the maximum treatment period used (7 days). The plasma steady state concentration would be at least 1.5-fold higher than the levels achieved after administration on days 1, 3 and 7 only. The relation of these kinetics to central nervous system concentrations is unknown.

3. Comments

3.1 Biochemical data

Pharmacokinetics data were obtained from the submitted safety, tolerability and pharmacokinetics study in humans (Lasseter, 2001). Twelve fasted subjects per dosing group were given a single oral dose of 30, 60, 90 or 120 mg ivermectin or multiple doses of either 30 or 60 mg ivermectin over 7 days. Pharmacokinetic analysis revealed that ivermectin concentrations in plasma were generally proportional up to a single oral dose of 120 mg in fasted subjects. Dose linearity of C_{max} and AUC was confirmed after dose normalization. There were no differences in pharmacokinetic variables between men and women. Minimal accumulation was observed with repeated dosing, and the elimination half-life ranged from

Table 3
Summary of ivermectin repeated-dose treatment regimens in selected human clinical efficacy studies in parasitized patients

First dose (µg/kg bw)	Subsequent dose(s) (µg/kg bw)	Frequency of treatment	Total no. of doses	Total no. of subjects	Reference
100	100	Every 2 weeks	6	30	Duke et al. (1991)
150	150	Monthly	4, 8 or 12	32	Duke et al. (1990)
150	150	Every 3 months	4, 8 or 11	28	Duke et al. (1992)
20	200 or 400	Days 1 and 5	2	18	Addiss et al. (1993)
20	200 or 400	Days 1 and 5	2	20	Kazura et al. (1993)
20	200 or 400	Days 1 and 5	2	21	Shenoy et al. (1993)
20	200 or 400	Days 1 and 5	2	22	Dreyer et al. (1995)
20	400	Days 1 and 4, then every 2 weeks	13	14	Ismail et al. (1996)
100	100, then 400	Every 6 months	6 (3 + 3)	92	Nguyen, Moulia-Pelat & Cartel (1996)
150	400, 600 or 800	Days 1 and 4	2	25	Awadzi et al. (1995, 1999)
150	400, then 800	Days 1 and ~60–90, then every 12 months	4	172	Gardon et al. (2002)
150	150 or 400, then 800	Every 3 months	13	319	Gardon et al. (2002)
800	800	Days 1 and 13	2	12	Awadzi et al. (1999)

18.8 to 20.1 hours in fasted subjects. The ivermectin AUC was 2.57-fold greater in fed versus fasted subjects receiving a single dose of 30 mg, but the elimination half-life was shorter, at 15.0 hours.

A literature search identified a number of oral dosing studies in dogs that had relevant pharmacokinetics data from a test group treated with ivermectin. The data indicate that for a 3-fold increase in ivermectin dose (81.5–250 µg/kg bw per day), there was approximately a 5.5-fold increase in the ivermectin C_{max} (24.0–132.6 ng/mL) and a 6-fold increase in the plasma ivermectin AUC (38.9–237 ng·d/mL). Dose normalization of the pharmacokinetics data from the oral dosing studies in dogs confirmed non-linear plasma pharmacokinetics of ivermectin in dogs. Furthermore, for this dose range, the elimination half-life ranged from 3.3 to 4.4 days (Daurio et al., 1992; Gokbulut et al., 2006; Al-Azzam et al., 2007; Dunn et al., 2011).

3.2 Toxicological data

Repeated-dose studies with ivermectin in laboratory animals have previously been evaluated by JECFA (Annex 1, reference *91*). The findings from the most relevant of these non-GLP-compliant studies are summarized in Table 4 and below.

Table 4
Studies relevant to 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)
Rat				
Reproductive toxicity study (oral)	0, 0.4, 0.8, 1.6	Increase in pup mortality	0.8	1.6
Multigeneration reproductive toxicity study (oral)	0, 0.05, 0.1, 0.2, 0.4	No adverse findings	0.4[a]	–
Dog				
Fourteen-week toxicity study (gastric intubation)	0, 0.5, 1.0, 2.0	**Mydriasis, retarded body weight gain**	**0.5***	1.0
Rhesus monkey				
Sixteen-day toxicity study (nasogastric intubation)	0, 0.3, 0.6, 1.2	No adverse findings	1.2[a]	–
Human				
Safety, tolerability and pharmacokinetics study in healthy subjects (oral)	0, 0.4, 0.8, 1.2, 1.5	**No adverse findings**	**1.5[a,**]**	–

* Pivotal study value for the derivation of the ADI (Merck & Co., Inc., 1978)
** Pivotal study value for the derivation of the ARfD (Lasseter, 2001)
[a] Highest dose tested.

In a 14-week study, ivermectin was given orally to rat pups 3–4 weeks of age, obtained from dams treated with ivermectin, at a dose of 0, 0.4, 0.8 or 1.6 mg/kg bw per day. Spleen enlargement and reactive bone marrow hyperplasia were observed in one animal at 0.8 mg/kg bw per day and in three animals at 1.6 mg/kg bw per day. Based on these observations, a lowest-observed-adverse-effect level (LOAEL) of 0.8 mg/kg bw per day was identified, and the NOAEL was 0.4 mg/kg bw per day (Merck & Co., Inc., 1979b). The Committee noted that the study design was not clearly explained and the findings were difficult to interpret.

In a 14-week study, ivermectin was given to dogs (four of each sex per group) by oral gastric intubation at a dose of 0, 0.5, 1.0 or 2.0 mg/kg bw per day. Controls received water or vehicle (sesame oil). At 2.0 mg/kg bw per day, mydriasis was observed in all animals; three males and one female developed tremors, ataxia, anorexia and dehydration, lost body weight (1.0–1.6 kg), were frequently found laterally recumbent and were ataxic when standing. Based on occasional mydriases and a retardation of weight gain in animals, the LOAEL was 1.0 mg/kg bw per day, and the NOAEL was 0.5 mg/kg bw per day (Merck & Co., Inc., 1978).

In a 16-day oral toxicity study, ivermectin was given to immature rhesus monkeys (13–21 months old) at a dose of 0, 0.3, 0.6 or 1.2 mg/kg bw by nasogastric intubation. Controls received vehicle (sesame oil). There were no

treatment-related effects noted in any of the treated animals. A NOAEL of 1.2 mg/kg bw per day, the highest dose tested, was identified (Merck & Co., Inc., 1986).

Long-term oral toxicity or carcinogenicity studies with ivermectin were not available, but the Committee at its thirty-sixth meeting concluded (Annex 1, reference *91*) that given the structural similarities and comparative toxicological profiles of ivermectin and abamectin, such studies were not required. In a 94-week dietary carcinogenicity study in mice using abamectin doses of 0, 2, 4 and 8 mg/kg bw per day, a NOEL (NOAEL) of 4.0 mg/kg bw per day was identified (Merck & Co., Inc., 1982). Furthermore, in a 105-week dietary carcinogenicity study in rats with abamectin doses of 0, 0.75, 1.5 and 2.0 mg/kg bw per day, a NOEL (NOAEL) of 1.5 mg/kg bw per day was identified (Merck & Co., Inc., 1983). The present Committee agrees with these conclusions.

In a reproductive toxicity study, ivermectin was given orally to female rats at a dose of 0, 0.4, 0.8 or 1.6 mg/kg bw per day from 15 days prior to mating until 20 days postpartum. Two control groups received the vehicle (sesame oil). Based on no adverse findings in the dams, a maternal toxicity NOAEL of 1.6 mg/kg bw per day, the highest dose tested, was identified. A statistically significant, treatment-related increase in pup mortality in the 1.6 mg/kg bw per day group was observed on day 1 and days 7–14 postpartum. An offspring toxicity LOAEL of 1.6 mg/kg bw per day was identified, and the offspring toxicity NOAEL was 0.8 mg/kg bw per day (Merck & Co., Inc., 1979a,b).

Three multigeneration reproductive toxicity studies were undertaken in rats. The first two studies failed to establish a NOAEL for ivermectin when given orally to rats at 0.4, 1.2 and 3.6 mg/kg bw per day or 2.0 mg/kg bw per day, respectively (Merck & Co., Inc., 1980, 1981). In a third multigeneration study, rats were given ivermectin orally at 0.05, 0.1, 0.2 or 0.4 mg/kg bw per day. A vehicle control group received sesame oil. Treatment-related effects were limited to a slight, but statistically significant, decrease in body weight gain during the post-weaning period in the F_{1b} females in the 0.4 mg/kg bw per day group and the F_{2b} males in the 0.2 and 0.4 mg/kg bw per day groups. There were no treatment-related effects on the reproductive performance of male or female rats in any dose group. There was no evidence of teratogenicity in the F_3 offspring. The NOAEL for parental, reproductive and offspring toxicity was 0.4 mg/kg bw per day, the highest dose tested (Merck & Co., Inc., 1980, 1981).

3.3 Observations in humans

In a double-blind, randomized, placebo-controlled, multiple-rising-dose study (0, 30, 60, 90 and 120 mg, equivalent to 0, 0.4, 0.8, 1.2 and 1.5 mg/kg bw, respectively, based on median body weight) to investigate the safety, tolerability

and pharmacokinetics of multiple doses of ivermectin, 12 healthy human subjects per dose group were administered oral doses of ivermectin of 30 or 60 mg on days 1, 4 and 7 or single doses of 90 or 120 mg. An additional four healthy human subjects per dose group were administered a placebo. All subjects were fasted prior to dosing. A group of the subjects who received 30 mg were allowed a 1-week washout and then fed prior to administration of a single oral dose of 30 mg ivermectin. All doses of ivermectin were well tolerated. No adverse effects on human health, in particular upon the neurological system, were identified. The NOAEL for acute oral toxicity of ivermectin was determined to be 120 mg, equivalent to 1.5 mg/kg bw, the highest dose tested, based on a median body weight of 77.9 kg (Lasseter, 2001).

Ivermectin has been administered to several million human patients for the treatment of onchocerciasis at a recommended oral dose level of 150 μg/kg bw administered once every 12 months. No signs of acute central nervous system toxicity have been reported. The adverse reactions that have been observed in treated patients have been described as allergic or inflammatory responses resulting from the killing of microfilariae, referred to as the "Mazotti reaction". No significant adverse effects on fetuses have been reported when pregnant women were inadvertently treated with ivermectin.

Ivermectin may also be used in the treatment of lymphatic filariasis, strongiloidiasis and scabies. The treatment of scabies generally requires a single oral dose of 200 μg/kg bw, but two or three repeated doses may be needed, separated by an interval of 1 or 2 weeks, to be fully effective (Dourmishev, Dourmishev & Schwartz, 2005). The sponsor identified a number of reported studies where parasitized patients received up to 13 oral doses of ivermectin (800 μg/kg bw) during the course of treatment. These studies reported that ivermectin was well tolerated and noted no serious adverse health effects. A recent review of the acute toxicity of macrocyclic lactones reported that adverse health effects of ivermectin treatment in patients with onchocerciasis were related not to the dosage of ivermectin, but to the skin microfilarial load (Yang, 2012).

3.4 Microbiological data

Considering the chemical structure and mode of action, the Committee did not anticipate any adverse effects of ivermectin residues on human gastrointestinal microbiota.

4. Evaluation

4.1 Acceptable daily intake

The Committee established an ADI of 0–10 μg/kg bw on the basis of a NOAEL of 0.5 mg/kg bw per day for neurological effects (mydriasis) and retardation of weight gain in a 14-week dog study, with application of an uncertainty factor of 50. The previous ADI of 0–1 μg/kg bw is withdrawn.

The Committee did not consider the human clinical data sufficient to assess the possible long-term effects of repeated exposure to ivermectin, such as would occur from its use as a veterinary drug. Therefore, the Committee identified the 14-week dog study as the most appropriate for use in establishing an ADI, given the non-relevance of effects in the CF-1 mouse and the neonatal rat due to their low expression of P-glycoprotein.

As the interspecies differences in pharmacokinetics between dogs and humans are such that humans would be exposed to less ivermectin at a given dose compared with dogs, a reduction in the interspecies uncertainty factor for pharmacokinetics would be appropriate. The quality of the information on pharmacokinetics in dogs was not sufficient to enable the Committee to calculate accurately a chemical-specific adjustment factor for interspecies differences in pharmacokinetics. A reduction by 50% was used as a conservative estimate. An uncertainty factor of 50, comprising a factor of 5 for interspecies differences and a factor of 10 for intraspecies differences, was therefore adopted.

4.2 Acute reference dose

As ivermectin may be administered to cattle in an injectable form, there is the possibility that humans may be exposed to animal tissue containing high concentrations of ivermectin from the injection site. For this scenario, the Committee evaluated the acute toxicity of the compound to determine the need for establishing an ARfD.

The Committee established an ARfD of 200 μg/kg bw, based on a NOAEL of 1.5 mg/kg bw, the highest dose tested in a safety, tolerability and pharmacokinetics study in healthy human subjects, with application of an uncertainty factor of 10 for intraspecies variability. The Committee identified the human study as the most appropriate study for use in establishing an ARfD, given the non-relevance of the embryo/fetal toxicity findings in juvenile rats due to their low expression of P-glycoprotein. The Committee noted that the ARfD was conservative, as an acute oral LOAEL for ivermectin has not been identified in humans.

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Sisapronil

First draft prepared by
Johan Schefferlie[1] and Leonard Ritter[2]

[1] Veterinary Medicinal Products Unit, Medicines Evaluation Board Agency, Utrecht, the Netherlands

[2] University of Guelph, Guelph, Ontario, Canada

1. Explanation

Sisapronil, formerly known as phenylpyrazole, is the proposed International Non-proprietary Name (INN) for 5-amino-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4-[2,2-difluoro-1-(trifluoromethyl) cyclopropyl]-1*H*-pyrazole-3-carbonitrile (International Union of Pure and Applied Chemistry), with Chemical Abstracts Service number 856225-89-3 (Fig. 1). It is a new member of the phenylpyrazole class of compounds. It is a long-acting subcutaneous injectable ectoparasiticide for control of cattle ticks. It also aids in the control of bot fly larvae, hornfly and screwworm. It is approved for use in cattle as a single subcutaneous injection of 2 mg/kg body weight (bw), with a withdrawal time of 120 days.

Fig. 1
Chemical structure of sisapronil
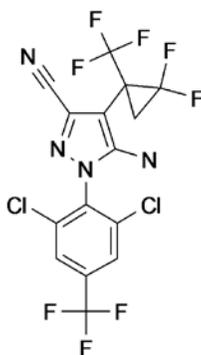
Sisapronil binds to ligand-gated chloride channels, in particular those gated by the neurotransmitter gamma-aminobutyric acid, thereby non-competitively blocking pre-synaptic and post-synaptic transfer of chloride ions across cell membranes in insects or acari. This mechanism of action results in uncontrolled activity of the central nervous system and death of the parasites.

Sisapronil has not previously been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The Committee evaluated sisapronil at the present meeting at the request of the Twenty-second Session of the Codex Committee on Residues of Veterinary Drugs in Foods (FAO/WHO, 2015), with a view to establishing an acceptable daily intake (ADI) and recommending maximum residue limits (MRLs) in cattle tissues.

Information submitted to the Committee included studies on acute, repeated-dose, reproductive and developmental toxicity, genotoxicity and neurotoxicity. Pivotal studies were conducted according to good laboratory practice (GLP) standards and in accordance with Organisation for Economic

Co-operation and Development (OECD) and/or International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) guidelines. A literature search provided no additional information.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

The pharmacokinetics of sisapronil was studied in rats, dogs and cynomolgus monkeys. Most of the investigations were done as part of the toxicological studies (see section 2.2).

(a) Rats

The concentration of sisapronil in plasma was investigated in all repeated-dose toxicity studies in rats, most extensively in the 28-day oral toxicity study (Hu, 2009) and the 90-day oral toxicity study (Rodríguez Gómez, 2012). Blood samples were taken at 4, 8 and 24 hours after gavage administration on day 1 in both studies. In addition, in the 28-day study, blood from animals from satellite groups was taken immediately after dosing on days 1, 4, 7, 14, 21, 28, 35, 38 and 42 and at 4, 8 and/or 24 hours after dosing on days 14 and 28. Remarkably, there was a 3- to 4-fold difference in the observed plasma concentrations at similar dose levels across the studies. The cause of these differences could not be determined. Nevertheless, the studies consistently showed that the plasma concentrations of sisapronil were dose dependent, but less than dose proportional. From the results, a plasma elimination half-life of approximately 14 days was estimated. The sponsor also cited a half-life of 20–39 days, derived from an exploratory oral study in rats with a sampling period of 14 days; however, that study was not provided. Owing to its slow elimination, sisapronil accumulated in plasma upon daily dosing. A time to steady state of approximately 100–150 days could be estimated from the time–concentration profile in plasma in rats from the 1-year repeated-dose toxicity study (Rodríguez Gómez, 2013). In that study, the mean sisapronil concentrations in plasma at the last time point were approximately 325, 567, 1172 and 3424 ng/mL for the 0.1, 0.3, 1.0 and 10 mg/kg bw per day dose groups, respectively.

Radioactive residues in liver and faeces were examined in male and female Sprague-Dawley rats orally dosed with ^{14}C-labelled sisapronil for 1 day at a dose of 50 mg/kg bw or 4 days at 50 mg/kg bw per day. The radioactivity in faeces

was approximately 98% of the total recovery from excreta, and the remainder was found in urine, suggesting limited absorption (Lineham, 2012).

(b) Dogs

The concentrations of sisapronil in plasma from Beagle dogs were measured in the 28-day and 90-day oral toxicity studies (Heward, 2011, 2012). The plasma samples were taken at only one time point after administration of sisapronil in capsules (8 hours) on days 1, 8, 15 and 28 of the 28-day study and on days 1, 30, 60 and 90 of the 90-day study; therefore, the kinetic profile could not be determined. Sisapronil accumulated in plasma up to 90 days, the last time point of sampling.

In a non-GLP-compliant study, groups of three male and three female fasted Beagle dogs were given a single intravenous sisapronil dose of 0.5, 1.5 or 5 mg/kg bw. Blood samples were taken over a period of 268 days. The plasma concentrations of sisapronil were dose dependent, but less than dose proportional. The plasma elimination half-life for all three doses was approximately 100 days (Fisher, 2008).

(c) Cynomolgus monkeys

The pharmacokinetic profile of sisapronil was studied in male and female cynomolgus monkeys. Four animals were given a single dose of sisapronil by oral gavage (2 mg/kg bw) or intravenously (0.5 mg/kg bw). Blood was collected from a femoral vein 0.25, 0.5, 1, 2, 4, 8, 24, 48, 96, 168, 240, 336, 504, 672, 840, 1008, 1344 and 1680 hours post-dosing. After oral administration, the sisapronil concentration in plasma reached its maximum of approximately 17 ng/mL at 24 hours. Following intravenous administration, the area under the concentration–time curve from time 0 to infinity ($AUC_{0-\infty}$) was 1230 ng·d/mL, and the plasma elimination half-life was 12.4 days. The oral bioavailability in cynomolgus monkeys was 6.8% (Stuhler, Peterson & Starch, 2012).

2.1.2 Biotransformation

In the study in which Sprague-Dawley rats were orally dosed with ^{14}C-labelled sisapronil for 1 day at a dose of 50 mg/kg bw or 4 days at 50 mg/kg bw per day (see section 2.1.1), the parent substance sisapronil accounted for more than 91% of the radioactivity in faeces at 24 hours after treatment, but the relative content of parent substance in faeces declined over the 24- to 144-hour study period. In liver, sisapronil was the primary residue. A metabolite was detected in the liver of rats, but could not be identified. It had the same retention time as the main metabolite found in cattle liver, which could not be identified because of the low quantities present (Lineham, 2012).

Table 1
Acute toxicity studies on sisapronil

Species	No. of each sex per group	Route	Dose (mg/kg bw)	LD$_{50}$ (mg/kg bw)	Reference
Mouse	3–4, sex not specified	Oral	3, 10, 30	>30	Mill (2003)
Rat	9 F	Oral	175, 550, 2 000	552	Beerens-Heijnen (2011a)
Rat	2 M	Oral	100, 250, 500, 1 000, 1 500	Not estimated	Gagnon (2012a)
Rat	3 M	Oral	10	Not applicable	Gagnon (2012b)
Rat	20 M	Oral	0, 100, 500, 1 000	Not estimated	Ryan (2011)
Mouse	4, sex not specified	Intravenous	0.3, 1, 3	>3	Mill (2003)

bw: body weight; F: females; LD$_{50}$: median lethal dose; M: males

2.2 Toxicological studies

2.2.1 Acute toxicity

(a) Lethal doses

The single-dose toxicity of sisapronil was investigated in mice and rats using the oral (mice and rats) and intravenous (mice) routes of administration (Table 1).

In a non-GLP-compliant study, groups of three or four mice (strain and sex not specified) were given sisapronil at a single oral dose of 3, 10 or 30 mg/kg bw. All animals appeared symptomatically normal (Mill, 2003).

In a non-GLP-compliant dose range–finding study, groups of two male Wistar rats were given sisapronil by gavage at a single oral dose of 100, 250, 500, 1000 or 1500 mg/kg bw. Body weights, feed consumption and clinical signs were recorded for 13 days after dosing. The animals that were found dead or euthanized moribund were examined histopathologically. Doses of 100 and 250 mg/kg bw were well tolerated, but rats dosed with 500 mg/kg bw and above showed observable clinical effects. Observations of hyperreactivity (to noise or cage opening) and sensitivity to touch were observed as early as 3 days post-treatment. Tremors upon stimulation were observed in 4/6 animals dosed at 500 mg/kg bw and above. At doses of 500 mg/kg bw and above, 4/6 animals were found dead, whereas the other two were euthanized moribund. The thymus of one animal at each dose of 500, 1000 and 1500 mg/kg bw had mild to moderate lymphoid depletion. In addition, the only animal examined histopathologically at the highest dose showed diffuse, mild vacuolation of the adrenocortical cells. A median lethal dose (LD$_{50}$) was not estimated (Gagnon, 2012a).

Another non-GLP-compliant study was conducted to compare the toxicity of sisapronil in Sprague-Dawley and Wistar rats given a single oral gavage dose of 10 mg/kg bw. The animals were examined twice daily for clinical signs, including mortality; however, the observation period was not specified, and the

study report did not contain raw data. It was stated that all animals appeared clinically normal (Gagnon, 2012b).

Sisapronil was tested for acute oral toxicity in female Wistar Crl:WI rats in a GLP-compliant study. Nine rats were given sisapronil by oral gavage at a dose of 175, 550 or 2000 mg/kg bw, following the up-and-down procedure with dosing intervals of 5 days and an observation period of 15 days for each rat. Hunched posture and piloerection were observed at all dose levels. Tremors were seen in mid-dose animals, whereas animals dosed at 2000 mg/kg bw additionally showed lethargy, chromodacryorrhoea, lean appearance and/or hypersensitivity to touch during the observation period until they died. The oral LD_{50} was estimated to be 552 mg/kg bw (Beerens-Heijnen, 2011a).

In a non-GLP-compliant study, groups of four mice (strain and sex not specified) received sisapronil intravenously at a single dose of 0.3, 1 or 3 mg/kg bw. No animals died. One mouse of the high-dose group showed a slight and transient depression in body temperature (Mill, 2003).

(b) Dermal irritation

Three New Zealand White rabbits were exposed to 0.5 g of sisapronil moistened with 50% watery ethanol by application onto clipped skin for 4 hours using a semi-occlusive dressing. Skin reactions were assessed 1, 24, 48 and 72 hours after exposure. Very slight erythema was observed in the treated skin areas of the three rabbits, which resolved within 24–72 hours after exposure. Only one animal showed very slight oedema on day 1. No oedema was noted in the remaining two animals. No staining of the treated skin by the test substance was observed, and no test substance remnants were seen. On the basis of this study, sisapronil is considered not irritating to the skin of rabbits (Beerens-Heijnen, 2011b).

(c) Ocular irritation

Seventy milligrams of sisapronil was instilled into the conjunctival sac of one eye of each of three New Zealand White rabbits. Instillation of the test substance resulted in irritation of the conjunctivae, which consisted of redness and discharge. The irritation had completely resolved within 48 hours in two animals and within 72 hours in the other animal. Based on these results, sisapronil is considered not irritating to the eyes of rabbits (Beerens-Heijnen, 2011c).

(d) Dermal sensitization

In a local lymph node assay, groups of five female CBA/J mice were given sisapronil at a concentration of 0%, 0.5%, 1% or 2.5% weight per weight (w/w) on 3 consecutive days by open application on the ears. Three days after the last exposure, all animals were injected with [³H-methyl]thymidine; after 5 hours,

the draining (auricular) lymph nodes were excised and pooled for each animal. No irritation of the ears was observed in any of the animals examined. All auricular lymph nodes of the animals of the experimental and control groups were considered normal in size, except for one animal at 2.5%, which showed larger lymph nodes. The stimulation index values calculated for the substance concentrations 0.5%, 1% and 2.5% were 0.9, 0.8 and 1.3, respectively. As there was no indication that the test substance elicits a stimulation index of 3 or higher when tested up to 2.5%, sisapronil was considered not to be a skin sensitizer (Beerens-Heijnen, 2011d).

2.2.2 Short-term studies of toxicity

(a) Rats

In a non-GLP-compliant study, sisapronil was given to groups of 10 male and 10 female Crl:CD(SD) rats by oral gavage at a dose of 0, 0.1, 1 or 10 mg/kg bw per day for 28 days. Observations included mortality, clinical signs, body weights, feed consumption, ophthalmic examinations, hormone analysis, clinical pathology and histology (only control and high-dose animals).

No animals died, and there were no effects on clinical signs. No treatment-related alterations in mean body weight, mean body weight gain or mean feed consumption were noted for animals given up to 10 mg/kg bw per day. No visible lesions were observed at ophthalmic examination. High-dose males had a decreased lymphocyte count and reticulocyte count. Higher plasma levels of thyroid stimulating hormone (TSH) and lower plasma levels of thyroxine (T_4) were observed in high-dose males and mid- and high-dose females. Higher levels of cholesterol and decreased levels of bilirubin were noted in all high-dose animals. Increased absolute and relative liver weights were observed in males and females of the high-dose group. In high-dose males, the absolute and relative weights of the thyroids were also increased, and the absolute and relative weights of the spleens were decreased. The effect on absolute lymphocyte count may have been associated with reduced spleen weight (no associated pathological findings). The effects on TSH, T_4, cholesterol and total bilirubin levels were consistent with metabolic enzyme induction and correlated with higher liver weights and hepatocellular hypertrophy, observed microscopically for animals given 10 mg/kg bw per day. The increased thyroid weights observed in high-dose males were accompanied by hypertrophy/hyperplasia of the thyroid follicular epithelium, characterized by a predominance of small follicles with decreased amounts of luminal colloid and increased height of thyroid epithelium at this dose level (Hu, 2009).

Short-term toxicity was further investigated in a non-GLP-compliant dose range–finding study. Groups of three female Wistar rats were given sisapronil by oral gavage at a dose of 0, 20, 40, 60, 80 or 100 mg/kg bw per day for 60 days. On day 27, animals previously assigned to the control group were reassigned to a 15 mg/kg bw per day dose group to further define dose tolerance. Body weight and feed consumption were measured weekly. Animals were observed multiple times daily for clinical signs of toxicity, including mortality.

At 40 mg/kg bw per day and higher, animals either were found dead or had to be euthanized. One animal of the 20 mg/kg bw per day group was euthanized on day 8; the other animals of that group and the animals of the 0/15 mg/kg bw per day group were terminal at day 60. Nineteen days after switching controls to 15 mg/kg bw per day, these animals started showing effects, including tense posture and hyperactivity, which continued throughout the study. Tense posture and hyperreactivity/aggressiveness were also observed at doses of 20–60 mg/kg bw per day. Additionally, tremors/seizures were noted at 40 mg/kg bw per day and higher, and decreased activity, red staining (face, paws) and occasional twitch were seen in animals of the 40, 60 and 100 mg/kg bw per day dose groups. Body weights were reduced at 40 mg/kg bw per day and above, and feed consumption was decreased at 60 mg/kg bw per day and above (Gagnon, 2012c).

In a GLP-compliant study, groups of 10 male and 10 female Wistar rats were given sisapronil by oral gavage at a daily dose of 0 (vehicle), 0.1, 0.3, 1.0 or 10 mg/kg bw per day for 13 weeks. Satellite groups were used to study the plasma kinetics of sisapronil (see section 2.1.1). The animals were checked for morbidity and mortality daily, whereas clinical signs were recorded weekly. A functional observational battery was performed on all animals prior to necropsy at the end of the study. Other observations included body weight, feed consumption, ophthalmology, haematology, clinical chemistry, urine analysis, gross pathology and histopathology (only the highest-dose animals and the controls).

No mortality occurred during the study. No treatment-related clinical signs were noted, including ophthalmology. Body weight and feed consumption were normal in all groups. The functional observational battery revealed no treatment-related effects. Blood parameters were also unaffected. Increased plasma levels of TSH and decreased plasma levels of T_4 were observed at 10 mg/kg bw per day (statistically significant and more pronounced in males). These changes were only slight and not statistically significant at 1.0 mg/kg bw per day. At lower doses, these parameters appeared unaffected. Males and females of the highest-dose group had slightly increased (<10%) levels of total protein and globulin. At 10 mg/kg bw per day, the absolute and relative (to body weight and brain weight) weights of the liver were increased in both sexes. This was also seen in females

of the 1.0 mg/kg bw per day group, but the differences relative to controls failed to attain statistical significance. The increase in liver weights was correlated with hepatocellular hypertrophy in 10/10 males and 5/10 females at the highest dose. Liver-specific clinical parameters, such as aspartate aminotransferase and alanine aminotransferase, were not affected. Thyroid follicular cell hypertrophy, observed in 9/10 males and 10/10 females of the highest-dose group, was characterized by a predominance of small follicles with decreased amount of luminal colloid and increased height of the thyroid epithelium. This hypertrophy was correlated with increased thyroid weights (absolute and relative to body weight and brain weight), decreased plasma levels of T_4 and increased plasma levels of TSH in animals in the highest-dose group. The study authors postulated that the thyroid hypertrophy may be secondary to the induction of thyroid metabolizing enzymes, such as uridine diphosphate-glucuronosyltransferase (UGT), occurring with hepatocellular hypertrophy in the liver.

The no-observed-adverse-effect level (NOAEL) in this study was 1.0 mg/kg bw per day, based on effects on the liver and thyroid at 10 mg/kg bw per day (Rodríguez Gómez, 2012).

In a 1-year oral toxicity study in rats, groups of 30 male and 30 female Wistar rats received sisapronil by daily gavage at a dose of 0 (vehicle), 0.1, 0.3, 1.0 or 10 mg/kg bw per day for 52 weeks. Satellite groups were added to investigate the plasma profile of sisapronil (see section 2.1.1). Animals were checked daily for mortality and morbidity, whereas clinical signs, body weight and feed consumption were recorded weekly. Haematological, clinical biochemistry and urine analysis examinations were carried out in 10 male and 10 female animals per group at 180 days and in the surviving animals at termination. Following gross pathology on all animals, tissues from animals in the control and highest-dose groups plus liver, thyroid and parathyroid glands, and macroscopic lesions from the remaining groups were evaluated by histopathological examination.

There was no mortality (except from deaths related to gavage trauma), and no clinical signs were noted. Body weights were unaffected, and feed consumption was increased in females in the highest-dose group only. Haematology and urine analysis revealed no treatment-related effects. Blood cholesterol levels appeared higher in 10 mg/kg bw per day animals, but this reached statistical significance in females only. Males and females of the 1.0 and 10 mg/kg bw per day dose groups had increased absolute and relative liver weights. These livers showed centrilobular hepatocellular hypertrophy and vacuolation. Increased relative thyroid weights were observed at 1.0 mg/kg bw per day in males only, and increased absolute and relative thyroid weights were seen at 10 mg/kg bw per day in both sexes. This was accompanied by a decrease in plasma levels of triiodothyronine (T_3) and T_4. Upon histopathology, thyroid follicular cell hypertrophy was noted in

all male dose groups, including controls, but the incidence was higher in males and females of the 1.0 and 10 mg/kg bw per day groups than in controls. Thyroid follicular cell hyperplasia was observed at 0.1, 0.3, 1.0 and 10 mg/kg bw per day in males, but only at 1.0 and 10 mg/kg bw per day in females. Thyroid follicular cell adenomas were observed at 0.1, 0.3, 1.0 and 10 mg/kg bw per day in both males and females, as well as in one male in the vehicle control group; the incidence was notably higher for the 10 mg/kg bw per day males. However, any observations at 0.1 and 0.3 mg/kg bw per day were small and not statistically significant. Although lung tissue was affected, this could be attributed to gastric reflux and aspiration following gavage.

The NOAEL in this study was 0.3 mg/kg bw per day, based on changes in the liver and thyroid at 1.0 mg/kg bw per day (Rodríguez Gómez, 2013).

The data from the 1-year rat study were used for benchmark dose (BMD) modelling. The analysis was performed using the Benchmark Dose Software (BMDS) from the United States Environmental Protection Agency (USEPA). A number of variables were used for the BMD analysis: TSH, total T_4, free T_3, relative (to body and brain weights) liver and thyroid weights as well as the occurrence of bilateral thyroid adenoma and hypertrophy. A benchmark response (BMR) of 10% extra risk above the background was selected as the basis for estimating BMDs. For the variables measured on a continuous scale (TSH, T_3, T_4, organ weights), all the available continuous variable models in BMDS were fit: exponential (models 2–5), Hill, linear, power and polynomial (limited to a quadratic) models. Similarly, for the variables measured on a dichotomous scale, all dichotomous models were fit: gamma, logistic, log-logistic, multistage, probit, log-probit, Weibull and quantal linear. Consistent with the guidance provided by the European Food Safety Authority (EFSA), the best-fitting models were chosen based on the nesting structure, smallest Akaike information criterion (AIC), small-scaled residuals, especially near the BMD and the control dose, parameter feasibility and visual check of the model fit. The outcome of this analysis is presented in Table 2.

Among the variables analysed, the BMDLs ranged from 0.26 to 0.79 mg/kg bw per day, which are supportive of the 0.3 mg/kg bw per day NOAEL identified in this study (Boucher, 2013).

(b) Dogs

Groups of four male and four female Beagle dogs were given sisapronil in capsules at a daily dose of 0, 1, 5 or 25 mg/kg bw per day for 28 days. Observations included morbidity, mortality, injury, feed and water intake, clinical signs, body weight, ophthalmology, physical/neurological examinations and functional observational

Table 2
Summary of BMD/BMDL results

Variable	Sex	BMR	BMD	BMDL
TSH	M/F	10%	0.921	0.631
T_3	M/F	10%	2.39	0.786
T_4	M/F	10%	1.65	0.547
Liver/body weight	M/F	5%	0.626	0.447
Thyroid/body weight	M/F	10%	0.919	0.454
Liver/brain weight	M/F	10%	1.29	0.448
Thyroid/brain weight	M/F	10%	0.486	0.298
Thyroid hypertrophy	M/F	10%	0.440	0.257
Thyroid adenoma	M	10%	2.10	0.305

BMD: benchmark dose; BMDL: lower 95% confidence limit on the benchmark dose; BMR: benchmark response; F: females; M: males; T_3: triiodothyronine; T_4: thyroxine; TSH: thyroid stimulating hormone
Source: Boucher (2013)

batteries (in weeks 2 and 4), and clinical pathology. At the end of the study, the animals were necropsied, organ weights were recorded and tissues were collected for microscopic examination (controls and high dose only). Additionally, the thyroid, thymus (female only) and liver of animals in all groups were examined histopathologically.

No mortality occurred, and no treatment-related clinical signs were noted. Ophthalmology, functional observational battery and neurological examinations revealed no effects. A trend to decreased body weight was apparent in high-dose males and in mid- and high-dose females, but those changes were slight and did not attain statistical significance. A slight and non-significant decrease in feed consumption was noted in the same groups. The tested parameters of haematology, clinical chemistry and urine analysis were unaffected. Relative liver weights were increased in high-dose males and females, accompanied by increased glycogen in hepatocytes. Absolute and relative thymus weights were decreased in females of the 5 and 25 mg/kg bw per day groups. These decreases were statistically significant in females of the 5 mg/kg bw per day group only. Microscopically, the decreased thymus weights correlated with an increased incidence of minimal to mild decreased cellularity of the lymphoid tissue.

The NOAEL in this study was 1 mg/kg bw per day, based on changes in the thymus at 5 mg/kg bw per day (Heward, 2011).

In a 90-day repeated-dose oral toxicity study, groups of four male and four female Beagle dogs were given sisapronil in capsules at a dose of 0 (vehicle), 0.3, 1 or 10 mg/kg bw per day. Observations included mortality, feed and water consumption, body weight, haematology, clinical chemistry, urine analysis,

ophthalmoscopy examinations, physical/neurological examinations, functional observational batteries, clinical signs, organ weights, gross pathology and histopathology (animals at 0 and 10 mg/kg bw per day and selected tissues for animals at 0.3 and 1 mg/kg bw per day).

In all treated male dogs, a dose-dependent increase in soft faeces was noted. There were no mortalities, and feed intake and body weight were not affected. Ophthalmology, physical/neurological tests and functional observational batteries revealed no effects. An increase in absolute and relative liver weights in high-dose males and females was noted. Two males and one female of this group showed vacuolar degeneration of individual hepatocytes in the centrilobular to midzonal regions. Males and females of the 1 and 10 mg/kg bw per day groups had an increased incidence of glycogen in hepatocytes. Minimal thyroid follicular cell hypertrophy was observed in 2/4 males of the mid-dose group and 4/4 males and 1/4 females of the high-dose group. One of four high-dose females had minimal thyroid follicular cell hypertrophy. Thyroid follicular cell hypertrophy was characterized by cuboidal to tall cuboidal follicular cells, frequently associated with increased vacuolation in the apical cytoplasm. This hypertrophy occurred without increased thyroid weight or changes in thyroid hormone levels in blood.

A NOAEL of 0.3 mg/kg bw per day was identified, based on minimal thyroid follicular cell hypertrophy and increased glycogen in hepatocytes at 1 mg/kg bw per day (Heward, 2012).

2.2.3 Long-term studies of toxicity and carcinogenicity

No 2-year toxicity studies or carcinogenicity studies were provided. The study with the longest duration was the 1-year repeated-dose oral toxicity study in rats (see section 2.2.2).

2.2.4 Genotoxicity

For the evaluation of the genotoxic properties of sisapronil, an in vitro bacterial reverse mutation assay, an in vitro test for chromosomal aberrations in mammalian cells and an in vivo micronucleus assay in mice (bone marrow) were available (Table 3). In addition to those experimental studies, an in silico analysis by Frank (2013) was submitted, using the DEREK software. No relevant structural alerts were identified.

2.2.5 Reproductive and developmental toxicity

(a) Multigeneration reproductive toxicity

A two-generation reproductive toxicity study was performed in rats in accordance with OECD Test Guideline 416. Three groups of Wistar Han rats (25 of each sex per dose) received sisapronil by daily oral gavage starting at least 70

Table 3
Genotoxicity studies on sisapronil

Test system	Test object	Concentration/dose	Results	References
In vitro				
Ames reverse mutation assay[a,b]	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537; and *Escherichia coli* WP2 uvrA pKM101	15–5 000[c] μg/plate	Negative; not mutagenic	Cheung (2011)
Chromosomal aberration assay[a,d]	Human peripheral blood lymphocyte primary cell cultures	9.73–1 000 μg/mL	Negative; not clastogenic	Gunther (2011)
In vivo				
Micronucleus induction[e]	Mouse bone marrow	62.5, 125 and 250 mg/kg bw[f]	Negative; not clastogenic	Engel (2011)

bw: body weight; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] With and without Aroclor 1254–induced rat liver S9 fraction.

[b] Positive controls were sodium nitrite, 9-aminoacridine, 2-nitrofluorene, nitrofurantoin and *N*-ethyl-*N*'-nitro-nitrosoguanidine in the absence of metabolic activation; and 2-anthramine in the presence of metabolic activation.

[c] Precipitation at 50 μg/plate and higher.

[d] Mitomycin C was used as a positive control without metabolic activation, and cyclophosphamide was used as a positive control in the presence of metabolic activation.

[e] Cyclophosphamide was used as a positive control.

[f] The maximum tolerated dose was 250 mg/kg bw. There was a dose-related decrease in the percentage of polychromatic erythrocytes.

days before mating (or starting at weaning for the F_1 animals). The doses tested were 0 (vehicle), 0.3, 2.0 and 15 mg/kg bw per day for the F_0 generation and 0.3 and 2.0 mg/kg bw per day for the F_1 generation. Because of the test substance–related effects on F_1 postnatal survival, an insufficient number of F_1 animals was available in the 15 mg/kg bw per day group to assign to the F_1 generation. Therefore, all pups in this group were euthanized on postnatal day 21, resulting in no F_1 generation at this dose level. The F_0 and F_1 females continued to receive the test substance throughout mating, gestation and lactation and through the day prior to euthanasia. F_1 and F_2 litters were culled to four pups of each sex, when possible, after weaning at postnatal day 21, and the non-selected pups were necropsied. Observations included clinical signs, body weight, feed consumption, reproductive parameters, functional observational battery, organ weights, gross pathology and histopathology.

There were no test substance–related deaths in the F_0 or F_1 adult generation. Sisapronil had no effect on body weight in the F_0 generation. Although the reproductive performance of the F_0 generation was not affected at any dose level, low fertility in males and females, decreased male copulation, decreased female conception indices and decreased ovarian follicle counts were noted in the F_1 generation at 2.0 mg/kg bw per day. Enlarged thyroid glands were observed in F_0 males at 2.0 mg/kg bw per day and in F_0 males and females at 15 mg/kg bw per day. This finding corresponded to higher mean thyroid gland weights and follicular cell hypertrophy and hyperplasia in the males and females of these groups. Thyroid follicular cell hypertrophy and hyperplasia were also noted at

0.3 mg/kg bw per day, although the finding was not statistically significant. In addition, nodular hyperplasia and follicular cell adenoma were observed in males in the 15 mg/kg bw per day group. Higher absolute and relative liver weights were observed in the 2.0 and 15 mg/kg bw per day groups. At 15 mg/kg bw per day, this was associated with hepatocellular hypertrophy, vacuolation, single-cell necrosis and, in males only, multinucleated hepatocytes. Whereas the F_1 postnatal survival was severely affected in the high-dose group, the survival, pup body weight and body weight gain were unaffected at 0.3 and 2.0 mg/kg bw per day.

The NOAEL for reproductive toxicity was 0.3 mg/kg bw per day, the NOAEL for offspring toxicity was 2.0 mg/kg bw per day and the NOAEL for parental toxicity was 0.3 mg/kg bw per day (Edwards, 2013a).

(b) Developmental toxicity

(i) Rats

Sisapronil was given to groups of 25 pregnant Crl:WI (Han) rats by daily gavage on gestation days 6 through 20. The doses were 0 (vehicle), 0.3, 2.0 and 20 mg/kg bw per day. On gestation day 21, a laparohysterectomy was performed on each female. The uteri, placentae and ovaries were examined, and the numbers of fetuses, early and late resorptions, total implantations and corpora lutea were recorded. Gravid uterine weights were recorded, and net body weights and net body weight changes were calculated. The fetuses were weighed, sexed and examined for external, visceral and skeletal malformations and developmental variations.

The only treatment-related effect noted was a lower mean body weight gain with corresponding reduced feed consumption at 20 mg/kg bw per day during the first week of treatment. The pups of these dams also had a lower body weight (up to 5.7%).

Based on these effects, the NOAEL for both maternal and embryo/fetal toxicity was 2.0 mg/kg bw per day (Edwards, 2012).

(ii) Rabbits

Groups of 25 pregnant New Zealand White (Hra:(NZW)SPF) rabbits were given sisapronil by daily gavage on gestation days 7 through 28 at a dose of 0 (vehicle), 0.3, 2.0 or 12.5 mg/kg bw per day. On gestation day 29, a laparohysterectomy was performed on each surviving female. The uteri, placentae and ovaries were examined, and the numbers of fetuses, early and late resorptions, total implantations and corpora lutea were recorded. Gravid uterine weights were recorded, and net body weights and net body weight changes were calculated. The fetuses were weighed, sexed and examined for external, visceral and skeletal malformations and developmental variations.

The dams of the high-dose group showed severe body weight loss and reduced feed intake, resulting in moribundity, abortion and premature delivery in several dams. The fetuses at this dose had lower mean body weights (up to 12.2%). No substance-related effects were noted at the low and middle dose levels.

The NOAEL for both maternal and embryo/fetal toxicity was 2.0 mg/kg bw per day (Edwards, 2013b).

2.2.6 Special studies

(a) Acute neurotoxicity

The neurofunctional effects of sisapronil were evaluated following the administration of a single oral dose of 0, 100, 500 or 1000 mg/kg bw to groups of 20 male rats. Ten animals from each group underwent the functional observational battery, and the remaining 10 animals were tested for effects on motor activity.

Six high-dose animals were found dead on study day 3. Animals of the 500 and 1000 mg/kg bw groups ate less, lost weight and showed hindlimb retraction. Additionally, at 1000 mg/kg bw, the animals had tremors and showed signs of reduced grooming. Functional observational battery parameters were also affected at 500 and 1000 mg/kg bw, in particular reduced gait, tremors, convulsions (high dose), hyperactivity, vocalization, laboured breathing, reduced foot splay, reduced grip strength and altered reflex responses. The motor activity parameters were significantly affected in both 500 and 1000 mg/kg bw animals, in a dose-dependent manner. The animals of the 100 mg/kg bw group showed no effects on the functional observational battery or motor activity tests.

A NOAEL of 100 mg/kg bw was identified for single-dose neurotoxicity (Ryan, 2011).

(b) Mode of action for effects on the thyroid in rats

In a study specifically designed to reveal the mode of action for liver and thyroid toxicity, groups of 30 male Han Wistar/Crl:W1(Han) rats were given sisapronil daily by oral gavage at a dose of 0, 1, 7.5 or 15 mg/kg bw per day for 30 days. From each group, 10 animals were euthanized on day 8, 10 animals on day 15, and the remaining 10 on day 31. The observations included clinical signs, body weights, body weight changes, feed consumption, thyroid hormones (free and total T_3, free and total T_4, and TSH), UGT1A-mediated T_4 glucuronide formation, gross necropsy findings, organ weights and histopathological examinations.

Sisapronil produced no clinical signs of systemic toxicity and had no influence on body weight or feed consumption. Statistically significantly lower levels of free and total T_4 in plasma were recorded on day 15 at 15 mg/kg bw per day and on day 31 at 7.5 and 15 mg/kg bw per day. In addition, statistically significantly higher levels of TSH in plasma were recorded at 7.5 mg/kg bw per

day (day 31) and at 15 mg/kg bw per day (days 15 and 31). Liver weights were increased in high-dose animals at all euthanasia time points and in mid-dose animals only on day 31. The thyroid weights were increased in mid- and high-dose animals, but only at the end of the study. Induction of UGT1A-mediated T_4 glucuronide formation was observed in mid-dose animals on days 15 and 31 and in high-dose animals at all time points. Time- and dose-related follicular cell hypertrophy was noted in treated animals. In addition, centrilobular and diffuse hepatocyte hypertrophy was observed on day 31 in treated animals. Mitotic activity of the thyroid increased with both time and dose level (Robertson, 2012).

(c) Microbiological effects

Considering the chemical structure and the mode of action of sisapronil, the Committee did not anticipate any adverse effects of sisapronil residues on the human gastrointestinal microbiota.

2.3 Observations in humans

No data in humans are available. Sisapronil is not used in human medicine.

3. Comments

3.1 Biochemical data

The pharmacokinetics of sisapronil was studied in rats, dogs and cynomolgus monkeys. Most of the investigations were done as part of the toxicological studies.

Twenty-four hours after administration of ^{14}C-labelled sisapronil by oral gavage to rats at a dose of 50 mg/kg bw, the radioactivity in faeces was approximately 98% of the total recovery from excreta, and the remainder was found in urine, suggesting limited absorption (Lineham, 2012).

An oral bioavailability of 6.8% was determined in cynomolgus monkeys by comparing the pharmacokinetics data in plasma following a single oral administration with those following a single intravenous administration (Stuhler, Peterson & Starch, 2012).

There was little information on the distribution and metabolism of sisapronil in laboratory animals. In a radiolabel study in rats, the parent sisapronil accounted for more than 91% of the radioactivity in faeces at 24 hours after oral administration. In addition, sisapronil was identified as the primary residue in the liver. A metabolite was detected in the liver of rats, but could not be identified. It had the same retention time as the main metabolite found in cattle liver, which could not be identified because of the low quantities present (Lineham, 2012).

These data suggest that sisapronil undergoes minimal metabolism and that the metabolite found in cattle is also present in the rat and therefore is inherently tested in the toxicity studies on rats.

In rats, the elimination half-life of sisapronil in plasma was approximately 14 days. This half-life was determined in a 28-day repeated-dose toxicity study in which blood samples were taken at 4, 8 and 24 hours post-dosing on several study days, including day 1 (Hu, 2009). The Committee considered that this estimated half-life in rats should be viewed with great caution, because it was based on only three time points and involved a large time extrapolation. The sponsor cited a half-life of 20–39 days, derived from an exploratory oral study in rats with a sampling period of 14 days; however, that study was not provided. The Committee noted that a time to steady state of approximately 100–150 days could be estimated from the time–concentration profile in plasma in rats from the 1-year repeated-dose toxicity study (Rodríguez Gómez, 2013). In the 1-year rat study, the mean concentrations of sisapronil in plasma at the last time point were approximately 325, 567, 1172 and 3424 ng/mL for the 0.1, 0.3, 1.0 and 10 mg/kg bw per day dose groups, respectively. The increase in sisapronil concentration in plasma was dose dependent, but less than dose proportional. Across the repeated-dose toxicity studies in rats, substantial differences in sisapronil concentrations in plasma were noted, but the cause of this could not be determined.

In a non-GLP-compliant study, dogs were given a single intravenous sisapronil dose of 0.5, 1.5 or 5 mg/kg bw. Blood samples were taken over a period of 268 days. The plasma elimination half-life for all three doses was approximately 100 days (Fisher, 2008).

The concentration of sisapronil in plasma was studied in cynomolgus monkeys over a period of 70 days after a single intravenous administration of 0.5 mg/kg bw. From this study, a plasma elimination half-life of 12.4 days was calculated (Stuhler, Peterson & Starch, 2012).

3.2 Toxicological data

Critical toxicity studies are summarized in Table 4.

Single-dose oral toxicity studies with sisapronil were conducted in mice and rats. The oral LD_{50} in rats was 552 mg/kg bw (Beerens-Heijnen, 2011a). In mice, no mortality was observed at 30 mg/kg bw, the highest dose tested (Mill, 2003).

Studies in rabbits showed that sisapronil was not irritating to the skin (Beerens-Heijnen, 2011b) or the eyes (Beerens-Heijnen, 2011c). Sisapronil was not a skin sensitizer in the murine local lymph node assay (Beerens-Heijnen, 2011d).

Repeated-dose oral toxicity studies were conducted with sisapronil in rats (gavage) and dogs (capsules). The main studies in rats included a 90-day

Table 4
Studies relevant to 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)
Rat				
Three-month study of toxicity (gavage)	0, 0.1, 0.3, 1.0, 10	Hepatocellular hypertrophy; thyroid follicular cell hypertrophy	1.0	10
One-year study of toxicity (gavage)	0, 0.1, 0.3, 1.0, 10	Centrilobular hepatocellular hypertrophy; thyroid follicular cell hypertrophy, hyperplasia and adenoma	0.3	1.0
Two-generation reproductive toxicity study (gavage)	0, 0.3, 2.0, 15	Parental toxicity: Follicular cell hypertrophy and hyperplasia; increased liver weights	0.3	2.0
		Reproductive toxicity: Low fertility in males and females, decreased male copulation, decreased female conception indices and decreased ovarian follicle counts in F_1 generation	0.3	2.0
		Offspring toxicity: Decreased survival	2.0	15
Developmental toxicity study (gavage)	0, 0.3, 2.0, 20	Embryo/fetal toxicity: Lower pup weight due to lower body weight gain and lower feed consumption in dams	2.0	20
Rabbit				
Developmental toxicity study (gavage)	0, 0.3, 2.0, 12.5	Maternal toxicity: Severe body weight loss, reduced feed intake, moribundity, abortion and premature delivery	2.0	12.5
		Embryo/fetal toxicity: Reduced pup weight	2.0	12.5
Dog				
Three-month study of toxicity[a] (capsule)	0, 0.3, 1, 10	Increased glycogen in hepatocytes; thyroid follicular cell hypertrophy	0.3	1

[a] A plasma half-life of approximately 100 days was found in a non-GLP-compliant study in dogs.

study and a 1-year study. Studies in dogs included a range-finding 28-day study and a 90-day study.

The main target organs for toxicity in rats and dogs were the liver and thyroid.

In rats given an oral gavage sisapronil dose of 0, 0.1, 0.3, 1.0 or 10 mg/kg bw per day for 90 days, increased levels of TSH and decreased levels of T_4 in plasma were observed at the highest dose. Animals of this group also showed increased absolute and relative liver and thyroid weights, hepatocellular hypertrophy and thyroid follicular cell hypertrophy. The NOAEL in this study was 1.0 mg/kg bw per day, based on effects on the liver and thyroid at 10 mg/kg bw per day (Rodríguez Gómez, 2012).

In rats given an oral gavage sisapronil dose of 0, 0.1, 0.3, 1.0 or 10 mg/kg bw per day for 1 year, decreases in levels of T_3 and T_4 in plasma were observed at

the highest dose. Increased absolute and relative liver weights were observed in both sexes at 1.0 and 10 mg/kg bw per day. Increased relative thyroid weights were observed at 1.0 mg/kg bw per day in males only, and absolute and relative thyroid weights were seen at 10 mg/kg bw per day in both sexes. The livers of the animals at 1.0 and 10 mg/kg bw per day showed centrilobular hepatocellular hypertrophy and vacuolation. Thyroid follicular cell hypertrophy, hyperplasia and adenomas were statistically significantly increased at doses of 1.0 and 10 mg/kg bw per day. The NOAEL in this study was 0.3 mg/kg bw per day, based on changes in the liver and thyroid at 1.0 mg/kg bw per day (Rodríguez Gómez, 2013).

In a range-finding 28-day oral toxicity study, dogs were given sisapronil in capsules at a dose of 0, 1, 5 or 25 mg/kg bw per day. Relative liver weights were increased in high-dose males and females, accompanied by increased glycogen in hepatocytes. Absolute and relative thymus weights were decreased in females of the 5 and 25 mg/kg bw per day groups. These decreases were statistically significant in females of the 5 mg/kg bw per day group only. Microscopically, the decreased thymus weights correlated with an increased incidence of minimal to mild decreased cellularity of the lymphoid tissue. The NOAEL in this study was 1 mg/kg bw per day, based on changes in the thymus at 5 mg/kg bw per day (Heward, 2011).

In a 90-day repeated-dose oral toxicity study, dogs were given sisapronil in capsules at a dose of 0, 0.3, 1 or 10 mg/kg bw per day. Dogs had increased absolute and relative liver weights and vacuolar degeneration of individual hepatocytes at the highest dose. At 1 and 10 mg/kg bw per day, an increase in glycogen in hepatocytes was observed. Minimal thyroid follicular cell hypertrophy was observed at 1 and 10 mg/kg bw per day. Thyroid follicular cell hypertrophy was characterized by cuboidal to tall cuboidal follicular cells, frequently associated with increased vacuolation in the apical cytoplasm. This hypertrophy occurred without increased thyroid weight or changes in thyroid hormone levels in blood. A NOAEL of 0.3 mg/kg bw per day was identified, based on minimal thyroid follicular cell hypertrophy and increased glycogen in hepatocytes at 1 mg/kg bw per day (Heward, 2012).

The genotoxicity of sisapronil was investigated in an adequate array of in vitro and in vivo tests (Cheung, 2011; Engel, 2011; Gunther, 2011). No evidence of genotoxicity was found. In silico analysis revealed no structural alerts for genotoxicity (Frank, 2013). The Committee concluded that sisapronil is not genotoxic.

No specific 2-year toxicity studies or carcinogenicity studies were provided. The study with the longest duration was the 1-year repeated-dose oral toxicity study in rats.

The only test article–related proliferative lesions in the 1-year oral toxicity study in rats described above were thyroid hyperplasia and thyroid tumours

(adenomas). The NOAEL for these thyroid effects was 0.3 mg/kg bw per day. A study was conducted to determine a possible mode of action for thyroid hormone changes and to evaluate the microscopic liver and thyroid changes observed in rats (Robertson, 2012). This study supported a mode of action for sisapronil-induced thyroid tumour formation involving the disruption of homeostasis of the hypothalamic–pituitary–thyroid (HPT) axis by an extrathyroidal mechanism. Specifically, sisapronil induces UGT, which increases the conjugation of thyroid hormones (T_3 and T_4) in the liver. In response to the hypothyroid state, TSH synthesis and release are stimulated, and this is the key event that leads to thyroid follicular cell growth and hyperplasia. In response to the hypothyroid state and increased TSH levels, the thyroid gland is stimulated to produce more T_4, and, over time, there is compensatory thyroid gland enlargement. For sisapronil, the available data provide compelling evidence that increased hepatic clearance of T_3 and T_4 causes chronic stimulation of the HPT axis, seen acutely as an increase in TSH secretion, with long-term stimulation of the thyroid gland as a chronic consequence leading to follicular cell adenoma development.

The data from the 1-year rat study were used for BMD modelling to assist in the analysis of the proposed mode of action. A number of variables were used for the BMD analysis: TSH, total T_4, free T_3, relative (to body and brain weights) liver and thyroid weights as well as the occurrence of bilateral thyroid adenoma and hypertrophy. A BMR of 10% extra risk above the background was selected as the basis for estimating BMDs. Among the variables analysed, the BMDLs ranged from 0.26 to 0.79 mg/kg bw per day, which are supportive of the 0.3 mg/ kg bw per day NOAEL identified in this study.

Although the Committee recognized that a specific carcinogenicity study had not been conducted, it concluded that sisapronil is not genotoxic and that the thyroid tumours in rats are a result of an indirect perturbation of the HPT axis by a mode of action not considered relevant to humans. Therefore, the Committee concluded that sisapronil does not pose a risk of carcinogenicity to humans at doses relevant to residues of veterinary drugs.

A two-generation reproductive toxicity study was conducted in rats given sisapronil by oral gavage at a dose of 0, 0.3, 2.0 or 15 mg/kg bw per day. The 15 mg/kg bw per day dose group was discontinued for the F_1 generation because of the high pup mortality in that group. Systemic toxicity was evidenced by significant effects on the liver (increased absolute and relative liver weights) and thyroid (follicular cell hypertrophy and hyperplasia) at 2.0 mg/kg bw per day and higher. Although the reproductive performance of the F_0 generation was not affected at any dose level, low fertility in males and females, decreased male copulation, decreased female conception indices and decreased ovarian follicle counts were noted in the F_1 generation at 2.0 mg/kg bw per day. The NOAEL for reproductive toxicity was 0.3 mg/kg bw per day, the NOAEL for offspring toxicity

was 2.0 mg/kg bw per day and the NOAEL for parental toxicity was 0.3 mg/kg bw per day (Edwards, 2013a).

Developmental toxicity studies were conducted in rats and rabbits. The rat study used oral gavage doses of 0, 0.3, 2.0 and 20 mg/kg bw per day. The only effects seen in this study were reduced body weight gain and feed consumption in dams at the high dose, which resulted in lower pup weights in that group. The NOAEL for both maternal toxicity and embryo/fetal toxicity was 2.0 mg/kg bw per day (Edwards, 2012).

The developmental toxicity study in rabbits used oral gavage doses of 0, 0.3, 2.0 and 12.5 mg/kg bw per day. The dams of the high-dose group showed reduced feed intake and severe body weight loss, resulting in moribundity, abortion and premature delivery in several dams. The fetuses of this dose group had lower mean body weights. The NOAEL for both maternal and embryo/fetal toxicity was 2.0 mg/kg bw per day (Edwards, 2013b).

The Committee concluded that sisapronil is not teratogenic.

The acute neurotoxicity of sisapronil was investigated in rats given an oral gavage dose of 0, 100, 500 or 1000 mg/kg bw. There was mortality at the highest dose, and animals of the 500 and 1000 mg/kg bw per day groups showed reduced gait, tremors, convulsions (high dose), hyperactivity, vocalization, laboured breathing, reduced foot splay, reduced grip strength and altered reflex responses. Motor activity parameters were also affected in these groups. The NOAEL in this study was 100 mg/kg bw per day (Ryan, 2011).

3.3 Microbiological data

Considering the chemical structure and the mode of action of sisapronil, the Committee did not anticipate any adverse effects of sisapronil residues on the human gastrointestinal microbiota.

4. Evaluation

The main findings in the livers of rats and dogs treated with sisapronil included increased liver weights, centrilobular hepatocellular hypertrophy, vacuolation and increased glycogen in hepatocytes. Hepatocellular hypertrophy and increased liver weights in rats are considered to be related to hepatic drug metabolizing enzyme induction, an adaptive response. It is well known that rats are particularly sensitive to developing thyroid tumours according to a mode of action, described above, that is considered not to be relevant to humans.

The Committee noted that the lowest relevant NOAEL was 0.3 mg/kg bw per day in both the 90-day oral toxicity study in dogs and the two-generation reproductive toxicity study in rats. However, the Committee considered this 90-

day study in dogs to be unsuitable to address long-term toxicity, in view of the very long elimination half-life of sisapronil in dogs (i.e. 100 days). Longer-term oral toxicity studies with sisapronil in dogs were not available. It remains uncertain how the toxic effects in dogs might progress and whether or not other effects might be triggered upon longer-term exposure. The Committee also concluded that the NOAEL from the reproductive toxicity study in rats may not be sufficient to protect against long-term effects of sisapronil.

The Committee concluded that a toxicological ADI could not be established because the Committee had no basis upon which to determine a suitable uncertainty factor to accommodate the lack of a long-term toxicity study.

4.1 Additional information that would assist in the further evaluation of the compound

- Data to address long-term toxicity relevant to humans (e.g. 1-year dog study)
- Comparative pharmacokinetics studies and an explanation of interspecies differences in the pharmacokinetic profiles.

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Teflubenzuron

First draft prepared by
Shiva C. Ghimire[1] and Kevin Greenlees[2]

[1] Veterinary Drugs Directorate, Health Canada, Ottawa, Ontario, Canada

[2] Office of New Animal Drug Evaluation, Center for Veterinary Medicine, Food and Drug Administration, Department of Health and Human Services, Rockville, Maryland, United States of America (USA)

1. Explanation

Teflubenzuron (International Union of Pure and Applied Chemistry name: 1-(3,5-dichloro-2,4-difluorophenyl)-3-(2,6-difluorobenzoyl)urea; Chemical Abstracts Service no. 83121-18-0; Fig. 1) is an insecticide belonging to the benzoylurea group of compounds. Its mode of action is through inhibition of the synthesis of chitin, and hence it is most effective on the developmental stages of insects. Insects are killed as a result of the disruption of their moulting. Teflubenzuron is approved in many countries as an insecticide for use in plant production as well as for control of sea lice (*Lepeophtheirus salmonis* and *Caligus rogercresseyi*) in aquaculture. Teflubenzuron is used as a premix coated onto non-medicated fish feed pellets to achieve an intended dose of 10 mg/kg body weight (bw) per day for 7 consecutive days. The withdrawal periods range from 7 to 11 days and from 45 to 96 degree-days.

Fig. 1
Chemical structure of teflubenzuron
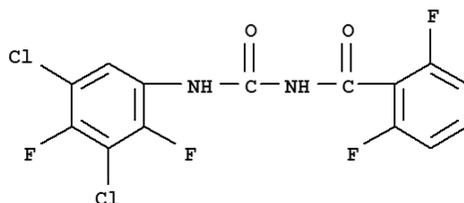
Teflubenzuron has not previously been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), although it was evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) as a pesticide in 1994 and 1996 (FAO/WHO, 1995, 1997) and is scheduled for periodic re-evaluation at JMPR's September 2016 meeting. JMPR established an acceptable daily intake (ADI) of 0–0.01 mg/kg bw on the basis of a lowest-observed-adverse-effect level (LOAEL) of 2.1 mg/kg bw per day in a mouse carcinogenicity study with the application of an uncertainty factor of 200, including an additional factor of 2 to account for the use of a LOAEL instead of a no-observed-adverse-effect level (NOAEL).

The Committee evaluated teflubenzuron at the present meeting at the request of the Twenty-second Session of the Codex Committee on Residues of Veterinary Drugs in Foods (FAO/WHO, 2015), with a view to establishing an ADI and recommending maximum residue limits (MRLs) in finfish tissues.

For this assessment, a data package was submitted by a sponsor. Additionally, the following databases of published literature were searched using the search term "teflubenzuron": Agricola (1970 – September 2015), CAB

Abstracts (1973 – 2015 week 38), Embase (1974 – September 2015), Food Science and Technology Abstracts (1969 – 2015 September week 3), Global Health (1973 – 2015 week 38), International Pharmaceutical Abstracts (1970 – September 2015) and Medline (1946 – September 2015). In total, 876 articles were retrieved, of which 271 were removed as duplicates; the titles and abstracts of the remaining 605 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 teflubenzuron in laboratory animals or humans were considered relevant for the assessment. Identified relevant articles and those submitted by the sponsor were reviewed. Most of the original studies provided by the sponsor were performed in the 1980s and conform to the good laboratory practice (GLP) guidelines, although the GLP-compliant status of a few studies could not be verified. Overall, available studies were considered sufficient for the evaluation of teflubenzuron.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

(a) Rats

In a GLP-compliant study, groups of Wistar (Chbb:THOM) rats (five of each sex per group) were administered uniformly ^{14}C aniline ring–labelled teflubenzuron (radiochemical purity >97%) in aqueous solutions of 1% Tylose and 1% Tween 80 by gavage at a single dose of 25 or 750 mg/kg bw. Serial blood samples were collected for up to 168 hours after treatment. Concentrations of radioactivity in plasma were determined by liquid scintillation counting. In the low-dose group, nearly constant concentrations of 0.38–0.46 μg teflubenzuron equivalents per millilitre in males and 0.22–0.25 μg/mL in females were maintained between 1 and 8 hours, which declined thereafter to below 0.01 μg/mL by 168 hours post-treatment. In the high-dose group, nearly constant concentrations of 0.98–1.43 μg/mL were detected between 20 minutes and 24 hours in female rats. In the males, with the exception of a distinct peak (3.27 μg/mL) at 24 hours, nearly constant concentrations of 1.17–1.72 μg/mL were maintained between 40 minutes and 24 hours. In both dose groups, the concentration of radioactivity in whole blood was slightly lower than that in plasma, but the time course was comparable. A 30-fold dose increase led to only a 4- to 6-fold increase in the concentration of the drug in plasma in animals of both sexes, suggesting a difference in absorption rates at different doses (Schlüter, 1986).

In another GLP-compliant study, concentrations of teflubenzuron in plasma in groups of five male and five female Wistar (KFM-Han) rats were determined after feeding the rats a diet medicated with teflubenzuron (purity 92.4%) at a nominal concentration of 0, 500, 1000, 2000, 4000, 8000, 16 000 or 32 000 mg/kg feed (equal to 0, 39, 77, 154, 305, 624, 1259 and 2520 mg/kg bw per day for males and 0, 38, 78, 158, 320, 607, 1313 and 2544 mg/kg bw per day for females, respectively) for a period of 28 days. Blood samples were collected on day 28, and plasma was analysed by high-performance liquid chromatography (HPLC). The concentrations reached a plateau in male rats (0.37 μg/mL) at teflubenzuron concentrations of 1000 mg/kg feed and higher (equivalent to ≥77 mg/kg bw per day) and in female rats (0.43 μg/mL) at teflubenzuron concentrations of 2000 mg/kg feed and higher (equivalent to ≥158 mg/kg bw per day) (Ellgehausen et al., 1986).

In a GLP-compliant study intended to address carcinogenicity and chronic toxicity, Wistar (KFM-Han) rats were administered teflubenzuron (purity 92.4%) at a nominal concentration of 0, 20, 100 or 500 mg/kg feed (equal to 0, 1.1, 5.4 and 27.6 mg/kg bw per day for males and 0, 1.3, 6.5 and 32.0 mg/kg bw per day for females, respectively). Plasma concentrations, determined on surviving animals in the chronic toxicity study (10 of each sex per group) at week 107, reflected the dietary dose: 0, 0.02, 0.06 and 0.27 μg/mL in males and 0, 0.2, 0.04 and 0.15 μg/mL in females, respectively (Suter et al., 1987a).

In a second GLP-compliant chronic toxicity and carcinogenicity study, Wistar (KFM-Han) rats were administered teflubenzuron (purity 92.4%) at a nominal concentration of 0, 2500 or 10 000 mg/kg feed (equal to 0, 123 and 487 mg/kg bw per day for males and 0, 154 and 615 mg/kg bw per day for females, respectively), and plasma concentrations were determined at weeks 4, 26, 52 and 104. In males, plasma concentrations of teflubenzuron increased from 0.18 μg/mL at week 4 to 0.79 μg/mL at week 104 of treatment at the low dose and from 0.23 μg/mL at week 4 to 0.70 μg/mL at week 104 at the high dose. In females, plasma concentrations of teflubenzuron increased from 0.18 μg/mL at week 4 to 0.60 μg/mL at week 104 at the low dose and from 0.14 μg/mL at week 4 to 0.43 μg/mL at week 104 at the high dose (Tennekes et al., 1989).

In a study described previously (Schlüter, 1986), additional groups of five male and five female Wistar (Chbb:THOM) rats were administered uniformly ^{14}C-aniline ring–labelled teflubenzuron (aqueous solution in 1% Tylose and 1% Tween 80) by gavage at either a single dose of 25 or 750 mg/kg bw or 14 daily doses of unlabelled teflubenzuron at 25 mg/kg bw per day followed by the same dose of radiolabelled compound 24 hours after the last dose. Urine and faeces were collected for 8 days post-treatment, after which the animals were killed and radioactivity in the carcass was determined. Irrespective of sex, dose or duration of treatment, most radioactivity (91–95% of the total dose) was excreted

in the faeces, predominantly (>85%) within the first 24 hours, and only small quantities (0.6–0.9% of the total dose for the low-dose group and 0.15–0.16% for the high-dose group) were excreted in urine. Radioactivity remaining in the carcass was negligible (low-dose group, 0.04–0.08% of the total dose; high-dose group, <0.01% of the total dose). Total recoveries of radioactive residues were 91–96%. Additionally, the radioactivity was measured in the expired air collected over a 24-hour period in one male and one female rat treated with ^{14}C-labelled teflubenzuron by gavage at 25 mg/kg bw. No radioactivity was detected in the expired air (Schlüter, 1986).

In a preliminary non-GLP-compliant study, nine male and nine female Wistar (Chbb:THOM) rats were administered uniformly ^{14}C-aniline ring–labelled teflubenzuron (radiochemical purity >99%) via gavage in dimethyl sulfoxide (DMSO) at a dose of 25 mg/kg bw per day for 7 consecutive days. Radioactivity in excreta was assessed in four rats of each sex during the dosing period and for 8 days following the last dose, after which the rats were killed to determine the remaining radioactivity in the carcass. Of the remaining animals, one rat of each sex was killed at 1, 6, 24, 48 and 120 hours after the last of the seven doses, and radioactivity in organs and tissues was determined. Excretion of radioactivity was comparable between sexes. Total recovery of radioactivity was 90–93% of the total dose in faeces, 2–3% in urine and less than 0.1% in the carcass (Schlüter, 1984). The excretion pattern of teflubenzuron was comparable after a single or a multiple oral administration (Schlüter, 1984, 1986).

In a GLP-compliant study, groups of bile duct–cannulated Wistar rats (three of each sex) were dosed by gavage with ^{14}C-aniline ring–labelled teflubenzuron (radiochemical purity >98%) in an aqueous solution containing 1% Tylose and 1% Tween 80 at 25 or 750 mg/kg bw. In the low-dose group, between 0 and 48 hours after dosing, 15.3–17% of the total dose was excreted in bile, 1.3–1.5% in urine and 45.4–46.6% in faeces. At 48 hours, 19.3–26.6% of the total dose was measured in the gastrointestinal tract, 0.4% in liver and 1.3–1.9% in the rest of the carcass. In contrast, in the high-dose group, only a small proportion of the total dose (1.6–2.1%) was excreted in bile, 0.3–0.4% in urine and 57.9–71.8% in the faeces between 0 and 48 hours post-treatment. The remaining radioactivity at 48 hours accounted for 12.2–25.0%, 0.06% and 1.0–1.4% of the total dose in the gastrointestinal tract, liver and the rest of the carcass, respectively. If the sum of the radioactivity in urine, bile, liver and the rest of the carcass is considered as an indication of absorption, 18.3–20.8% of the total dose was absorbed at the low dose compared with 3.0–4.0% at the high dose, suggesting a dose-dependent absorption of this drug from the gastrointestinal tract (Hawkins & Mayo, 1988).

2.1.2 **Biotransformation**

The nature of the radioactive residues in urine and faeces of male and female rats treated orally for 7 consecutive days with ^{14}C-aniline ring–labelled teflubenzuron at a dose of 25 mg/kg bw in DMSO was investigated in a study described above (Schlüter, 1984). No difference was noted in drug metabolism between males and females. Metabolites in faeces were identified by thin-layer chromatography (TLC). The parent compound represented the majority of radioactivity in faeces (73.3% of the administered dose, 80.2% of faecal radioactivity). The remainder was represented by at least 15 unidentified metabolites, each representing less than 1% of the administered dose. Metabolites in urine were identified by TLC and mass spectroscopy (MS). Three metabolites were characterized in urine. Two were identified as structural isomers of hydroxylated (benzoyl ring positions 3 and 4) teflubenzuron (metabolites I and II, see Fig. 2 below). The third metabolite was formed by substitution of a fluoride atom by a hydroxide group (metabolite III, see Fig. 2). Each of these metabolites represented less than 1% of the dose (Schlüter, 1985).

The biotransformation of teflubenzuron in Wistar rats was also investigated in a study described above (Schlüter, 1986) by analysing the urine and faeces after a single oral dose of 25 (low) or 750 (high) mg/kg bw ^{14}C-labelled compound or daily oral doses of 25 mg/kg bw of unlabelled compound for 14 consecutive days followed by a single dose of labelled compound. Samples were analysed by TLC, HPLC, ultraviolet spectroscopy and MS. No difference in metabolism was noted between the sexes or between animals administered a single or multiple doses of the drug. Most radioactivity excreted in faeces (>90% in single-dose groups and >95% in repeated-dose groups) co-chromatographed with the parent compound. Trace amounts of diverse, mostly polar, compounds were detected in faeces of all treatment groups. One of these metabolites was identified as (3,5-dichloro-2,4-difluorophenyl)urea (metabolite IV, see Fig. 2). This metabolite in faeces represented less than 0.2% of the dose in the high-dose group and less than 1% of the dose in the low-dose group. Given the low amounts present, no other metabolites in faeces were characterized. In urine, the radioactivity was due mainly to very polar compounds, although traces of parent compound were also reported. However, the possibility that the parent compound present in urine was due to cross-contamination by faecal particles was not ruled out. Urine from the high-dose group also contained a trace amount of the same metabolite, (3,5-dichloro-2,4-difluorophenyl)urea (metabolite IV), identified in faeces. Some polar compounds in urine were identified to be conjugated metabolites, although exact identification was not made (Schlüter, 1986).

In another study using the bile duct–cannulated rats described above (Hawkins & Mayo, 1988), metabolites in faeces, urine and bile were analysed by TLC. In 0- to 48-hour pooled faecal samples, most radioactivity (>97% at 25 mg/kg bw and 87% at 750 mg/kg bw) was identified as the parent teflubenzuron. In 0- to 48-hour urine, most radioactivity (>81%) was associated with unidentified polar material. Hydrolysis of urine indicated the presence of conjugates of metabolites that co-chromatographed with (3,5-dichloro-2,4-difluorophenyl)-urea (metabolite IV), its corresponding substituted aniline metabolite (metabolite VII) and the meta-hydroxybenzoyl derivative (metabolite I) of teflubenzuron (see Fig. 2). In 0- to 48-hour bile, most radioactivity (>85%) was represented by unidentified polar material, and only <1–5% of radioactivity co-chromatographed with (3,5-dichloro-2,4-difluorophenyl)urea (metabolite IV) and 1% with teflubenzuron (see Fig. 2). Hydrolysis of bile extracts indicated the presence of conjugates of (3,5-dichloro-2,4-difluorophenyl)urea (metabolite IV), the corresponding substituted aniline (metabolite VII) and the *meta*-hydroxybenzoyl derivative of teflubenzuron (metabolite I, see Fig. 2). As the biliary excretion contained only a low proportion of teflubenzuron, the unchanged teflubenzuron in faeces likely represented the non-absorbed drug (Hawkins & Mayo, 1988).

In a non-GLP-compliant study, the metabolites of teflubenzuron in rats were analysed using ^{19}F nuclear magnetic resonance (NMR) spectral analysis. Adult male Wistar rats weighing 350–400 g (number not specified) were administered 20 mg (approximately 55 mg/kg bw) teflubenzuron by gavage in olive oil with 20% DMSO, and urine (0–24 and 24–48 hours) and faeces (0–48 hours) were collected and analysed by the NMR technique after an appropriate extraction procedure. The identity of metabolite conjugates was analysed after treating the extracted samples with acid, β-glucuronidase or arylsulfatase. Teflubenzuron metabolism was supplemented with additional metabolism studies in rats separately administered four scission products (metabolites IV–VII, see Fig. 2) formed by hydrolytic cleavage of the phenylurea bridge of teflubenzuron – namely, (a) (3,5-dichloro-2,4-difluorophenyl)urea (metabolite IV), (b) 2,6-difluorobenzoic acid (metabolite V), (c) 2,6-difluorobenzamide (metabolite VI) and (d) 3,5-dichloro-2,4-difluoroaniline (metabolite VII). When rats were administered the scission products of teflubenzuron, most of the administered chemicals were absorbed and excreted via urine. In contrast, in rats administered teflubenzuron, more than 90% of the dose was excreted in faeces, primarily as the parent compound (91%). NMR spectral analysis could not identify any metabolites in faeces irrespective of whether the teflubenzuron or its scission products were administered. Analyses of urine samples identified a number of metabolites: scission products, other metabolites and/or their conjugates (see Fig. 2). Most of the urinary metabolites were characterized. Only a small portion (12% of the urinary metabolites, approximately 1% of the total

dose) could not be identified, but these were postulated to be compounds with an intact teflubenzuron structure (Koerts et al., 1997).

The proposed biotransformation pathway of teflubenzuron in rats is presented in Fig. 2.

Fig. 2
Proposed biotransformation pathway of teflubenzuron in rats
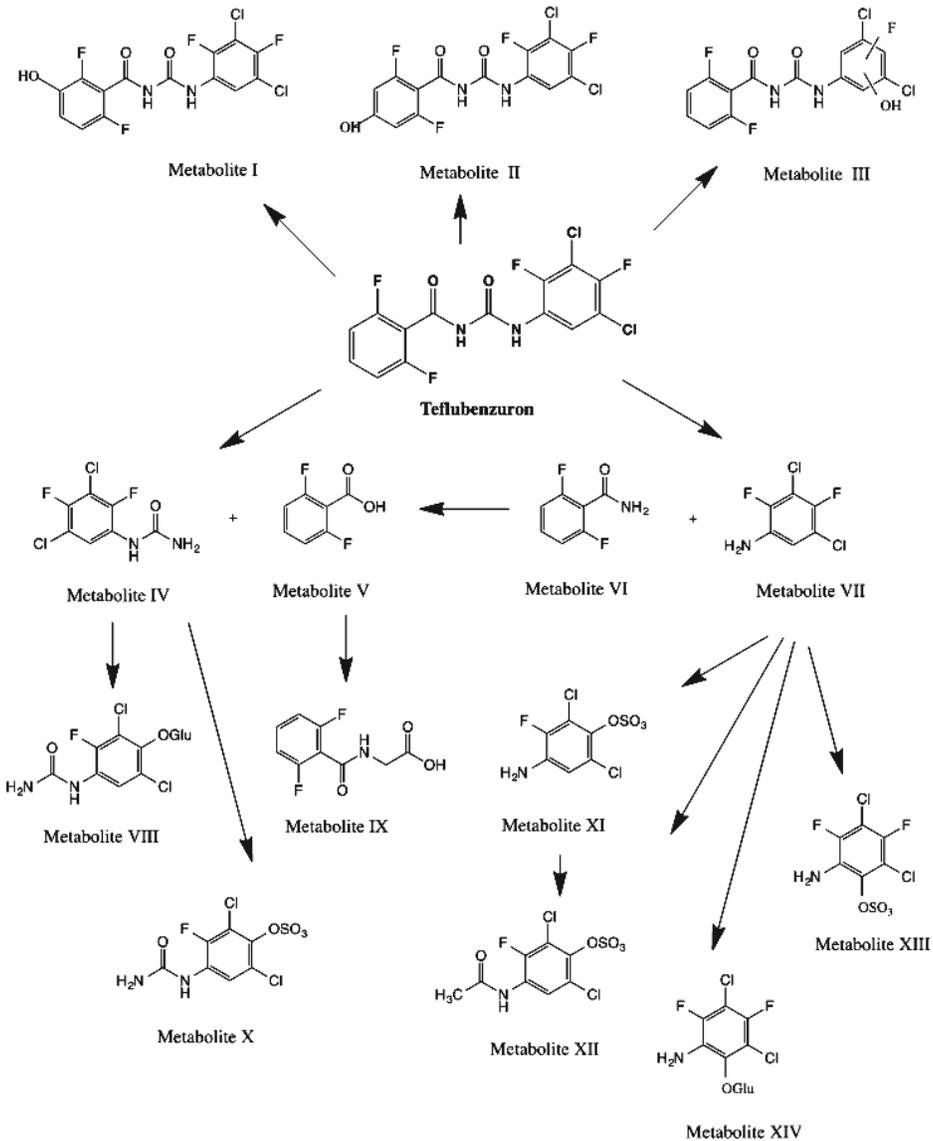
Two pathways for the hydrolytic cleavage of the phenylurea bridge of teflubenzuron are proposed: pathway 1 yields (3,5-dichloro-2,4-difluorophenyl)-urea (metabolite IV) and 2,6-difluorobenzoic acid (metabolite V), and pathway 2 yields 2,6-difluorobenzamide (metabolite VI) and 3,5-dichloro-2,4-difluoroaniline (metabolite VII). The benzoate part of teflubenzuron was mainly excreted as 2,6-difluorobenzoic acid and, to a minor extent, as 2,6-difluorobenzoylglycine (metabolite IX) and 2,6-difluorobenzamide. The aniline part of teflubenzuron was excreted as the glucuronidated (metabolite VIII) or sulfated (metabolite X) conjugates of (4-hydroxy-3,5-dichloro-2-fluorophenyl)urea, the sulfated conjugate of 4-amino-2,6-dichloro-3-fluorophenol (metabolite XI), the sulfated conjugate of 4-acetamido-2,6-dichloro-3-fluorophenol (metabolite XII) and the sulfated (metabolite XIII) or glucuronidated (metabolite XIV) conjugates of 2-amino-3,5-difluoro-4,6-dichlorophenol (Fig. 2). The relative distribution of various metabolites in the urine of rats orally dosed with teflubenzuron is presented in Table 1.

Table 1

Relative distribution of metabolites in male rat urine (0–24 hours post-dosing) administered teflubenzuron by gavage

Metabolite	% of total metabolites	Metabolite designation[a]
2,6-Difluorobenzoic acid	81.4 ± 3.8	V
2,6-Difluorobenzamide	2.0 ± 0.3	VI
(3,5-Dichloro-2-fluoro-4-phenylglucuronidephenyl)urea	5.2 ± 2.4	VIII
2,6-Difluorobenzoylglycine	4.0 ± 0.5	IX
(3,5-Dichloro-2-fluoro-4-phenylsulfatephenyl)urea	0.3 ± 0.6	X
4-Amino-2,6-dichloro-3-fluorophenylsulfate	1.2 ± 0.4	XI
4-Acetamido-2,6-dichloro-3-fluorophenylsulfate	0.6 ± 0.5	XII
2-Amino-3,5-difluoro-4,6-dichlorophenylsulfate	2.8 ± 0.5	XIII
2-Amino-3,5-difluoro-4,6-dichlorophenylglucuronide	1.2 ± 0.3	XIV
Unidentified	1.3 ± 0.7	–

[a] See Fig. 2.
Source: After Koerts et al. (1997)

2.2 Toxicological studies

2.2.1 Acute toxicity

(a) Lethal doses

The results of acute toxicity studies with teflubenzuron conducted according to Organisation for Economic Co-operation and Development (OECD) test guidelines (GLP compliant or quality assured) are summarized in Table 2.

Table 2

Single-dose toxicity studies with teflubenzuron in laboratory animals

Species	No. of animals per sex per group	Route	Dose (mg/kg bw) / concentration (mg/m³)	LD_{50} (mg/kg bw) / LC_{50} (mg/m³)	Reference
Mouse	5	Oral	1 000, 5 000	> 5 000	Ullmann (1983d)
Rat	5	Oral	1 000, 5 000	> 5 000	Ullmann (1983e)
Rat	5	Oral	5 000	> 5 000	Ullmann, Sacher & Vogel (1988)
Rat	5	Dermal	2 000	> 2 000	Ullmann (1983b)
Rat	5	Inhalation	5 038 mg/m³	> 5 038 mg/m³	Ullmann (1983a)
Rat	5	Intraperitoneal	300, 500, 800, 1 200, 2 000	> 2 000	Ullmann (1983c)

bw: body weight; LC_{50}: median lethal concentration; LD_{50}: median lethal dose

NMRI mice or Wistar (KFM-Han) rats (five of each sex per group) were gavaged once with teflubenzuron (purity 96.5–97.5%) in either 2% carboxymethyl cellulose (one rat study) or polyethylene glycol (PEG) 400 (one rat and one mouse study) at 1000 or 5000 mg/kg bw and were observed for 14 days. At 1000 mg/kg bw, no symptoms were observed in mice, but ruffled fur was observed in a few rats. Ruffled fur, dyspnoea (in both rats and mice), sedation and hunched posture (only in rats) were observed in animals dosed at 5000 mg/kg bw, which resolved by 2–4 days post-treatment. At necropsy, the only abnormality noted was mottled (dark red) lungs in three male rats of the low-dose group. No mortality was observed in either species, suggesting a median lethal dose (LD_{50}) of greater than 5000 mg/kg bw in mice and rats (Ullmann, 1983d,e; Ullmann, Sacher & Vogel, 1988).

Teflubenzuron (purity 96.5%) in PEG 400 at a dose of 2000 mg/kg bw was applied once on the clipped skin surface of Wistar (KFM-Han) rats (five of each sex), and the area was covered with a dressing pad. Skin reaction was assessed 24 hours after the treatment, and animals were observed for a period of 14 days, followed by a full necropsy examination. Clinical examination revealed no adverse skin reactions, and no abnormality was detected in necropsy, suggesting an LD_{50} for dermal exposure of greater than 2000 mg/kg bw (Ullmann, 1983b).

Wistar (KFM-Han) rats (five of each sex) were exposed via inhalation to teflubenzuron (purity not specified) at a measured concentration of 5038 (4508–5244) mg/m³ air for 4 hours and observed for 14 days, followed by a full necropsy. Dyspnoea and ruffled fur were observed in all rats starting within 2 hours of exposure and lasting until 24 hours post-treatment. No mortality or macroscopic changes were noted in treated animals, suggesting a median lethal concentration (LC_{50}) of greater than 5000 mg/m³ air (Ullmann, 1983a).

Wistar (KFM-Han) rats (five of each sex per group) were injected intraperitoneally (300, 500, 800, 1200 or 2000 mg/kg bw) with teflubenzuron (purity 96.5%) in PEG 400 and observed for 14–22 days, followed by a necropsy of surviving animals. During the first week after dosing, slight to moderate clinical signs occurred in all dose groups, which consisted of ataxia, dyspnoea, sedation, hunched appearance and ruffled fur. Mortality at different doses ranged from 0% to 30% and was not dose dependent. Postmortem examination revealed extensive peritonitis in all dose groups. The LD_{50} was greater than 2000 mg/kg bw per day (Ullmann, 1983c).

(b) Dermal irritation

The skin irritating potential of teflubenzuron (purity 96.5%) was investigated in three adult New Zealand White rabbits. The drug was applied to intact clipped skin on the dorsum of each rabbit. Half a gram of test article, moistened with tap water, was applied to the skin under a patch of surgical gauze and was held in place for 4 hours. The skin reaction at the application site was observed at 1, 24, 48 and 72 hours after removal of the patch. Under the conditions of the test, there was no evidence of skin irritation by teflubenzuron (Ullmann, 1983g).

(c) Ocular irritation

The eye irritating potential of teflubenzuron (purity 96.5%) was investigated in three adult New Zealand White rabbits. The drug (0.1 g) was deposited into the conjunctival sac of the left eye, and the eyelid was held closed briefly to prevent the loss of the drug. The right eye served as control. Eye irritation was recorded at 1, 24, 48 and 72 hours post-treatment. Slight reddening of the ventral conjunctivae and sclera was observed at 1 hour, but there was no sign of eye irritation on subsequent observations (Ullmann, 1983f), suggesting that teflubenzuron was not an eye irritant under the conditions of the test.

(d) Dermal sensitization

The delayed hypersensitivity potential of teflubenzuron was investigated using the guinea-pig (Dunkin-Hartley) maximization test. Ten animals of each sex were used as test animals, and five animals of each sex served as controls. After initial induction by intradermal injection and topical patch application, animals received a percutaneous application of either 25% teflubenzuron (purity 93.5%) in a 1:1 mixture of propylene glycol and normal saline or the vehicle alone. At 24 hours after removal of the medicated pad, 25% (5/20) of the treated animals had a positive mild skin sensitizing reaction, compared with 30% (3/10) of the controls. A second reading performed 48 hours after removal of the medicated pad showed a positive response in 5% (1/20) of the treated animals compared

with none (0/10) of the controls. In a second challenge study conducted 1 week after the first study, none of the treated or control animals had a positive skin reaction at 24 and 48 hours after removal of the medicated pad (Ullmann, 1984), suggesting that teflubenzuron did not sensitize skin under the test conditions.

2.2.2 Short-term studies of toxicity

(a) Mice

In a study conducted according to Japan Ministry of Agriculture, Forestry and Fisheries guidelines (GLP status unknown, study protocol unavailable), groups of CD-1 mice (12 of each sex per group) were administered teflubenzuron (purity not reported) at a concentration of 0, 100, 1000 or 10 000 mg/kg feed (equal to 0, 12, 115 and 1213 mg/kg bw per day for males and 0, 14, 142 and 1450 mg/kg bw per day for females, respectively) for 13 weeks.

No differences in feed consumption, body weight gain, mortality, clinical observations, or ophthalmoscopic, urine analysis and haematological parameters were noted between the groups. Biochemical changes noted included significantly increased alkaline phosphatase activity in males and alanine transaminase activity in females, increased total cholesterol level in females, decreased blood glucose level in males and increased blood glucose level in females at 10 000 mg/kg feed. Observations at necropsy were enlargement (both sexes) and discoloration (males only) of the liver at the middle and high doses. Absolute and relative liver weights were increased at the middle and high doses in both sexes. Significant histopathological changes noted were centrilobular hepatocellular swelling (both sexes) and fatty change (males only) at the middle and high doses.

The NOAEL was 100 mg/kg feed (equal to 12 mg/kg bw per day), based on liver changes in groups treated at 1000 mg/kg feed (equal to 115 mg/kg bw per day) (Takahashi et al., 1987).

(b) Rats

In a GLP-compliant study, groups of Wistar (KFM-Han) rats (10 of each sex per group) were administered teflubenzuron (purity 96.5%) at a concentration of 0, 100, 1000 or 10 000 mg/kg feed (equal to 0, 8, 82 and 809 mg/kg bw per day for males and 0, 9, 94 and 942 mg/kg bw per day for females, respectively) for 13 weeks. Additional satellite groups of five rats of each sex were included in the control and the high-dose groups, which, after 13 weeks of treatment, were maintained for an additional 4 weeks on unmedicated diet to determine the reversibility of effects.

There were no deaths or treatment-related signs of toxicity. Body weights, feed consumption, urine analysis, haematology and ophthalmoscopy did not reveal any meaningful treatment-related changes. Clinical biochemistry revealed

increased activities of ornithine transcarbamylase (in males at the high dose at week 6 and in both sexes at the high dose at week 13), aspartate transaminase (in males at the middle and high doses at week 6 and in both sexes at the same doses at week 13), alanine transaminase (in males at the middle and high doses at week 6 and at the high dose at week 13) and alkaline phosphatase (in all treated males at week 6 and in males at the low and high doses at week 13). Increases were more pronounced in males than in females. Additionally, lactate dehydrogenase activity was significantly elevated in males of the high-dose group at week 13. After 4 weeks of drug withdrawal, clinical biochemistry parameters returned to control levels. The only abnormal gross pathological and histopathological findings were the increased absolute and relative weights of liver in females and of testes in males at the high dose. In the satellite group, 4 weeks after cessation of treatment, absolute and relative thymus weights were higher in rats treated for 13 weeks at a teflubenzuron concentration of 10 000 mg/kg feed.

Being less than 1.5-fold above the control value and within the normal physiological range, the change in alkaline phosphatase activity in male rats at the low dose was considered not to be biologically relevant. Based on effects on liver enzyme activities at 1000 mg/kg feed (equal to 82 mg/kg bw per day), a NOAEL of 100 mg/kg feed (equal to 8 mg/kg bw per day) was identified (Suter et al., 1987c).

(c) Dogs

The short-term toxicity of teflubenzuron in dogs was assessed in two 13-week feeding studies and a 52-week feeding study.

In the first GLP-compliant study, groups of Beagle dogs (four of each sex per group) were fed a diet containing teflubenzuron (purity 96.5%) at a nominal concentration of 0, 100, 1000 or 10 000 mg/kg feed (equal to 0, 3.5, 33.7 and 318.2 mg/kg bw per day for males and 0, 4.0, 42.8 and 417.1 mg/kg bw per day for females, respectively) for 13 weeks.

No mortality or signs of toxicity were observed during the study. There were no changes in feed consumption or body weight gain, and a hearing test, ophthalmoscopic examination, haematology analysis and urine analysis did not reveal any changes of toxicological significance. Clinical biochemistry revealed that in a male and a female dog of the high-dose group, activities of alanine transaminase (male: weeks 4, 8 and 13; female: week 4), aspartate transaminase (male and female: week 4), alkaline phosphatase (male: weeks 4 and 8; female: week 4) and ornithine transcarbamylase (male: weeks 4 and 8; female: week 4) were elevated. Additionally, serum ornithine transcarbamylase activity was increased in another male of the same dose at week 4. Absolute and relative liver

weights were elevated in animals of both sexes at the high dose. Gross pathology revealed an increased incidence of nodular foci in the pyloric or fundic region of dogs in the high-dose group. Isolated dark red foci were also noted in the pyloric region of the stomach of two mid-dose and two high-dose dogs. A male dog at the high dose had a firm liver with irregular contour of the capsule. Microscopic changes identified were limited to hepatitis and gastritis in some dogs. One male at the low dose (100 mg/kg feed) had mild hepatitis, and another had moderate centrilobular necrosis of the liver. Slight round cell infiltration was noted in one male at each of the middle and high doses. A moderate chronic active hepatitis was diagnosed in one animal of each sex at the high dose (10 000 mg/kg feed), which was characterized by single-cell necrosis, cell infiltration and collapsed stroma. Focal gastritis was observed in females receiving teflubenzuron at a concentration of 1000 (one animal) or 10 000 (two animals) mg/kg feed. Follicular hyperplasia of the pyloric mucosa was noted in most animals of both sexes at a concentration of 10 000 mg/kg feed.

Although mild microscopic changes in liver were noted in two dogs at the lowest dose (100 mg/kg feed), given the lack of a clear dose–response relationship, 100 mg/kg feed (equal to 3.5 mg/kg bw per day) was identified as the NOAEL, based on stomach lesions at 1000 mg/kg feed (equal to 33.7 mg/kg bw per day) (Bathe et al., 1985).

A supplementary GLP-compliant study was conducted to clarify the effects on liver in groups of Beagle dogs (four of each sex per group) that were fed a diet containing teflubenzuron (purity 92.4%) at a nominal concentration of 0, 30 or 100 mg/kg feed (equal to 0, 1.2 and 4.4 mg/kg bw per day for males and 0, 1.5 and 5.1 mg/kg bw per day for females, respectively) for 13 weeks.

There was no mortality, and there were no treatment-related signs of toxicity. Feed consumption and body weight gain were not affected by treatment, and no treatment-related toxicological changes were noted in the hearing test, ophthalmoscopic examination, haematology, clinical biochemistry, urine analysis, organ weights, and macroscopic or microscopic examination of various organs.

The highest dose of 100 mg/kg feed (equal to 4.4 mg/kg bw per day) was identified as the NOAEL (Bathe et al., 1987).

Groups of Beagle dogs (four of each sex per group) were fed diets containing teflubenzuron (purity 92.4%) at a concentration of 0, 30, 100 or 500 mg/kg feed (equal to 0, 1.0, 3.2 and 17.3 mg/kg bw per day for males and 0, 1.2, 4.0 and 18.0 mg/kg bw per day for females, respectively) for 52 weeks under GLP-compliant study conditions.

There were no deaths or treatment-related signs of toxicity. No treatment-related differences were noted in feed consumption, body weight gain, auditory test (pretest and week 52), ophthalmic examinations (pretest and week 52), haematology (pretest and weeks 13, 26 and 52), clinical biochemistry (pretest and weeks 13, 26 and 52) or urine analysis (pretest and weeks 13, 26 and 52). At necropsy, the only notable change observed was an increase in absolute liver weight in males at the highest dose (500 mg/kg feed). No treatment-related changes were noted in gross pathological or histopathological examinations.

The NOAEL from this study was identified as 100 mg/kg feed (equal to 3.2 mg/kg bw day), based on the liver weight change in males at 500 mg/kg feed (equal to 17.3 mg/kg bw per day) (Sachsse et al., 1986).

The Committee identified an overall NOAEL of 100 mg/kg feed (equal to 4.4 mg/kg bw per day) from the three short-term studies in dogs, based on the findings of adverse effects in liver at a dose of 500 mg/kg feed (equal to 17.3 mg/kg bw per day).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Mice

In a GLP-compliant carcinogenicity study, teflubenzuron (purity 92.4%) was administered in the diet to groups of NMRI mice (60 of each sex per group) at a concentration of 0, 15, 75 or 375 mg/kg feed (equal to 0, 2.1, 10.5 and 53.6 mg/kg bw per day for males and 0, 3.1, 15.4 and 71.7 mg/kg bw per day for females, respectively) for 78 weeks (18 months). Ten animals of each sex in each group were killed at 12 months (interim kill), and the remainder were killed at 18 months (terminal kill). Animals were observed for clinical signs of toxicity, and feed consumption and body weights were recorded regularly. Ophthalmoscopic examinations, haematology and clinical biochemistry were evaluated at prespecified intervals. Animals that died prior to or on their scheduled killing dates were subjected to gross pathological and histopathological examinations.

There were no treatment-related signs of toxicity or changes in ophthalmoscopic or haematological parameters. Mortality in high-dose males (10%) was lower than that in the control (33%) and the low-dose (27%) males. The mortality was considered to be within biological variation and not treatment related. Body weight gain was reduced (9% for interim sacrifice group and 5% for terminal kill group) in males at the high dose. Compared with controls, in male mice treated at the high dose, activities of aspartate transaminase, alanine transaminase and ornithine transcarbamylase were elevated at both weeks 52 and 78, whereas lactate dehydrogenase and alkaline phosphatase activities were elevated only at week 52. In females treated at this dose, only the alanine

transaminase activity was elevated significantly. Slight, but non-significant, increases in the activities of alanine transaminase and ornithine transcarbamylase were noted in males treated at the middle dose at week 78 and in females treated at the high dose in both weeks 52 and 78, respectively.

At interim and terminal kill necropsy, the absolute and relative liver weights were higher in both sexes of the high-dose group. At interim kill, relative liver weight was slightly increased for males at the middle dose. Macroscopic examinations revealed an increased incidence of hepatic nodules (30% versus 16.7% in controls) in males at the high dose. Histopathological investigations in terminal kill animals indicated an increased incidence of hepatocellular adenomas in males at the middle and high doses. The incidence of hepatocellular adenoma was 12% in controls, 10% at the low dose, 22% at the middle dose and 32% at the high dose. The incidence of hepatocellular adenoma recorded among controls in this study (12%) was similar to the mean incidence of 10.9% (range 4.5–16.4%) recorded in male NMRI mice used in four long-term studies (concurrent historical controls) in the same laboratory. Therefore, the incidence of hepatocellular adenoma in both the mid- and high-dose groups was higher than the highest range observed in the historical controls. No hepatocellular carcinoma was identified in females. Among the males, the incidence of hepatocellular carcinoma was not affected by the treatment, being 3/50, 5/50, 4/50 and 5/50 in the control, low-dose, mid-dose and high-dose groups, respectively.

Several treatment-related, dose-dependent non-neoplastic hepatic changes were reported in animals of both sexes at both the interim and terminal kills. They included hepatocellular hypertrophy in combination with single-cell necrosis, diffuse Kupffer cell proliferation, phagocytic cell foci, lipofuscin accumulation and patchy glycogen storage. These changes were more pronounced in males than in females; significant changes were detected in females only in the high-dose group. The proportions of male mice with dose-dependent non-neoplastic hepatic lesions in the control, low-, mid- and high-dose groups, respectively, were as follows: hepatocellular hypertrophy – 12/60, 29/60, 46/60 and 56/60; single-cell necrosis – 13/60, 26/60, 42/60 and 56/60; phagocytic cell foci – 17/60, 21/60, 43/60 and 54/60; and lipofuscin accumulation – 8/60, 11/60, 20/60 and 27/60. In addition, in the mid- and high-dose males, an increased incidence of nodular hepatic hyperplasia was observed at terminal kill. A slight bile duct proliferation was observed in males at the middle (3/50) and high (10/50) doses, with a concomitant decrease in normal centrilobular fat storage. In the high-dose females, patchy fatty change was noted in the liver.

In the low-dose males, although the incidence of non-neoplastic hepatic lesions was significantly increased, the severity of these lesions was comparable with that observed in the controls. For example, the severity grades in low-dose and control males were, respectively, 2.3 and 2.1 for hepatocellular hypertrophy

and 1.9 and 1.9 for single-cell necrosis. The sponsor considered non-neoplastic hepatic changes observed in the low-dose group to be common background findings in mice of this age and strain and proposed the lowest dose (15 mg/kg feed, equal to 2.1 mg/kg bw per day) as the NOAEL (Suter et al., 1987b).

The non-neoplastic lesions (based on the re-evaluation of the liver sections) from male mice in the terminal kill group from the carcinogenicity study reported above were analysed using a linear extrapolation approach, and a point of departure of 1 mg teflubenzuron per kilogram feed was proposed (Tennekes, 1989). However, given that better dose–response modelling approaches are currently available, the Committee did not agree with the modelling approach.

Histopathological sections of liver from male mice in the above study were subjected to re-evaluation by an independent pathologist. In total, seven slides (one routine and six additional slides) from each liver were examined for nodular liver lesions: hepatocellular hyperplastic nodules, adenomas and carcinomas, based on predefined and more detailed diagnostic criteria. The pathologist identified hepatocellular hyperplastic nodules in 2/60, 0/60, 6/60 and 12/60 animals, hepatocellular adenomas in 8/60, 5/60, 13/60 and 13/60 animals, and hepatocellular carcinoma in 2/60, 6/60, 3/60 and 3/60 animals in the control, low-, mid- and high-dose groups, respectively. Based on these data, a dose-related increase in the incidence of hepatocellular hyperplastic nodules and a slight but statistically non-significant increase in the hepatocellular adenomas were proposed. The pathologist's conclusion was that whereas teflubenzuron increased the incidence of non-neoplastic hepatocellular nodules at the high dose (375 mg/kg feed), it did not manifest either the tumorigenic effect (enhancement of hepatocellular adenomas) or carcinogenic effect (enhancement of hepatocellular carcinomas) (Vesselinovitch, 1988).

Considering all available information, the Committee concluded that teflubenzuron induced hyperplastic proliferation in liver of mice by an unknown mechanism and considered the non-neoplastic lesions observed in the low-dose group as adverse. The lowest dietary concentration of 15 mg/kg feed (equal to 2.1 mg/kg bw per day) was identified as the LOAEL.

In the absence of a NOAEL, to better characterize the point of departure, the Committee conducted a dose–response analysis of these data using the benchmark dose (BMD) approach (see section 2.2.7 below). The Committee considered the lower confidence limit on the benchmark dose for a 10% response over the controls ($BMDL_{10}$) of 0.54 mg/kg bw per day for the BMD of 0.73 mg/kg bw per day, estimated by the multistage model for hepatocellular hypertrophy, the most toxicologically relevant effect, as the most appropriate point of departure for this study.

(b) **Rats**

In a GLP-compliant chronic toxicity and carcinogenicity study, teflubenzuron (purity 92.4%) was administered to groups of Wistar (KFM-Han) rats (70 of each sex per group) for a period of 120 weeks at a dietary concentration of 0, 20, 100 or 500 mg/kg feed (equal to 0, 1.0, 4.8 and 24.8 mg/kg bw per day for males and 0, 1.2, 5.9 and 29.9 mg/kg bw per day for females, respectively). Ten rats of each sex were killed at week 53 (interim kill 1) and 107 (interim kill 2), and the remainder were killed at week 120 (terminal kill). Animals were observed for clinical signs of toxicity, and feed consumption and body weight were recorded regularly. Ophthalmoscopic examinations, a hearing test, haematology, urine analysis and clinical biochemistry were evaluated at prespecified intervals. Animals that died prior to or on their scheduled killing dates were subjected to gross pathological and histopathological examinations.

There was no effect of treatment on mortality; it ranged in different groups from 5% to 20% among the animals killed at weeks 53 and 107 and from 40% to 50% among the animals killed at week 120. There were no signs of toxicity or treatment-related effects on feed consumption or on ophthalmoscopic, hearing, haematological or urine analysis parameters. The body weight gain in males at the middle and high doses was slightly, but non-significantly, reduced.

Clinical biochemistry revealed increased activities (1.5- to 3-fold) of aspartate and alanine transaminases at weeks 14, 26, 53 and 78, of alanine transaminase at week 120 and of ornithine transcarbamylase at weeks 53 and 78 in males treated at the high dose.

At terminal kill, absolute and relative liver weights were increased in males at the high dose. No treatment-related changes were noted in gross pathology. Several non-neoplastic microscopic changes were noted in different organs, mainly the endocrine and large parenchymatous organs. However, they were not treatment related. The only statistically significant increases noted in the trend analysis of the incidence of neoplastic lesions were haemangiomas in mesenteric lymph nodes in males treated at the high dose (8/47 versus 1/48 controls) and pancreatic exocrine carcinoma in males at the same dose (2/47 versus 0/50 in controls). However, when compared with the historical controls, the incidence of this neoplasm in the high-dose males was not significantly different. Also, the number of animals with pancreatic exocrine carcinoma was too small to allow a meaningful conclusion to be drawn (Suter et al., 1987a). It was noted that the study used a rat strain prone to a high incidence of spontaneous neoplasms in a number of organs. For example, among the controls, 40% of females had mammary gland fibroadenoma, 35% of male rats and 78% of female rats had pituitary adenoma, and 18% of male rats and 12% of female rats had various skin neoplasms. The

Committee considered that high rates of spontaneous neoplasms in control rats significantly reduced the sensitivity of the study to identify carcinogenic effects.

Based on the effect on liver enzymes and liver weight at 500 mg/kg feed (equal to 24.8 mg/kg bw per day), 100 mg/kg feed (equal to 4.8 mg/kg bw per day) was identified as the NOAEL from this study (Suter et al., 1987a).

In a supplemental GLP-compliant carcinogenicity study, groups of Wistar (KFM-Han) rats (60 of each sex per group) were administered teflubenzuron (purity 92.4%) at a concentration of 0, 2500 or 10 000 mg/kg feed (equal to 0, 123 and 487 mg/kg bw per day for males and 0, 154 and 615 mg/kg bw per day for females, respectively) for a period of 111 weeks. Ten rats of each sex were killed at week 104 (interim kill), and the remainder were killed at the end of the study. Animals were observed for clinical signs of toxicity, and feed consumption and body weight were recorded regularly. Haematology, urine analysis and clinical biochemistry were evaluated at prespecified intervals. Animals were subjected to gross pathological and histopathological examinations.

There were no signs of toxicity or treatment-related changes in feed consumption. Mortality was significantly reduced in the high-dose males (28%) compared with the controls. There was no treatment-related difference in mortality (39–44%) among females or between the low-dose and control males. However, a non-significant reduction in body weight gain was noted in females at both the low (6%) and high (7.5%) doses. No treatment-related changes were observed in haematology or urine analysis parameters. Clinical biochemistry revealed increased activities of alanine transaminase and aspartate transaminase in males at both treatment doses. Aspartate transaminase activity was also elevated in females at the high dose.

At interim kill, compared with controls, the absolute and relative liver and kidney weights were increased in males treated at the high dose. Females in the same group also had a slight, but statistically non-significant, increase in absolute and relative liver weights. At terminal kill, absolute and relative liver weights were increased in the high-dose males. On gross pathology, dose-dependent increases in the incidence of diffuse, clay-coloured discoloration and focal and multifocal discoloration of the liver were observed in treated males, the incidences being higher at the high dose than at the low dose. Treatment-related non-neoplastic microscopic changes were noted in the liver of both sexes at both doses tested. These lesions were more frequent and more severe in males than in females. They included increased incidence of fatty changes and centrilobular hepatocellular hypertrophy in both sexes at both dose levels, increased incidence of mixed cell and basophilic cell foci in males at both dose levels, increased incidence of focal hepatocellular hyperplasia and spongiosis hepatis in males at the high dose and increased incidence of basophilic cell foci in females at the high dose (Tennekes et

al., 1989). The incidences of mesenteric lymph node haemangioma and pancreatic exocrine carcinoma in males, which were identified in the previous study, were not different between the treated rats and untreated controls in this study. As in the previous study, some of the experimental animals (including controls) had a high incidence of spontaneous neoplasms in various organs, which were not treatment related, but likely reduced the sensitivity of the test.

A NOAEL could not be identified from this study because of elevated activities of liver enzymes, macroscopic liver findings and non-neoplastic microscopic hepatic changes in treated rats at both dose levels.

2.2.4 Genotoxicity

To examine the genotoxic potential of teflubenzuron, four in vitro and two in vivo studies were conducted. The results of these studies are summarized in Table 3. All studies were conducted under GLP-compliant conditions, except the reverse mutation study in *Salmonella typhimurium*. The latter, however, was audited for quality assurance.

Table 3

Results of genotoxicity assays on teflubenzuron

Test system	Test object	Concentration/dose	Results	Reference
Bacterial reverse mutation (Ames test)	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538	125, 250, 500, 1 250, 2 500 and 5 000 µg/plate	Negative[a]	Kramer (1982)
Point mutation (*hgprt* locus) assay	Chinese hamster V79 cells	5, 10, 25 and 50 µg/mL	Negative[a]	Heidemann (1986)
Chromosomal aberration assay	Chinese hamster V79 cells	4, 25 and 50 µg/mL	Negative[a]	Heidemann (1985)
Unscheduled DNA synthesis	Wistar CF HB male rat hepatocytes	1, 3.3, 10, 33.3 and 100 µg/mL	Negative	Müller (1986)
In vivo mouse micronucleus assay	NMRI mice (both sexes)	5 000 mg/kg bw	Negative	Guenard (1984)
In vivo DNA covalent binding assay	NMRI male mice	40 mg/kg bw	Negative	Kugler-Steigmeier, Lutz & Schlatter (1988)

bw: body weight; DNA: deoxyribonucleic acid; hgprt: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction from rat liver homogenate
[a] In the presence and absence of metabolic activation (S9).

The potential of teflubenzuron (purity not reported) to induce reverse mutation was tested in vitro in five histidine-dependent strains of *Salmonella typhimurium* – namely, TA100, TA1535 (base mutation–sensitive strains), TA98, TA1537 and TA1538 (frameshift mutation–sensitive strains). A direct plate incorporation method was used to test six concentrations (125, 250, 500, 1250, 2500 and 5000 µg/plate) of the test substance in four replicate plates with or without a metabolic activation system (S9) and with appropriate negative and positive controls. The test substance was dissolved in DMSO. Higher concentrations

(1250–5000 µg/plate) of teflubenzuron precipitated in the top agar layer. Under the conditions of the test, there was no evidence that teflubenzuron induced reverse mutations in the test strains of *S. typhimurium* in both the presence and absence of S9 (Kramer, 1982).

The potential of teflubenzuron to induce a point mutation (*hgprt* locus) in Chinese hamster V79 cells was investigated in vitro in two independent experiments. The cells were exposed to the test substance (purity 92.4%) dissolved in DMSO for 4 hours at a final concentration of 5, 10, 25 or 50 µg/mL in either the presence or absence of S9. Appropriate negative and positive controls were used. The positive control used for testing without metabolic activation was ethylmethanesulfonate, and that for testing with metabolic activation was 9,10-dimethyl-1,2-benzanthracene. Concentrations of teflubenzuron higher than 50 µg/mL precipitated in the medium. At all concentrations of the test substance, there was no increase in mutation rate in teflubenzuron-treated cells when compared with negative controls in either the presence or absence of S9. The sensitivity of the test system was demonstrated by the enhanced mutation rates found in cells treated with the positive controls. Under the test conditions used, there was no evidence that teflubenzuron induces point mutations in the *hgprt* locus in Chinese hamster V79 cells (Heidemann, 1986).

Chromosomal aberration rates were determined in Chinese hamster V79 cells treated with teflubenzuron (purity 92.4%) solubilized in DMSO at a final concentration of 4, 25 or 50 µg/mL of medium for 4 hours. Experiments were conducted with or without S9. Appropriate negative and positive controls were used. The positive control used for testing without metabolic activation was ethylmethanesulfonate, and that for testing with metabolic activation was cyclophosphamide. Structural aberrations recorded included gaps, breaks, exchanges and partial or total disintegrations of chromosomes. At all concentrations of the test substance, there was no increase in chromosomal aberration rate in teflubenzuron-treated cells when compared with negative controls in either the presence or absence of S9. The sensitivity of the test system was demonstrated by the enhanced chromosomal aberration rates in cells treated with positive controls. Under the test conditions used, there was no evidence that teflubenzuron induces chromosomal aberrations (Heidemann, 1985).

The ability of teflubenzuron (purity 92.4%) to induce unscheduled DNA synthesis in primary rat hepatocytes (male Wistar rats) in vitro was evaluated. Five concentrations of teflubenzuron (1, 3.3, 10, 33.3 or 100 µg/mL) dissolved in acetone were tested. The test system included both negative and positive (7,12-dimethylbenz(*a*)anthracene) controls. Rat hepatocytes were incubated at 37 °C in the culture medium for 1 hour followed by treatment with the test or control substance for 3 hours in the presence of [³H]thymidine and incubated further for an additional 3 hours. The extracted DNA was analysed for [³H]-

thymidine incorporation by liquid scintillation counting. There was no increased incorporation of radioactivity in test groups when compared with negative controls, although a 2.1-fold increase was noted in the positive control. Under the conditions of the assay, there was no evidence that teflubenzuron induced unscheduled DNA synthesis in rat hepatocytes (Müller, 1986).

The mutagenic potential of teflubenzuron (purity 96.5%) was tested in an in vivo mouse micronucleus assay. Groups of NMRI mice (18 of each sex per treatment) were administered, by gavage, teflubenzuron in 2% carboxymethyl cellulose at 5000 mg/kg bw, the vehicle (negative control) or cyclophosphamide in 0.9% saline solution at 50 mg/kg bw (positive control). Six mice of each sex per group were killed at 24, 48 and 72 hours post-treatment, and bone marrow was collected and analysed. At all time points examined, no significant treatment-related increase in micronucleated polychromatic erythrocytes was observed in mice of either sex treated with teflubenzuron. In contrast, increased micronucleated cells were observed in mice treated with the positive control. Thus, teflubenzuron was negative in the in vivo mouse micronucleus assay (Guenard, 1984).

To investigate whether the tumorigenic activity of teflubenzuron in liver was associated with its potential to covalently bind to DNA, an in vivo study was conducted in male NMRI mice. Six mice (two groups of three each) were dosed by gavage with either aniline ring–labelled [^{14}C]teflubenzuron (radiochemical purity >98%) or benzoyl ring–labelled [^{14}C]teflubenzuron (radiochemical purity >99%) at a nominal dose of 40 mg/kg bw. Three mice were used as the negative controls. Mice were killed 24 hours post-treatment, and DNA was purified from the liver. Radioactivity in purified liver DNA from the treated animals was below the limit of detection, with a calculated upper limit of covalent binding index of 0.1, suggesting that teflubenzuron did not form DNA adducts in this in vivo test (Kugler-Steigmeier, Lutz & Schlatter, 1988).

2.2.5 Reproductive and developmental toxicity

(a) Multigeneration reproductive toxicity

Groups of Sprague-Dawley rats (25 of each sex per group) were fed a diet containing teflubenzuron (purity 92.4%) at a concentration of 0, 20, 100 or 500 mg/kg feed (for males, equal to 0, 1.5, 7.4 and 36.9 mg/kg bw per day in the parental generation and 0, 1.9, 9.6 and 48.2 mg/kg bw per day in the F$_1$ generation; for females, equal to 0, 1.6–3.6, 7.9–18.5 and 39.5–89.3 mg/kg bw per day [the range represents mean drug intakes for the parental and F$_1$ generations prior to mating, during gestation and during lactation], respectively) starting at the age of 6–7 weeks. At 10 weeks of age, a male and a female (parental generation) of the same dose group were housed together until a successful mating or for a maximum

of 14 days. Females were allowed to litter and rear pups to weaning (21 days), when the parents were killed for necropsy. From each litter, one or two pups (F_1 generation) of each sex were selected (minimum 25 of each sex per group) for further breeding (F_1 parent), and the rest of the pups were necropsied at weaning. Selected F_1 parents continued to receive the same teflubenzuron-medicated diet as their dams and were individually mated at the age of 12 weeks. As the parental generation, F_1 parents also littered and reared their pups until weaning, when both the parents and their pups were necropsied. All breeding rats and each litter born to both generations were observed daily for signs of toxicity. Other reproductive and general health parameters recorded were body weight, feed consumption, mating performance, fertility rate, duration of gestation, pinna unfolding, generalized hair growth, tooth eruption, eye opening, litter size, pup weight, pup mortality and sex ratio. Additionally, F_1 pups underwent functional tests, such as pupillary reflex, startle response and ability to learn the use of water maze. Histopathological examinations were limited to sex organs of parents of both generations receiving teflubenzuron at concentrations of 0 and 500 mg/kg feed.

The sponsor's study report suggested that there was no evidence of toxicity and no effects of teflubenzuron on reproductive performance (Osterburg, 1989). Closer examination of the necropsy findings in F_1 generation pups revealed a dose-related increase in the incidence of unilateral and bilateral dilatation of the renal pelvis; the incidences were 0.9% (2/214) in controls, 1.9% (5/262) at the low dose, 3.3% (7/215) at the middle dose and 6.8% (15/221) at the high dose, with the incidence at the high dose being statistically different from the control incidence. No such effect was seen in the F_2 generation. No other signs of maternal or offspring toxicity were observed.

The NOAEL for offspring toxicity was 100 mg/kg feed (equal to 7.4 mg/kg bw per day), based on the increased incidence of renal pelvic dilatation seen at 500 mg/kg feed (equal to 36.9 mg/kg bw per day). The NOAEL for parental and reproductive toxicity was 500 mg/kg feed (equal to 36.9 mg/kg bw per day), the highest dose tested.

(b) Developmental toxicity

(i) Rats

In a GLP-compliant study, teflubenzuron (purity 92.4%) in 0.5% aqueous carboxymethyl cellulose was administered by gavage at a dose of 0, 10, 50 or 250 mg/kg bw per day to groups of pregnant Wistar rats (25 per group) from day 6 to day 15 of gestation following natural mating. Treated rats were killed on day 20 of gestation, and fetuses were removed by caesarean section and examined for developmental abnormalities.

There were no deaths or adverse clinical findings in dams, and feed consumption and body weight were not affected by treatment. The number of corpora lutea and implantation rate were similar between the groups. At necropsy, there were no dead fetuses and no treatment-related organ or skeletal malformations. The proportion of dams having spontaneous abortion (compared with total bred or pregnant), implantation rates, number of resorptions and number of congenital runts were not different between the treatment groups, although the absolute number of rats (n = 4) with complete abortion was higher in the high-dose group. There was a dose-related decrease in the absolute number of live pups: 240, 236, 223 and 208 in the 0, 10, 50 and 250 mg/kg bw per day dose groups, respectively. Consequently, the number of live pups per dam was significantly reduced in the high-dose group when compared with the controls.

Under the test conditions described, teflubenzuron had weak embryo/fetal toxicity. Therefore, from this study, a NOAEL of 50 mg/kg bw per day was identified for embryo/fetal toxicity, based on a reduction in the number of live pups per dam at 250 mg/kg bw per day. The NOAEL for maternal toxicity was 250 mg/kg bw per day, the highest dose tested (Gleich, Weisse & Unkelbach, 1986).

Teflubenzuron (purity 92.1%) in 0.5% aqueous carboxymethyl cellulose was administered by gavage to groups of 25 pregnant Wistar rats at 0, 100, 300 or 1000 mg/kg bw per day during days 7–17 of gestation, and the effect on dams and fetuses was observed. The highest dose tested was the upper "limit" recommended according to the Japanese standard guidelines followed in the study. Treated animals were killed on day 20 of gestation.

In dams, no treatment-related changes were noted in general condition, body weight, feed consumption or necropsy. No treatment-related changes were observed in conditions of pregnancy, including number of corpora lutea, number of implantations, proportions of dead or resorbed fetuses, number of live fetuses and placental weight. No difference was noted in sex ratio or body weight of live fetuses, and no external abnormalities were observed. Additionally, no visceral or skeletal abnormalities were observed in fetuses.

The NOAEL for both maternal and embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Ishida et al., 1987).

(ii) Rabbits

Groups of naturally mated and considered pregnant Himalayan rabbits (15 per group) were gavage dosed with teflubenzuron (purity 92.4%) in 0.5% carboxymethyl cellulose at 0, 10, 50 or 250 mg/kg bw per day from day 6 to day 18 of gestation under GLP-compliant conditions. Clinical observations, body weight and feed consumption were recorded. On day 29 of gestation, animals were

killed and examined macroscopically, and the ovaries and uterus were removed. The numbers of corpora lutea, implantations, live and dead fetuses, resorptions and congenital runts, fetal weights and fetal sex were recorded. All live fetuses were observed for a 24-hour period in an incubator to determine early viability. Subsequently, all fetuses were examined for malformations by X-ray and given a macroscopic as well as microscopic examination.

There were no signs of maternal toxicity at any dose, and none of the rabbits died during the study. The frequency distribution of corpora lutea, implantations and live fetuses per dam as well as the early and late resorption rates did not differ between the groups. There were no treatment-related effects on abortions, fetal deaths, fetal weight, sex ratio, number of congenital runts, and visceral or skeletal malformations. The only significant effect noted was decreased survival of offspring during the first 24 hours in the low-dose (94%) and high-dose (88.5%) groups compared with the controls (100%). However, the survival of offspring in the low-dose group within the first 24 hours was not different from survival in historical controls (94.9%).

Whereas the study author concluded that the compound was not embryotoxic, maternally toxic or teratogenic at the highest dose tested (Gleich et al., 1985), the Committee considered it to exhibit slight embryo/fetal toxicity at the highest dose tested (based on decreased survival of offspring in the first 24 hours after birth) and identified the middle dose of 50 mg/kg bw per day as the NOAEL for embryo/fetal toxicity. The NOAEL for maternal toxicity was 250 mg/kg bw per day, the highest dose tested.

To further elucidate whether teflubenzuron has an embryotoxic effect in rabbits, a non-GLP-compliant supplementary study was conducted. Groups of female Himalayan rabbits (five per group) considered to be pregnant after natural mating were gavaged with teflubenzuron (purity not reported) in 0.5% carboxymethyl cellulose at 0, 250 or 500 mg/kg bw per day (two groups at the high dose) on days 6–18 of gestation. All animals, except the second group at the high dose, were killed on day 19, and their livers were removed, weighed, homogenized and assayed for cytochrome P450 and *O*- and *N*-demethylase activities. Additionally, the numbers of corpora lutea, live embryos and resorptions were recorded. The remaining animals of the high-dose group were killed on day 29 of gestation, and their livers were examined as described above. Fetuses were examined for skeletal and visceral malformations as described in the previous study.

The only changes noted were a few early resorptions and a reduced total number of living embryos in treated animals. The study author considered teflubenzuron not to be embryotoxic or teratogenic in rabbits, as the observed

effects could be a chance finding due to the small number of animals used (Gleich, 1985). A closer analysis of the data, however, suggested a treatment-related increase in the number of early resorptions (0 in control versus 3 and 4 in the 250 and 500 mg/kg bw per day groups, respectively) and a decrease in the number of live fetuses (32 in controls versus 20 in each treatment group), showing a potential embryo/fetal toxic effect of teflubenzuron. The Committee, however, considered the findings of this study to be of limited significance for risk assessment.

In a GLP-compliant study, 22 pregnant New Zealand White rabbits were dosed by gavage with teflubenzuron (purity 92.4%) in 0.5% carboxymethyl cellulose at 1000 mg/kg bw per day during days 6–18 of pregnancy, and the effects of teflubenzuron on maternal and fetal health were compared with the effects in 16 controls. Rabbits were killed on gestation day 28 and examined for gross pathology and reproductive performance, including effects on developing fetuses.

No treatment-related changes in the behaviour or clinical conditions of the dams were noted. Treated rabbits had a higher incidence of macroscopic liver lesions – grossly granulated cut surface (8/22 in treated rabbits versus 1/16 in controls). Body weight, feed consumption and number, weight or sex of fetuses as well as preimplantation or postimplantation losses were not affected by treatment. There were no treatment-related developmental abnormalities.

In view of the observed liver lesions, no NOAEL could be identified for maternal toxicity, but the only dose tested (1000 mg/kg bw per day) was identified as a NOAEL for embryo/fetal toxicity in rabbits (Osterburg, 1987). However, this study did not evaluate the offspring survival during the first 24 hours, and hence the Committee cannot discount the effects seen in the previous study.

2.2.6 Special studies

(a) Haematological toxicity

The influence on haematological parameters, including the potential to form methaemoglobin, of five benzoyl urea insecticides, including teflubenzuron, was investigated in groups of 10 adult Wistar rats (sex and GLP compliance not specified). Ten rats served as controls. Animals received aqueous solutions of the active ingredient by gavage at 100 mg/kg bw per day for 28 days, and blood samples were collected at the end of the study.

No overt clinical signs of toxicity were observed during treatment. Compared with controls, teflubenzuron did not cause any changes in haemoglobin, haematocrit, red blood cell count, mean corpuscular volume or mean corpuscular haemoglobin concentration. Reticulocyte count was increased in all treated rats. Methaemoglobin levels were elevated in rats treated with diflubenzuron

and triflumuron, but not in those treated with flufenoxuron, hexaflumuron or teflubenzuron. Red blood cells and the haemoglobin concentrations in blood were not affected in rats treated with teflubenzuron when compared with controls (Tasheva & Hristeva, 1993).

(b) Effects on mammalian cells in vitro

The effects of teflubenzuron and other pesticides on the human intestinal Caco-2 cell line were investigated in vitro in a cell culture model. Among others, the effects of pesticides on cell viability, morphological changes, cell monolayer integrity, alkaline phosphatase activity, pesticide-induced oxidative stress and pesticide-induced cell death mechanism were investigated. The results indicated that various adverse effects could be observed on the in vitro cell culture at teflubenzuron concentrations of 5 μmol/L and above (Ilboudo et al., 2014). However, the Committee considered that the findings of this study were of limited relevance to the risk assessment of teflubenzuron as a veterinary drug.

(c) Microbiological effects

Considering the chemical structure and mode of action of teflubenzuron, the Committee did not anticipate any adverse effects of teflubenzuron residues on human gastrointestinal microbiota.

2.2.7 Dose–response analysis

The Committee considered that of the non-neoplastic changes observed in the liver of male mice in the carcinogenicity study (Suter et al., 1987b), hepatocellular hypertrophy was the most relevant end-point for the dose–response analysis. To better characterize the point of departure, the Committee conducted a dose–response analysis of these data using the United States Environmental Protection Agency's Benchmark Dose (BMD) Software. The BMD and a lower confidence limit on the BMD for a 10% response over the controls (BMD_{10} and $BMDL_{10}$, respectively) for hepatocellular hypertrophy were determined using nine different dichotomous models. A summary of these analyses is presented in Table 4.

The LogLogistic, LogProbit and Multistage models provided acceptable fits based on statistical considerations. However, the BMD_{10} and $BMDL_{10}$ estimated by the LogLogistic and LogProbit models were much lower than the lowest dose used in the study. Furthermore, the Multistage model provided a better fit of the BMDL value for the benchmark response at the low end of the observed range of the data (Fig. 3). Therefore, the Committee considered the $BMDL_{10}$ of 0.54 mg/kg bw per day for the BMD_{10} of 0.73 mg/kg bw per day estimated by the Multistage model as the most appropriate point of departure for this study.

Table 4

BMD$_{10}$ and BMDL$_{10}$ for hepatocellular hypertrophy[a] in male mice in the carcinogenicity study

Model	BMD$_{10}$ (mg/kg bw per day)	BMDL$_{10}$ (mg/kg bw per day)	P-value	AIC	Scaled residual for	
					Treatment	Control
Gamma	1.66	1.22	0.000 8	254.8	1.362	−1.861
Logistic	3.4	2.6	0	263.8	0.798	−3.089
LogLogistic	0.37	0.082	0.997	243.7	0	0
LogProbit	0.38	0.086	0.850	243.8	0.012	0.012
Multistage	0.73	0.54	0.248	245.1	−0.45	−0.45
Multistage-Cancer	1.7	1.2	0.000 8	254.8	1.362	−1.861
Probit	4.0	3.2	0	265.9	0.714	−3.258
Weibull	1.7	1.2	0.000 8	254.8	1.362	−1.861
Quantal-Linear	1.7	1.2	0.000 8	254.8	1.362	−1.861

AIC: Akaike information criterion; BMD$_{10}$: benchmark dose for a 10% response; BMDL$_{10}$: lower 95% confidence limit on the benchmark dose for a 10% response
[a] Proportion affected: 12/60, 29/60, 46/60 and 56/60 at control, low, middle and high doses, respectively.

2.3 Observations in humans

No information was available on the toxicity of teflubenzuron in humans.

3. Comments

The Committee considered data on the pharmacokinetics, short- and long-term toxicity, reproductive and developmental toxicity, genotoxicity and carcinogenicity of teflubenzuron. In addition to a sponsor's submission, relevant studies retrieved from the published literature were evaluated. Most studies submitted by the sponsor were conducted under GLP-compliant conditions. Those that were not conducted under GLP-compliant conditions are identified below.

3.1 Biochemical data

Orally administered teflubenzuron was only partially, but relatively quickly, absorbed in rats. Following administration of a single oral dose of radiolabelled teflubenzuron at 25 mg/kg bw, approximately 20% of the radioactivity was absorbed; only 4% was absorbed when rats were dosed at 750 mg/kg bw, suggesting a dose-dependent absorption (Schlüter, 1986; Hawkins & Mayo, 1988). Peak plasma concentrations were reached within 1–2 hours post-dosing and were maintained at similar levels for up to 8 hours (low dose) or 24 hours (high dose) (Schlüter, 1986). In repeatedly dosed animals, there was some evidence of a dose-dependent plateau in plasma concentration (Ellgehausen et al., 1986).

Fig. 3
Benchmark dose–response curve for hepatocellular hypertrophy in mice for 10% extra risk for the BMD and BMDL: A. LogLogistic model, B. LogProbit model, and C. Multistage model
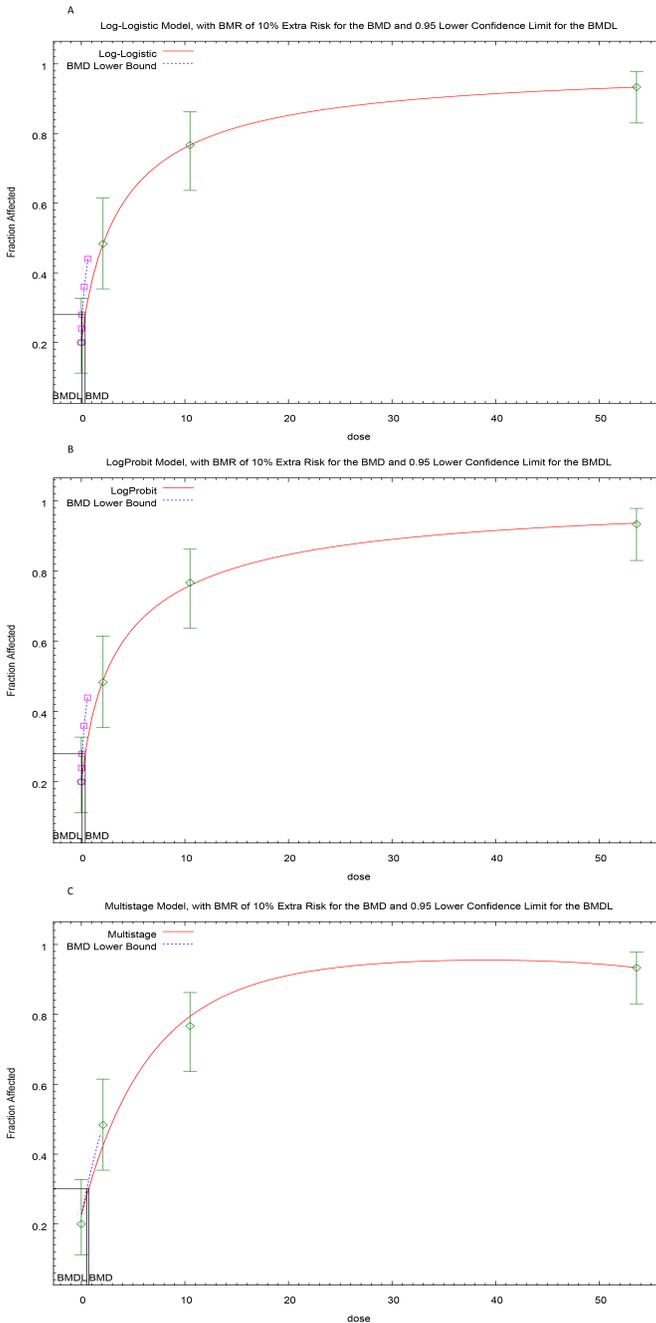
Most (90–95%) radiolabelled teflubenzuron administered by gavage to rats (single dose at 25 or 750 mg/kg bw or 14 daily doses of 25 mg/kg bw of unlabelled drug followed by a single dose of 25 mg/kg bw radiolabelled drug) was excreted in faeces, primarily as the parent compound. More than 85% of the drug was excreted within the first 24 hours of the dosing. Only a small fraction (0.15–3%) of the total oral dose of teflubenzuron was excreted in the urine. There was no difference in excretion pattern between sexes or between animals dosed with a single or multiple doses of the drug (Schlüter, 1984, 1986; Hawkins & Mayo, 1988). Absorbed teflubenzuron was mostly excreted through bile, predominantly as polar materials. Only negligible residues of teflubenzuron were detected in tissues and organs (<2% of the dose), with no evidence of accumulation (Schlüter, 1984, 1986; Hawkins & Mayo, 1988).

Metabolites identified in bile and urine were benzoyl or aniline ring hydroxylated teflubenzuron and conjugates of (3,5-dichloro-2,4-difluorophenyl)-urea and 3,5-dichloro-2,4-difluoroaniline (Schlüter, 1985; Hawkins & Mayo, 1988). Several polar metabolites were detected in faeces, but the only metabolite characterized was (3,5-dichloro-2,4-difluorophenyl)urea (Schlüter, 1986). Hydrolytic cleavage of the phenylurea bridge was identified as the predominant pathway of teflubenzuron metabolism in a non-GLP-compliant study in which rats were gavaged once with approximately 55 mg/kg bw of the drug. The scission products thus produced were either excreted unmodified or further metabolized and excreted (Koerts et al., 1997).

3.2 Toxicological data

Critical studies relevant to the risk assessment of teflubenzuron are summarized in Table 5. Most of the toxicity studies were conducted under GLP-compliant conditions, and the purity of the substance used, where specified, was greater than 90%.

Teflubenzuron was shown to have low acute toxicity in laboratory animals. The oral LD_{50} in mice and rats was greater than 5000 mg/kg bw (Ullmann, 1983d,e; Ullmann, Sacher & Vogel, 1988). The dermal LD_{50} in rats was greater than 2000 mg/kg bw (Ullmann, 1983b), and the inhalation LC_{50} in rats was greater than 5000 mg/m³ air (Ullmann, 1983a). Teflubenzuron was not irritating to the skin (Ullmann, 1983g) or eyes (Ullmann, 1983f) of rabbits, and it did not cause skin sensitization in the guinea-pig maximization test (Ullmann, 1984).

Short-term toxicity studies of teflubenzuron in which the drug was administered in diet were conducted in mice (one 13-week study), rats (one 13-week study) and dogs (two 13-week studies and one 52-week study). In all three

Table 5

Studies relevant to 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				
Eighteen-month carcinogenicity study (diet)	0, 2.1, 10.5, 53.6	Hepatocellular adenoma	2.1	10.5
		Hepatocellular hypertrophy	–	2.1[a]
			BMDL$_{10}$: 0.54*	–
Rat				
Two-year toxicity and carcinogenicity study (diet)	0, 1.0, 4.8, 24.8	Increased activity of liver enzymes, and increased liver weights in males	4.8[b]	24.8[c]
Two-generation reproductive toxicity study (diet)	0, 1.5, 7.4, 36.9	Unilateral and bilateral dilatation of the renal pelvis in F$_1$ pups	Offspring toxicity: 7.4	36.9
			Parental toxicity: 36.9[d]	–
			Reproductive toxicity: 36.9[d]	–
Developmental toxicity studies[e] (gavage)	Study 1: 0, 10, 50, 250	Decreased number of live pups per dam	Embryo/fetal toxicity: 50 (study 1)	250 (study 1)
	Study 2: 0, 100, 300, 1 000		Maternal toxicity: 1 000[b,d]	–
Rabbit				
Developmental toxicity studies[e] (gavage)	Study 1: 0, 10, 50, 250	Decreased survival of offspring within 24 h of birth	Embryo/fetal toxicity: 50[b]	250[c]
	Study 2: 0, 250, 500		Maternal toxicity: 500[b,d]	–
	Study 3: 0, 1 000			
Dog				
Thirteen-week studies of toxicity[e] (diet)	Study 1: 0, 3.5, 33.7, 318.2	Increased liver weight	4.4[b]	17.3[c]
	Study 2: 0, 1.2, 4.4			
One-year study of toxicity (diet)	0, 1.0, 3.2, 17.3			

* **Pivotal study value for the derivation of an ADI (Suter et al., 1987b)**
[a] Lowest dose tested.
[b] Overall NOAEL.
[c] Overall LOAEL.
[d] Highest dose tested.
[e] Two or more studies combined.

species, liver was identified as the target organ for toxic effects, as evidenced by elevated enzyme activities and/or adaptive cellular changes.

In a study whose GLP-compliant status could not be verified, mice were administered teflubenzuron at a concentration of 0, 100, 1000 or 10 000 mg/kg diet (equal to 0, 12, 115 and 1213 mg/kg bw per day for males and 0, 14, 142

and 1450 mg/kg bw per day for females, respectively) for 13 weeks. In the high-dose group, activities of liver enzymes (alkaline phosphatase in males, alanine transaminase in females) and total cholesterol levels (females) were elevated, and blood glucose levels (both sexes) were altered. In the mid- and high-dose groups, absolute and relative liver weights were increased, with centrilobular hepatocellular swelling (both sexes) and microscopic fatty changes (in males). The NOAEL was identified as 100 mg/kg feed (equal to 12 mg/kg bw per day), based on liver changes at 1000 mg/kg feed (equal to 115 mg/kg bw per day) (Takahashi et al., 1987).

Rats were administered teflubenzuron at a concentration of 0, 100, 1000 or 10 000 mg/kg feed (equal to 0, 8, 82 and 809 mg/kg bw per day for males and 0, 9, 94 and 942 mg/kg bw per day for females, respectively) for 13 weeks. Some animals from the control and high-dose groups were observed for an additional 4 weeks without treatment. Increased activities of several enzymes, notably aspartate transaminase (middle and high doses, both sexes), ornithine transcarbamylase (high dose, both sexes), alanine transaminase (mid- and high-dose males), lactate dehydrogenase (high-dose males) and alkaline phosphatase (all treated males), were observed, which returned to normal levels after 4 weeks of drug withdrawal. At necropsy, increased absolute and relative weights of liver in females and of testes in males were observed at the high dose. The statistically significant change in alkaline phosphatase activity in male rats of the low-dose group was not considered to be biologically relevant, being less than 1.5-fold above the control value and within the normal physiological range. Based on effects on liver enzymes at 1000 mg/kg feed (equal to 82 mg/kg bw per day), a NOAEL of 100 mg/kg feed (equal to 8 mg/kg bw per day) was identified (Suter et al., 1987c).

In a non-GLP-compliant study, the potential of teflubenzuron to form methaemoglobin and exert other effects on haematological parameters in rats was investigated in a comparative study that investigated five benzoylurea insecticides. Although reticulocyte count was increased in rats treated with teflubenzuron (100 mg/kg bw per day for 28 days), it was not associated with anaemia or the formation of methaemoglobin (Tasheva & Hristeva, 1993).

In dogs administered teflubenzuron at a concentration of 0, 100, 1000 or 10 000 mg/kg feed (equal to 0, 3.5, 33.7 and 318.2 mg/kg bw per day for males and 0, 4.0, 42.8 and 417.1 mg/kg bw per day for females, respectively) for 13 weeks, activities of alanine transaminase, aspartate transaminase, alkaline phosphatase and ornithine transcarbamylase were increased in both sexes at the high dose. At necropsy, absolute and relative liver weights were elevated and the incidence of nodular foci in the pyloric or fundic region of the stomach was increased at the high dose. Isolated dark red foci were noted in the pyloric region of the stomach at the middle and high doses. Focal gastritis was observed in females at the middle

and high doses, and follicular hyperplasia of the pyloric mucosa was noted in most animals at the high dose. Mild hepatitis in one male and centrilobular hepatic necrosis in another male were observed at the low dose. Also, moderate chronic active hepatitis was diagnosed in one animal of each sex at the high dose. Given the lack of a clear dose–response relationship for hepatitis, the mild microscopic hepatic changes noted in two dogs in the low-dose group were not considered to be a treatment-related adverse effect. The NOAEL was 100 mg/kg feed (equal to 3.5 mg/kg bw per day), based on stomach lesions at 1000 mg/kg feed (equal to 33.7 mg/kg bw per day) (Bathe et al., 1985).

In a supplementary study to clarify the effects on liver, dogs were administered teflubenzuron at a concentration of 0, 30 or 100 mg/kg feed (equal to 0, 1.2 and 4.4 mg/kg bw per day for males and 0, 1.5 and 5.1 mg/kg bw per day for females, respectively) for 13 weeks. There were no treatment-related adverse effects identified in clinical examination, laboratory testing, and macroscopic or microscopic examination of organs. The NOAEL was 100 mg/kg feed (equal to 4.4 mg/kg bw per day), the highest dose tested (Bathe et al., 1987).

In a 52-week study, dogs were administered teflubenzuron at a concentration of 0, 30, 100 or 500 mg/kg feed (equal to 0, 1.0, 3.2 and 17.3 mg/kg bw per day for males and 0, 1.2, 4.0 and 18.0 mg/kg bw per day for females, respectively). At necropsy, the absolute liver weight in males was increased at the high dose, but no treatment-related changes were noted in gross pathological or histopathological examinations. The NOAEL was 100 mg/kg feed (equal to 3.2 mg/kg bw per day), based on the liver weight change at 500 mg/kg feed (equal to 17.3 mg/kg bw per day) (Sachsse et al., 1986).

The Committee identified an overall NOAEL of 100 mg/kg feed (equal to 4.4 mg/kg bw per day) from the three short-term studies in dogs, based on the findings of adverse effects in liver at a dose of 500 mg/kg feed (equal to 17.3 mg/kg bw per day).

In a carcinogenicity study in mice, teflubenzuron was administered at a concentration of 0, 15, 75 or 375 mg/kg feed (equal to 0, 2.1, 10.5 and 53.6 mg/kg bw per day for males and 0, 3.1, 15.4 and 71.7 mg/kg bw per day for females, respectively) for 78 weeks, with an interim kill at week 52. Aspartate transaminase, alanine transaminase, ornithine transcarbamylase, lactate dehydrogenase and alkaline phosphatase activities were elevated in high-dose males, but only alanine transaminase activity was elevated in high-dose females. Absolute and relative liver weights were higher in both sexes at the high dose, and relative liver weight was slightly increased in the mid-dose males. The incidence of macroscopic hepatic nodules was increased in the high-dose males. Histopathology indicated an increased incidence of hepatocellular adenomas and nodular hepatic hyperplasia in males treated at the middle and high doses compared with both concurrent and historical controls, but there was no difference in the incidence

of hepatic carcinoma. Several treatment-related, dose-dependent non-neoplastic hepatic changes were also observed, which were more pronounced in males than in females. In particular, males in the control, low-dose, mid-dose and high-dose groups, respectively, had dose-dependent incidences of hepatocellular hypertrophy (12/60, 29/60, 46/60 and 56/60), single-cell necrosis (13/60, 26/60, 42/60 and 56/60), phagocytic cell foci (17/60, 21/60, 43/60 and 54/60) and lipofuscin accumulation (8/60, 11/60, 20/60 and 27/60). In the low-dose group, the incidence, but not the severity, of these non-neoplastic hepatic changes was significantly higher when compared with the controls (Suter et al., 1987b).

Histopathological sections of liver from male mice in this study were re-evaluated by an independent pathologist, with a focus on nodular liver lesions. The pathologist concluded that there was a dose-related increase in the incidence of hepatocellular hyperplastic nodules and a slight, but statistically non-significant, increase in hepatocellular adenoma (Vesselinovitch, 1988).

Given that only hepatic adenomas were observed and that the genotoxicity test results were negative (see below), the Committee considered that teflubenzuron was not carcinogenic in mice. However, the Committee concluded that teflubenzuron induced hyperplastic proliferation in liver of mice by an unknown mechanism. Based on the increased incidence of non-neoplastic hepatic changes observed in liver (e.g. hepatocellular hypertrophy, single-cell necrosis, phagocytic cell foci, lipofuscin accumulation) at all doses, no NOAEL could be identified. The lowest dietary concentration, 15 mg/kg feed (equal to 2.1 mg/kg bw per day), was identified as the LOAEL.

In the absence of a NOAEL, to better characterize the point of departure, the Committee conducted a dose–response analysis of these data using the BMD approach. Of several non-neoplastic hepatic changes identified, hepatocellular hypertrophy was considered to be the most toxicologically relevant effect for dose–response modelling. The BMD_{10} and $BMDL_{10}$ were determined using nine different dichotomous models. Three models (LogLogistic, LogProbit and Multistage) provided acceptable fits based on statistical considerations. However, the BMD_{10} and $BMDL_{10}$ estimated by the LogLogistic and LogProbit models were much lower than the lowest dose used in the study. Furthermore, the Multistage model provided a better fit of the BMDL value for the benchmark response at the low end of the observed range of the data. Therefore, the Committee considered the $BMDL_{10}$ of 0.54 mg/kg bw per day for the BMD_{10} of 0.73 mg/kg bw per day estimated by the Multistage model as the most appropriate point of departure for this study.

In a carcinogenicity study, rats were administered teflubenzuron at a concentration of 0, 20, 100 or 500 mg/kg feed (equal to 0, 1.0, 4.8 and 24.8 mg/kg bw per day for males and 0, 1.2, 5.9 and 29.9 mg/kg bw per day for females, respectively) for 120 weeks, with an interim kill at weeks 53 and 107. Mortality

ranged from 40% to 50% at week 120, which was not influenced by treatment. Increased (approximately 1.5- to 3-fold) activities of alanine transaminase, aspartate transaminase and ornithine transcarbamylase were noted in males in the high-dose group. Absolute and relative liver weights were increased in high-dose males. Several non-neoplastic microscopic changes were noted in different organs, but were not treatment related. Trend analysis identified increased incidences of haemangiomas in mesenteric lymph nodes and pancreatic exocrine carcinoma in the high-dose males. However, they were not significantly different when compared with historical controls. Also, the occurrence of pancreatic exocrine carcinoma was too infrequent (2/47 versus 0/50) to allow a meaningful comparison to be drawn. Based on the effect on liver enzymes and liver weight at 500 mg/kg feed (equal to 24.8 mg/kg bw per day), the NOAEL was 100 mg/kg feed (equal to 4.8 mg/kg bw per day) (Suter et al., 1987a).

In a supplemental carcinogenicity study, rats were administered teflubenzuron at a concentration of 0, 2500 or 10 000 mg/kg feed (equal to 0, 123 and 487 mg/kg bw per day for males and 0, 154 and 615 mg/kg bw per day for females, respectively) for 111 weeks, with an interim kill at week 104. Clinical biochemistry revealed increased activities of alanine transaminase and aspartate transaminase in males at both doses and of aspartate transaminase in females at the high dose. The absolute and relative liver (interim and terminal kill) and kidney (interim kill) weights were increased in males at the high dose compared with controls. Dose-dependent increases in the incidence of diffuse, clay-coloured discoloration and focal and multifocal discoloration of livers were observed in treated males (both doses). Also, treatment-related non-neoplastic microscopic changes (e.g. fatty changes, mixed cell and basophilic cell foci, focal hepatocellular hyperplasia, spongiosis hepatis) were noted in the liver of both sexes at both doses tested, lesions being more severe in males than in females. There was no compound-related increase in the incidence of any tumours observed in this study, including mesenteric lymph node haemangioma and pancreatic exocrine carcinoma in male rats, thus confirming the lack of association between substance administration and the occurrence of these tumours suggested from the previous study (Tennekes et al., 1989).

Although no NOAEL could be identified in the second study owing to non-neoplastic microscopic hepatic changes and elevated liver enzyme activities in both treatment groups, the Committee was able to identify an overall NOAEL of 100 mg/kg feed (equal to 4.8 mg/kg bw per day) from the two chronic toxicity and carcinogenicity studies in rats.

The Committee concluded that teflubenzuron is not carcinogenic in mice or rats.

The genotoxic potential of teflubenzuron was investigated in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was detected, and teflubenzuron was considered unlikely to be genotoxic.

In view of the lack of genotoxicity and the absence of carcinogenicity in mice and rats, the Committee concluded that teflubenzuron is unlikely to pose a carcinogenic risk to humans.

In a multigeneration reproductive toxicity study, teflubenzuron was administered to rats at a concentration of 0, 20, 100 or 500 mg/kg feed (equal to 0, 1.5, 7.4 and 36.9 mg/kg bw per day for males and 0, 1.6, 7.9 and 39.5 mg/kg bw per day for females, respectively). The only treatment-related adverse effect noted was a significant increase in the incidence of unilateral and bilateral dilatation of the renal pelvis in F_1 pups in the high-dose group (6.8%) when compared with the controls (0.9%). No such effect was seen in the F_2 generation. The NOAEL for offspring toxicity was 100 mg/kg feed (equal to 7.4 mg/kg bw per day), and the NOAEL for both parental and reproductive toxicity was 500 mg/kg feed (equal to 36.9 mg/kg bw per day), the highest dose tested (Osterburg, 1989).

The developmental toxicity of teflubenzuron was investigated in pregnant rats by gavage administration at 0, 10, 50 or 250 mg/kg bw per day from days 6 to 15 of gestation. The number of live pups per dam was significantly reduced at the high dose when compared with the controls. The NOAEL for maternal toxicity was 250 mg/kg bw per day, the highest dose tested, and the NOAEL for embryo/fetal toxicity was 50 mg/kg bw per day, based on a reduction in the number of live pups per dam at 250 mg/kg bw per day (Gleich, Weisse & Unkelbach, 1986).

In a second developmental toxicity study, teflubenzuron was administered by gavage to pregnant rats at 0, 100, 300 or 1000 mg/kg bw per day during days 7–17 of gestation. No treatment-related toxicity was observed in dams, for both general health and reproductive parameters. No external, visceral or skeletal abnormalities were observed in pups. The NOAEL for both maternal and embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Ishida et al., 1987).

An overall NOAEL of 1000 mg/kg bw per day for maternal toxicity was identified. No overall NOAEL for embryo/fetal toxicity could be identified, as the reason for the difference in NOAELs for embryo/fetal toxicity in these two developmental toxicity studies in rats was unknown.

Pregnant rabbits were dosed with teflubenzuron by gavage at 0, 10, 50 or 250 mg/kg bw per day from days 6 to 18 of gestation and killed on day 29 of gestation. No maternal or reproductive toxicity was observed, and there were no developmental abnormalities. The only significant effect noted in the offspring was decreased survival during the first 24 hours in the high-dose group (88.5%) compared with the controls (100%). The NOAEL for embryo/fetal toxicity was 50 mg/kg bw per day, based on decreased survival at 250 mg/kg bw per day, and the

NOAEL for maternal toxicity was 250 mg/kg bw per day, the highest dose tested (Gleich et al., 1985).

To further elucidate the embryotoxic effect in rabbits, a small supplementary study (five per group) was conducted by administering teflubenzuron by gavage at 0, 250, 500 (killed on day 19) or 500 mg/kg bw per day (killed at day 29) on days 6–18 of gestation. There was evidence of embryotoxicity in all treated animals, although no maternal toxicity was identified (Gleich, 1985).

In another developmental toxicity study in rabbits, pregnant animals were dosed with teflubenzuron by gavage at 0 or 1000 mg/kg bw per day during days 6–18 of pregnancy and killed on gestation day 28. Treated rabbits had a higher incidence of liver lesions compared with controls, but there were no treatment-related reproductive or developmental abnormalities. No NOAEL was identified for maternal toxicity, as effects were noted at the only dose tested. The NOAEL for embryo/fetal toxicity was 1000 mg/kg bw per day, the only dose tested. However, this study did not evaluate offspring survival during the first 24 hours, and hence the Committee cannot discount the effects seen in the previous study (Osterburg, 1987).

An overall NOAEL of 500 mg/kg bw per day was identified for maternal toxicity, and an overall NOAEL of 50 mg/kg bw per day was identified for embryo/fetal toxicity.

3.3 Microbiological data

Considering the chemical structure and mode of action of teflubenzuron, the Committee did not anticipate any adverse effects of teflubenzuron residues on human gastrointestinal microbiota.

4. Evaluation

An ADI of 0–5 µg/kg bw was established on the basis of a $BMDL_{10}$ of 0.54 mg/kg bw per day for hepatocellular hypertrophy in male mice observed in the carcinogenicity study, with application of an uncertainty factor of 100 to account for interspecies and intraspecies variability, and rounded to one significant figure.

The use profile of teflubenzuron as a veterinary drug is such that dietary exposure to teflubenzuron from a large portion is unlikely to be markedly greater than that from chronic consumption. The toxicological profile of teflubenzuron is such that it is unlikely to present an acute hazard. The Committee therefore concluded that it was not necessary to assess the acute risk from exposure to teflubenzuron when used as a veterinary drug.

5. References

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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).

9. Specifications for identity and purity and toxicological evaluation of some antimicrobials and antioxidants. FAO Nutrition Meetings Report Series, No. 38A, 1965; WHO/Food Add/24.65 (out of print).

10. Specifications for identity and purity and toxicological evaluation of food colours. FAO Nutrition Meetings Report Series, No. 38B, 1966; WHO/Food Add/66.25.

11. Specifications for the identity and purity of food additives and their toxicological evaluation: some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases (Ninth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 40, 1966; WHO Technical Report Series, No. 339, 1966 (out of print).

12. Toxicological evaluation of some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases. FAO Nutrition Meetings Report Series, No. 40A, B, C; WHO/Food Add/67.29.

13. Specifications for the identity and purity of food additives and their toxicological evaluation: some emulsifiers and stabilizers and certain other substances (Tenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 43, 1967; WHO Technical Report Series, No. 373, 1967.

14. Specifications for the identity and purity of food additives and their toxicological evaluation: some flavouring substances and non-nutritive sweetening agents (Eleventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 44, 1968; WHO Technical Report Series, No. 383, 1968.

15. Toxicological evaluation of some flavouring substances and non-nutritive sweetening agents. FAO Nutrition Meetings Report Series, No. 44A, 1968; WHO/Food Add/68.33.

16. Specifications and criteria for identity and purity of some flavouring substances and non-nutritive sweetening agents. FAO Nutrition Meetings Report Series, No. 44B, 1969; WHO/Food Add/69.31.

17. Specifications for the identity and purity of food additives and their toxicological evaluation: some antibiotics (Twelfth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 45, 1969; WHO Technical Report Series, No. 430, 1969.

18. Specifications for the identity and purity of some antibiotics. FAO Nutrition Meetings Series, No. 45A, 1969; WHO/Food Add/69.34.

19. Specifications for the identity and purity of food additives and their toxicological evaluation: some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances (Thirteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 46, 1970; WHO Technical Report Series, No. 445, 1970.

20. Toxicological evaluation of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances. FAO Nutrition Meetings Report Series, No. 46A, 1970; WHO/Food Add/70.36.

21. Specifications for the identity and purity of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other food additives. FAO Nutrition Meetings Report Series, No. 46B, 1970; WHO/Food Add/70.37.

22. Evaluation of food additives: specifications for the identity and purity of food additives and their toxicological evaluation: some extraction solvents and certain other substances; and a review of the technological efficacy of some antimicrobial agents (Fourteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 48, 1971; WHO Technical Report Series, No. 462, 1971.

23. Toxicological evaluation of some extraction solvents and certain other substances. FAO Nutrition Meetings Report Series, No. 48A, 1971; WHO/Food Add/70.39.

24. Specifications for the identity and purity of some extraction solvents and certain other substances. FAO Nutrition Meetings Report Series, No. 48B, 1971; WHO/Food Add/70.40.

25. A review of the technological efficacy of some antimicrobial agents. FAO Nutrition Meetings Report Series, No. 48C, 1971; WHO/Food Add/70.41.

26. Evaluation of food additives: some enzymes, modified starches, and certain other substances: Toxicological evaluations and specifications and a review of the technological efficacy of some antioxidants (Fifteenth report

of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 50, 1972; WHO Technical Report Series, No. 488, 1972.

27. Toxicological evaluation of some enzymes, modified starches, and certain other substances. FAO Nutrition Meetings Report Series, No. 50A, 1972; WHO Food Additives Series, No. 1, 1972.

28. Specifications for the identity and purity of some enzymes and certain other substances. FAO Nutrition Meetings Report Series, No. 50B, 1972; WHO Food Additives Series, No. 2, 1972.

29. A review of the technological efficacy of some antioxidants and synergists. FAO Nutrition Meetings Report Series, No. 50C, 1972; WHO Food Additives Series, No. 3, 1972.

30. Evaluation of certain food additives and the contaminants mercury, lead, and cadmium (Sixteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 51, 1972; WHO Technical Report Series, No. 505, 1972, and corrigendum.

31. Evaluation of mercury, lead, cadmium and the food additives amaranth, diethylpyrocarbamate, and octyl gallate. FAO Nutrition Meetings Report Series, No. 51A, 1972; WHO Food Additives Series, No. 4, 1972.

32. Toxicological evaluation of certain food additives with a review of general principles and of specifications (Seventeenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 53, 1974; WHO Technical Report Series, No. 539, 1974, and corrigendum (out of print).

33. Toxicological evaluation of some food additives including anticaking agents, antimicrobials, antioxidants, emulsifiers, and thickening agents. FAO Nutrition Meetings Report Series, No. 53A, 1974; WHO Food Additives Series, No. 5, 1974.

34. Specifications for identity and purity of thickening agents, anticaking agents, antimicrobials, antioxidants and emulsifiers. FAO Food and Nutrition Paper, No. 4, 1978.

35. Evaluation of certain food additives (Eighteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 54, 1974; WHO Technical Report Series, No. 557, 1974, and corrigendum.

36. Toxicological evaluation of some food colours, enzymes, flavour enhancers, thickening agents, and certain other food additives. FAO Nutrition Meetings Report Series, No. 54A, 1975; WHO Food Additives Series, No. 6, 1975.

37. Specifications for the identity and purity of some food colours, enhancers, thickening agents, and certain food additives. FAO Nutrition Meetings Report Series, No. 54B, 1975; WHO Food Additives Series, No. 7, 1975.

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ANNEX 2

Abbreviations used in the monographs

ABC	adenosine triphosphate binding cassette
ADI	acceptable daily intake
AIC	Akaike information criterion
ARfD	acute reference dose
AUC	area under the plasma concentration–time curve
$AUC_{0-\infty}$	area under the plasma concentration–time curve from time 0 to infinity
BMD	benchmark dose
BMD_{10}	benchmark dose for a 10% response over the controls
BMDL	lower 95% confidence limit on the benchmark dose
$BMDL_{10}$	lower 95% confidence limit on the benchmark dose for a 10% response over the controls
BMDS	Benchmark Dose Software
BMR	benchmark response
bw	body weight
CCRVDF	Codex Committee on Residues of Veterinary Drugs in Foods
C_{max}	peak concentration in plasma
CPU	4-chlorophenylurea
CYP	cytochrome P450
DNA	deoxyribonucleic acid
EFSA	European Food Safety Authority
eq	equivalents
F	female
F_0	parental generation
F_1	first filial generation
F_2	second filial generation
FAO	Food and Agriculture Organization of the United Nations
GC-MS	gas chromatography with mass spectrometry
GDWQ	*Guidelines for Drinking-water Quality*
H_2B_{1a}	22,23-dihydroavermectin B_{1a}
H_2B_{1b}	22,23-dihydroavermectin B_{1b}
hgprt	hypoxanthine–guanine phosphoribosyltransferase
HPLC	high-performance liquid chromatography
HPT	hypothalamic–pituitary–thyroid
INN	International Non-proprietary Name

JECFA	Joint FAO/WHO Expert Committee on Food Additives
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
LC_{50}	median lethal concentration
LC-MS	liquid chromatography with mass spectrometry
LC-MS/MS	liquid chromatography with tandem mass spectrometry
LD_{50}	median lethal dose
LOAEL	lowest-observed-adverse-effect level
LOD	limit of detection
LOQ	limit of quantification
LSC	liquid scintillation counting
M	male
MDR	multiple drug resistance
MRL	maximum residue limit
MS	mass spectrometry/spectroscopy
NA	not applicable
ND	no data
NMR	nuclear magnetic resonance
NOAEC	no-observed-adverse-effect concentration
NOAEL	no-observed-adverse-effect level
NOEL	no-observed-effect level
NTP	National Toxicology Program (USA)
OECD	Organisation for Economic Co-operation and Development
PCA	4-chloroaniline; *p*-chloroaniline
PEG	polyethylene glycol
S9	$9000 \times g$ supernatant fraction from rat liver homogenate
SD	standard deviation
$t_{1/2}$	elimination half-life
T_3	triiodothyronine
T_4	thyroxine
TLC	thin-layer chromatography
T_{max}	time to reach C_{max}
TSH	thyroid stimulating hormone
UGT	uridine diphosphate-glucuronosyltransferase
USA	United States of America
USEPA	United States Environmental Protection Agency
VICH	International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products
WHO	World Health Organization
WHOPES	WHO Pesticide Evaluation Scheme
w/w	weight per weight

ANNEX 3

Eighty-first meeting of the Joint FAO/WHO Expert Committee on Food Additives

Rome, Italy, 17–26 November 2015

Members

Professor A. Anadón, Department of Toxicology and Pharmacology, Faculty of Veterinary Medicine, Universidad Complutense de Madrid, Madrid, Spain

Dr J.O. Boison, Centre for Veterinary Drug Residues, Canadian Food Inspection Agency, Saskatoon, Saskatchewan, Canada (*Joint Rapporteur*)

Professor A.R. Boobis, Centre for Pharmacology & Therapeutics, Department of Experimental Medicine, Division of Medicine, Faculty of Medicine, Imperial College London, London, England, United Kingdom (*Vice-Chair*)

Dr L.G. Friedlander, Residue Chemistry Team, Division of Human Food Safety, Center for Veterinary Medicine, Food and Drug Administration, Department of Health and Human Services, Rockville, Maryland, United States of America (USA) (*Chair*)

Dr K.J. Greenlees, Office of New Animal Drug Evaluation, Center for Veterinary Medicine, Food and Drug Administration, Department of Health and Human Services, Rockville, Maryland, USA (*Joint Rapporteur*)

Professor S.H. Jeong, Department of Biomedical Science, College of Life and Health Science, Hoseo University, Asan City, Chungnam, Republic of Korea

Professor B. Le Bizec, Laboratoire d'Étude des Résidus et des contaminants dans les aliments (LABERCA), École Nationale Vétérinaire, Agroalimentaire et de l'Alimentation Nantes Atlantique (ONIRIS), Nantes, France

Professor J. Palermo-Neto, Department of Pathology, Faculty of Veterinary Medicine, University of São Paulo, São Paulo, Brazil

Professor Emeritus L. Ritter, University of Guelph, Guelph, Ontario, Canada

Dr P. Sanders, National Reference Laboratory for Veterinary Drug Residues and Antimicrobial Resistance, Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES), Fougères, France

Secretariat

Ms G. Brisco, Joint FAO/WHO Food Standards Programme, Food and Agriculture Organization of the United Nations, Rome, Italy (*Codex Secretariat*)

Dr A. Bruno, Joint FAO/WHO Food Standards Programme, Food and Agriculture Organization of the United Nations, Rome, Italy (*Codex Secretariat*)

Dr C.E. Cerniglia, Division of Microbiology, National Center for Toxicological Research, Food and Drug Administration, Department of Health and Human Services, Jefferson, Arkansas, USA (*WHO Expert*)

Dr A. Chicoine, Veterinary Drugs Directorate, Health Canada, Saskatoon, Saskatchewan, Canada (*FAO Expert*)

Dr H. Erdely, Residue Chemistry Team, Division of Human Food Safety, Center for Veterinary Medicine, Food and Drug Administration, Department of Health and Human Services, Rockville, Maryland, USA (*FAO Expert*)

Dr V. Fattori, Food and Agriculture Organization of the United Nations, Rome, Italy (*FAO Secretariat*)

Dr S. Ghimire, Veterinary Drugs Directorate, Health Canada, Ottawa, Ontario, Canada (*WHO Expert*)

Dr J.C. Leblanc, Food and Agriculture Organization of the United Nations, Rome, Italy (*FAO Secretariat*)

Dr M. Lipp, Food and Agriculture Organization of the United Nations, Rome, Italy (*FAO Joint Secretary*)

Dr J. MacNeil, Consultant, Food and Agriculture Organization of the United Nations, Rome, Italy (*FAO Technical Editor*)

Dr K. Ogawa, Division of Pathology, Biological Safety Research Center, National Institute of Health Sciences, Tokyo, Japan (*WHO Expert*)

Professor S. Rath, Department of Analytical Chemistry, University of Campinas, Campinas, São Paulo, Brazil (*FAO Expert*)

Dr R. Reuss, Food Standards Australia New Zealand, Canberra, Australian Capital Territory, Australia (*FAO Expert*)

Dr G.J. Schefferlie, Veterinary Medicinal Products Unit, Medicines Evaluation Board Agency, Utrecht, the Netherlands (*WHO Expert*)

Dr S. Scheid, Department of Veterinary Medicines, Federal Office of Consumer Protection and Food Safety, Berlin, Germany (*FAO Expert*)

Dr C. Schyvens, Scientific Assessment and Chemical Review, Australian Pesticides and Veterinary Medicines Authority, Kingston, Australian Capital Territory, Australia (*WHO Expert*)

Ms M. Sheffer, Orleans, Ontario, Canada (*WHO Editor*)

Dr A. Tritscher, Risk Assessment and Management, Department of Food Safety and Zoonoses, World Health Organization, Geneva, Switzerland (*WHO Secretariat*)

Dr S. Vaughn, Chair, Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF), Office of New Animal Drug Evaluation, Center for Veterinary Medicine, Food and Drug Administration, Department of Health and Human Services, Rockville, Maryland, USA (*CCRVDF*)

Dr P. Verger, Risk Assessment and Management, Department of Food Safety and Zoonoses, World Health Organization, Geneva, Switzerland (*WHO Joint Secretary*)

Ms Yong Zhen Yang,[1] Food and Agriculture Organization of the United Nations, Rome, Italy (*FAO JMPR Secretariat*)

Dr T. Zhou, Office of New Animal Drug Evaluation, Center for Veterinary Medicine, Food and Drug Administration, Department of Health and Human Services, Rockville, Maryland, USA (*WHO Expert*)

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[1] Attended session on dietary exposure assessment only.

ANNEX 4

Recommendations on the substances on the agenda

Diflubenzuron (insecticide)

Acceptable daily intake	In the absence of adequate information on exposure to 4-chloroaniline (PCA), a genotoxic and carcinogenic metabolite and/or degradate of diflubenzuron, the Committee was unable to establish an acceptable daily intake (ADI) for diflubenzuron because it was not possible to assure itself that there would be an adequate margin of safety from its use as a veterinary drug. The Committee also noted that it was not possible to calculate a margin of exposure for PCA in the absence of adequate information on exposure to PCA.
Maximum residue limits	The Committee was unable to recommend maximum residue limits (MRLs) for diflubenzuron, as an ADI could not be established.

Ivermectin (antiparasitic agent)

Acceptable daily intake	The Committee established an ADI of 0–10 µg/kg body weight on the basis of a no-observed-adverse-effect level (NOAEL) of 0.5 mg/kg body weight per day for neurological effects (mydriasis) and retardation of weight gain in a 14-week dog study, with application of an uncertainty factor of 50 (5 for interspecies differences based on pharmacokinetics studies in dogs and humans and 10 for intraspecies differences). The previous ADI of 0–1 µg/kg body weight was withdrawn.
Acute reference dose	The Committee established an acute reference dose (ARfD) of 0.2 mg/kg body weight, based on a NOAEL of 1.5 mg/kg body weight, the highest dose tested in a safety, tolerability and pharmacokinetics study in healthy human subjects, with application of an uncertainty factor of 10 for intraspecies variability.

Estimated chronic dietary exposure	The estimated daily intake (EDI) is 38 µg/person per day, based on a 60 kg individual, which represents 6% of the upper bound of the ADI.
	The global estimate of chronic dietary exposure (GECDE) for the general population is 0.9 µg/kg body weight per day, which represents 9% of the upper bound of the ADI.
	The GECDE for children is 1.5 µg/kg body weight per day, which represents 15% of the upper bound of the ADI.
	The GECDE for infants is 1.3 µg/kg body weight per day, which represents 13% of the upper bound of the ADI.
Estimated acute dietary exposure	The maximum values of residues found at injection sites led to global estimates of acute dietary exposure (GEADE) of 52 µg/kg body weight for the general population and 87 µg/kg body weight for children, corresponding, respectively, to 27% and 43% of the ARfD.
Residue definition	Ivermectin B_{1a}

Recommended maximum residue limits (MRLs)[a]

Species	Fat (µg/kg)	Kidney (µg/kg)	Liver (µg/kg)	Muscle (µg/kg)
Cattle	400	100	800	30

[a] No new data were provided for use of ivermectin in dairy cattle; therefore, the Committee did not recommend any revision to the MRL of 10 µg/kg for ivermectin in milk.

Lasalocid sodium (antiparasitic agent)

Following consideration of the issues raised in concern forms from the Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF), the Committee concluded that there would be no concern for colonization barrier disruption in the colon from acute exposure to residues of lasalocid. The ADI established and MRLs recommended at the seventy-eighth meeting of JECFA (WHO TRS No. 988, 2014) remain unchanged.

Sisapronil (ectoparasiticide)

Acceptable daily intake	The Committee concluded that a toxicological ADI could not be established because the Committee had no basis upon which to determine a suitable uncertainty factor to accommodate the lack of a long-term toxicity study.
Maximum residue limits	The Committee could not recommend MRLs, as an ADI could not be established.

Teflubenzuron (insecticide)

Acceptable daily intake	The Committee established an ADI of 0–5 µg/kg body weight on the basis of a lower 95% confidence limit on the benchmark dose for a 10% response (BMDL$_{10}$) of 0.54 mg/kg body weight per day for hepatocellular hypertrophy in male mice observed in a carcinogenicity study, with application of an uncertainty factor of 100 to account for interspecies and intraspecies variability.
Estimated chronic dietary exposure	The EDI is 42.9 µg/person per day, on the basis of a 60 kg individual, which represents approximately 14% of the upper bound of the ADI.
	The GECDE for the general population is 1.6 µg/kg body weight per day, which represents 31% of the upper bound of the ADI.
	The GECDE for children is 2.1 µg/kg body weight per day, which represents 43% of the upper bound of the ADI.
	The GECDE for infants is 0.9 µg/kg body weight per day, which represents 18% of the upper bound of the ADI.
Residue definition	Teflubenzuron

Recommended maximum residue limits (MRLs)

Species	Fillet[a] (µg/kg)	Muscle (µg/kg)
Salmon	400	400

[a] Muscle plus skin in natural proportion.

Zilpaterol hydrochloride (β_2-adrenoceptor agonist)

Acceptable daily intake	The Committee reaffirmed the ADI of 0–0.04 µg/kg body weight established at the seventy-eighth meeting (WHO TRS No. 988, 2014).
Acute reference dose	The Committee established an ARfD of 0.04 µg/kg body weight based on a lowest-observed-adverse-effect level (LOAEL) of 0.76 µg/kg body weight for acute pharmaco-logical effects observed in a single-dose human study, with application of an uncertainty factor of 20, comprising a default uncertainty factor of 10 for human individual variability and an additional uncertainty factor of 2 to account for use of a LOAEL for a slight effect instead of a NOAEL.
Residue definition	Zilpaterol (free base) in muscle, liver and kidney
Estimated acute dietary exposure	The GEADE is 1.9 µg/day for the general population, which represents approximately 80% of the ARfD.
	The GEADE is 0.57 µg/day for children, which represents approximately 94% of the ARfD.

Recommended maximum residue limits (MRLs)[a]

Species	Kidney (µg/kg)	Liver (µg/kg)	Muscle (µg/kg)
Cattle	3.3	3.5	0.5

[a] There were insufficient zilpaterol residue data to adequately consider exposure to residues in lungs and other edible offal of cattle apart from liver and kidney.

This volume contains monographs prepared at the eighty-first meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met in Rome, Italy, from 17 to 26 November 2015.

The toxicological monographs in this volume summarize data on the veterinary drug residues that were evaluated toxicologically by the Committee: diflubenzuron, ivermectin, sisapronil and teflubenzuron. Annexed to the report is a summary of the Committee's recommendations on these and other drugs discussed at the eighty-first 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 information 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.