IMAZALIL

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Explanation

Imazalil (synonym: enilconazole, a pharmaceutical) is the International Organization for Standardization–approved common name for 1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl]-1*H*-imidazole (International Union of Pure and Applied Chemistry), for which the Chemical Abstracts Service number is 33586-44-0.

Imazalil belongs to the group of imidazole fungicides used to control a wide range of fungi on fruits, vegetables and ornamentals. Its fungicidal mode of action is by inhibition of sterol biosynthesis.

Imazalil was previously evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1977, 1980, 1985, 1986, 1991, 2000, 2001 and 2005. In 1991, JMPR established an acceptable daily intake (ADI) of 0–0.03 mg/kg body weight (bw), based on a no-observed-adverse-effect level (NOAEL) for clinical signs, decreased body weight gain and feed consumption, decreased serum concentrations of calcium, increased alkaline phosphatase (ALP) activity and increased liver weight in a 12-month toxicity study in dogs. In 2000, JMPR reaffirmed the ADI and concluded that an acute reference dose (ARfD) was unnecessary.

In 2005, the Meeting established an ARfD of 0.05 mg/kg bw, using a NOAEL of 5 mg/kg bw per day for maternal and fetal toxicity in a study of developmental toxicity in rabbits and a safety factor of 100.

Imazalil was reviewed by the present Meeting under the periodic review programme of the Codex Committee on Pesticide Residues. New studies included a single-dose toxicity study in rats, an acute neurotoxicity study in rats, a 28-day toxicity study in rats, a 90-day toxicity study in mice, mechanistic studies to evaluate the human relevance of liver and thyroid tumours, published studies on evaluation of endocrine effects and neurodevelopmental effects, and toxicity studies on metabolites.

The evaluation of the biochemical and toxicological aspects of imazalil was based on previous JMPR evaluations, updated as necessary with additional information. All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with relevant national or international test guidelines, unless otherwise specified. A search of the open literature did not reveal any relevant publications that would have an impact on the evaluation.

Evaluation for acceptable intake

1. Biochemical aspects

Imazalil is rapidly absorbed, distributed, metabolized and excreted by rats.

1.1 Absorption, distribution and excretion

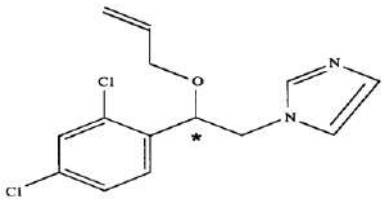
Wistar rats (five of each sex) were given ^{3}H-labelled imazalil sulfate in a single oral dose of 20 mg/kg bw. Almost 90% of the administered radioactivity was excreted within 96 hours, with approximately equal quantities detected in the urine and faeces. Tissue residues ranged from 5.4% to 6.1% of the administered radioactivity within 48 hours after dosing and from 1.8% to 3.5% within 96 hours after dosing. The highest levels of radioactivity were found in the liver, lung and kidneys. Analysis of the urine from dosed animals indicated that 4% of the tritium was volatile by lyophilization. Thus, tritium exchange apparently occurred to a minor extent (Heykants, Meuldermans & Hurkmans, 1975).

Five male rats were given ^{3}H-labelled imazalil sulfate in a single oral dose of 20 mg/kg bw to study the absorption, distribution, metabolism and excretion of imazalil. Approximately 90% of the administered radioactivity was excreted within 96 hours, with comparable quantities excreted in the urine and faeces. Similar results were found when a single oral dose of α-(2,4-diochlorophenyl)-1*H*-imidazole-1-ethanol (a metabolite of imazalil) was administered. In both these experiments, up to approximately 2% of the administered radioactivity was identified as tritiated water, indicating exchange of the tritium label. The relative amount of tritiated water found in the urine increased with time (Meuldermans et al., 1977b).

[^{14}C]Imazalil (purity 99.9%; Fig. 1) and unlabelled technical-grade imazalil (purity 98.63%) were administered to groups of five male and five female Wistar rats. A single dose of [^{14}C]imazalil at 1.25 mg/kg bw was administered intravenously to the first group. The second group received [^{14}C]imazalil at a single dose of 1.25 mg/kg bw by gavage. The third group received unlabelled imazalil at 1.25 mg/kg bw per day for 14 days by gavage followed by a single oral dose of

[^{14}C]imazalil at 1.25 mg/kg bw. The fourth group received [^{14}C]imazalil at a single dose of 20 mg/kg bw by gavage. The final specific activities of the labelled formulations were 1.1 MBq/mg at the low dose and 74 kBq/mg at the high dose. The animals were observed before dosing and at the end of each faecal collection. Urine was collected over the periods 0–4, 4–8, 8–24, 24–48, 48–72 and 72–96 hours. Faeces were collected 0–24, 24–48, 48–72 and 72–96 hours after dosing with radiolabelled imazalil. The animals were killed at 96 hours, and selected organs were removed.

Fig. 1. Structure of radiolabelled imazalil



Source: Mannens, Van Leemput & Heykants (1993)

After the single intravenous dose, 84% of the label was excreted by males and females within 24 hours and 89% within 96 hours. When imazalil was given at a single gavage dose of 1.25 mg/kg bw, 90% was excreted within 24 hours by males and 93% by females. After imazalil was given for 14 days at a dose of 1.25 mg/kg bw per day by gavage followed by a single oral dose of [^{14}C]imazalil, males excreted 94% within 24 hours and females excreted 84%; nearly all the label had been excreted in the urine and faeces of animals of both sexes by 96 hours. When imazalil was given at a single dose of 20 mg/kg bw by gavage, 90% and 89% were excreted within 24 hours by males and females, respectively; more than 95% was excreted in the faeces and urine by 96 hours. Somewhat more imazalil appeared in the urine than in the faeces with all dosing regimens, whereas there was no significant sex difference (Table 1).

At 96 hours after oral administration of [^{14}C]imazalil, tissue concentrations (including carcass) of radioactivity were about 1% of the administered dose. There was a dose-linear increase in the tissue levels after single oral low and high doses. There was no indication that accumulation occurred in any tissue after multiple dosing. Nearly 50% of the radiolabel retained in the body was found in the liver. Comparison of the excretion patterns after oral and intravenous dosing suggests that the bioavailability, and therefore the absorption, of imazalil given orally is high (Mannens, Van Leemput & Heykants, 1993).

1.2 Biotransformation

Studies involving administration of ^{3}H-labelled imazalil sulfate to rats in a single oral dose of 20 mg/kg bw indicate that extensive metabolism of imazalil occurs. In one study, α-(2,4-dichlorophenyl)-1*H*-imidazole-1-ethanol and 2,4-dichloromandolic acid were identified as the major metabolites in the urine. In this study, 10% of the radioactivity in the urine and 3% of the radioactivity in the faeces were identified as unchanged imazalil (Heykants, Meuldermans & Hurkmans, 1975).

The major metabolites identified in a more extensive metabolic study are shown in Fig. 2. The compound shown in brackets in Fig. 2 was not identified in the study. In contrast to the study described above, no unchanged imazalil was detected in the urine, and only 0.1% of the administered radioactivity was identified as unchanged imazalil in the faeces (Meuldermans al., 1977b).

Table 1. Distribution of radioactivity following administration of imazalil to rats

	% of administered radioactivity (standard deviation)							
	Single IV dose of 1.25 mg/kg bw		**Single oral dose of 1.25 mg/kg bw**		**Repeated oral dose of 1.25 mg/kg bw for 14 days**		**Single oral dose of 20 mg/kg bw**	
Sample	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Urine								
0–24 h	44.88	53.52	46.55	58.52	49.20	52.63	51.40	54.54
	(3.34)	(6.44)	(8.60)	(4.02)	(5.70)	(4.99)	(2.75)	(8.88)
0–96 h	46.31	55.17	48.66	59.96	50.91	55.10	53.14	56.86
	(3.39)	(6.72)	(8.76)	(3.77)	(6.01)	(6.01)	(2.97)	(8.84)
Faeces								
0–24 h	39.04	30.48	43.41	34.22	44.67	31.50	38.56	34.06
	(5.05)	(5.34)	(8.32)	(2.72)	(4.78)	(15.09)	(2.37)	(4.09)
0–96 h	41.74	32.41	46.07	36.11	47.55	41.67	42.77	37.71
	(5.00)	(5.93)	(8.36)	(2.29)	(5.59)	(3.83)	(2.59)	(5.40)
Cage wash	0.55	0.52	0.85	0.61	0.37	1.03	0.74	1.25
	(0.44)	(0.23)	(0.55)	(0.16)	(0.17)	(0.58)	(0.42)	(1.18)
Total	88.60	89.01	95.83	96.77	99.02	97.95	96.68	95.89
	(2.20)	(1.14)	(1.00)	(1.72)	(0.39)	(0.47)	(0.51)	(3.26)
Tissues	–	–	1.05	0.85	1.01	0.89	1.20	1.03
			(0.12)	(0.13)	(0.13)	(0.3)	(0.31)	(0.33)

bw: body weight; IV: intravenous
Source: Mannens, Van Leemput & Heykants (1993)

In a study described in section 1.1 above, little imazalil was excreted unchanged: less than 1% of the administered dose in the faeces, and trace amounts in the urine. The compound was metabolized to at least 25 metabolites. The metabolic profiles in the urine and faeces were largely comparable. Moreover, identical metabolites were recovered in the excreta from both sexes, and the intergroup similarities were remarkable. Three major metabolites were identified: (±)-1-[2-(2,4-dichlorophenyl)-2-(2,3-dihydroxypropyloxy)ethyl]-imidazolidine-2,5-dione (metabolite 8), (±)-1-[2-(2,4-dichlorophenyl)-2-(2,3-dihydroxypropyloxy)ethyl]-1*H*-imidazole (metabolite 10) and (±)-1-(2,4-dichlorophenyl)-2-imidazol-1-ylethanol (metabolite 11; see Fig. 2). The main routes of metabolism were epoxidation, epoxide hydration, oxidative *O*-dealkylation, imidazole oxidation and scission, and oxidative *N*-dealkylation. The metabolic pattern was similar after oral and intravenous administration and in animals of both sexes (Mannens, Van Leemput & Heykants, 1993).

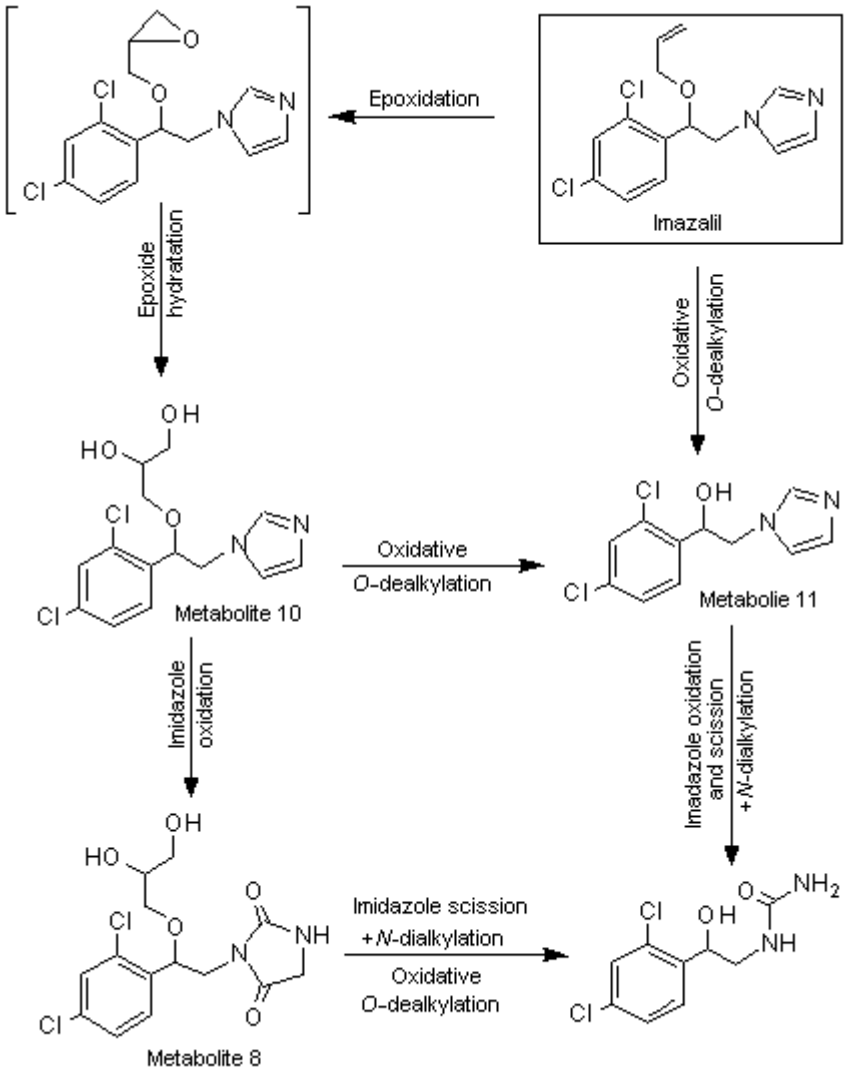
Tritium-labelled imazalil sulfate was incubated with rat liver homogenates to study the in vitro metabolism of imazalil. The two metabolites identified were α-(2,4-dichlorophenyl)-1*H*-imidazole-1-ethanol and 1-[2-(2,4-dichlorophenyl)-2-(2,3-dihydroxypropyloxy)ethyl]-1*H*-imidazole (Meuldermans et al., 1977a).

2. Toxicological studies

2.1 Acute toxicity

The results of studies on the acute toxicity of imazalil in rats and rabbits are summarized in Table 2.

Fig. 2. Metabolic pathways of imazalil in rats



Source: Mannens, Van Leemput & Heykants (1993)

Table 2. Acute toxicity of imazalil

Route	Species	Strain	Sex	LD_{50} (mg/kg bw)[a] (95% CI)	Purity (%)	Reference
Oral	Rat	Wistar	Male	343 (262–448)	>95 (nitrate)	Niemegeers (1979)
			Female	288 (221–377)		
			Male	343 (262–448)	100.4 (technical)	Niemegeers (1979); Goodwine (1990)
			Female	227 (174–297)		
			Male	355 (272–464)	102.7 (sulfate)	Niemegeers (1979)
			Female	309 (237–404)		
			Male	371 (284–485)	99.1 (acetate)	Niemegeers (1979)
			Female	309 (237–404)		

Route	Species	Strain	Sex	LD_{50} (mg/kg bw)[a] (95% CI)	Purity (%)	Reference
		Sprague Dawley	Male and female	664 (595–742)	NS (technical)	Dreher (1990a)
			Female	720 (212–2 160)	97.5 (technical)	Moore (2005a)
Dermal	Rat	Wistar	Male	4 200 (2 966–5 948)	NS	Marsboom & Van Ravestyn (1975)
			Female	4 880 (3 144–7 575)		
	Rat	Sprague Dawley	Male and female	>2 000	NS (technical)	Dreher (1990b)
	Rabbit	New Zealand white	Male and female	>2 000	97.6 (technical)	Teuns et al. (1990a)
Inhalation[b]	Rat	Wistar	Male and female	LC_{50} > 2 000 mg/m^3	Smoke from a candle containing 5.0 g imazalil	Appelman & Woutersen (1983)
Inhalation (dust, 27.3–39.6%; <4 µm, nose only)	Rat	Sprague Dawley	Male	LC_{50} = 2.88 (2.34–3.54) mg/L	NS (technical)	Blagden (1990)
			Female	LC_{50} = 1.84 (1.04–3.26) mg/L		
Primary skin irritation	Rabbit	New Zealand white	NS	Not irritating	98.9	Teuns & Marsboom (1987)
			Male and female	Mildly irritating	NS (technical)	Dreher (1990c)
			Male and female	Mildly irritating	97.46	Durando (2006)
Eye irritation	Rabbit	New Zealand white	Male and female	Severely irritating	97.6 (technical)	Teuns et al. (1990b)
			Male and female	Moderately irritating	NS (technical)	Dreher (1990d)
			Male and female	Moderately irritating	NS (technical)	Glaza (1996)
Skin sensitization	Guinea-pig	Dunkin-Hartley	Male	Not sensitizing (Magnusson & Kligman maximization)	NS (technical)	Dreher (1990e)
		Pirbright	Male	Slightly sensitizing (Magnusson & Kligman maximization)	98.5	Teuns et al. (1990c)
		Hartley albino	Male	Not sensitizing (Buehler method)	98.94	Wnorowski (1997)

bw: body weight; CI: confidence interval; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; NS, not stated

[a] Except where specified otherwise.

[b] Deployed as smoke from a smoke generator.

(a) Oral administration

The oral toxicities of the nitrate, sulfate and acetate salts of imazalil, as well as imazalil base, were investigated. Each compound was orally administered by gavage at 160, 320 or 640 mg/kg bw as an aqueous suspension at a dosing volume of 1 mL/100 g bw to groups of 10 male and 10 female Wistar rats. The median lethal dose (LD_{50}) values for males ranged from 343 to 371 mg/kg bw, and for females from 227 to 309 mg/kg bw. There was no mortality for any of the compounds at 160 mg/kg bw (although there were signs of toxicity, which included ataxia, piloerection, hypotonia, hypothermia and ptosis). Mortality started at 320 mg/kg bw (for all compounds) (Niemegeers, 1979).

Sprague Dawley rats (five of each sex per dose) were dosed with imazalil technical, administered as a suspension in polyethylene glycol, at 400, 438, 480 or 679 mg/kg bw. The only mortalities that occurred were at 679 mg/kg bw. The oral LD_{50} was calculated to be 664 mg/kg bw for both males and females (Dreher, 1990a).

In an up-and-down procedure, seven female Sprague Dawley rats were orally dosed with imazalil technical (purity 97.46%) administered as a 50% weight per weight (w/w) mixture in corn oil. Doses were 72 (one rat), 227 (two rats), 720 (three rats) and 2000 (one rat) mg/kg bw. Mortality occurred in 2/3 rats dosed at 720 mg/kg bw as well as the one rat dosed at 2000 mg/kg bw. Signs of toxicity (hypoactivity, hunched posture, piloerection) occurred only at 720 and 2000 mg/kg bw. Decedents had red intestines. The oral LD_{50} was calculated to be 720 mg/kg bw, with a 95% profile likelihood–based confidence interval of 212.4– 2160 mg/kg bw (Moore, 2005a).

(b) Dermal application

Rats

In a dermal toxicity study with Wistar albino rats, groups of 10 males and 10 females received 24-hour occluded exposure to test material at either 2560 or 5120 mg/kg bw. The post-exposure observation period was 7 days. At 2560 mg/kg bw, one male and one female died; at 5120 mg/kg bw, seven males and five females died. The report gives no information as to signs of toxicity, dermal irritation or gross necropsy results. The LD_{50} values are reported as 4200 (2966–5948) mg/kg bw for males and 4880 (3144–7575) mg/kg bw for females (Marsboom & Van Ravestyn, 1975).

In a dermal toxicity study, five male and five female Sprague Dawley rats were semi-occlusively exposed for 24 hours to imazalil at a dose of 2000 mg/kg bw. The application site was moistened with distilled water before the test material was applied. There were no mortalities, no signs of toxicity and no dermal irritation. All rats gained weight on days 0–7 and 7–14. No abnormalities were detected at gross necropsy. The dermal LD_{50} was greater than 2000 mg/kg bw (Dreher, 1990b).

Rabbits

In a dermal toxicity study with New Zealand white rabbits, five males and five females received 24-hour occluded exposure to undiluted technical imazalil (purity 97.6%) at 2000 mg/kg bw. There was no mortality. There was slight sedation in six (three of each sex) rabbits on day 1, but this was gone the following day. There was slight dermal (grade 1 erythema and/or oedema) irritation, which was gone by day 4. All rabbits gained weight from day −1 to day 7 and again from day 7 to day 14. At necropsy, one female had slightly thickened skin at the application site, and another had a slightly swollen and pale liver with pronounced lobulation; the report stated that this has also been observed in controls and therefore is of no relevance. The dermal LD_{50} was greater than 2000 mg/kg bw (Teuns et al., 1990a).

(c) Exposure by inhalation

Rats

In an inhalation study involving whole body exposure to imazalil-containing smoke generated from a candle containing 5.0 g imazalil, five male and five female specific pathogen–free (SPF)–reared Wistar rats were exposed for 4 hours to imazalil at a nominal concentration of 2 g/m^3 (calculated by dividing 5.0 g by the 2.5 m^3 volume of the chamber). According to the report: "During the exposure the following levels of Imazalil were measured by the sponsor: 20.67 g/m^3, 6.30 g/m^3, 8.55 g/m^3 and 7.39 g/m^3. All these values were much higher than the nominal concentration. An explanation for this discrepancy was not found." From the particle size distribution, 99.7% by weight had an aerodynamic diameter less than 3.6 µm. There was no mortality. During the first 30 minutes of exposure, the rats could not be observed because of the smoke. During the remainder of the exposure period, the rats were somewhat restless and had their eyes half closed. During the first 4 days of the observation period, all rats had laboured respiration. They recovered completely during the second week. All rats lost weight from the day of exposure to day 2, but all had gains from their pre-exposure weight by day 7, and all had good weight gains from day 7 to day 14. Gross necropsy did not reveal any abnormalities that could be ascribed to treatment (Appelman & Woutersen, 1983).

In an inhalation study, groups of five male and five female Sprague Dawley rats received 4-hour nose-only exposure to imazalil technical at 1.97, 3.15 or 4.57 mg/L. The respective mass median aerodynamic diameters were 6.2, 5.1 and 6.2 µm, and the respirable fractions (% particles < 4 µm) were 33.9%, 39.6% and 27.3%. All rats exposed to 4.57 mg/L died during the 4-hour exposure period. Eight (three males and five females) of the 10 rats exposed to 3.15 mg/L were found dead on the day following exposure, whereas at 1.97 mg/L, two of the five females died, with one euthanized in extremis following exposure and the other found dead the next day. The LC_{50} values were 2.88 (2.34–3.54) mg/L for males, 1.84 (1.04–3.26) mg/L for females and 2.43 (1.99–2.96) mg/L for the two sexes combined (Blagden, 1990).

In an inhalation feasibility study with imazalil (purity 97.46%), it was stated that the test substance (described as a yellow to brown crystalline mass of solidified oil) was extremely difficult to aerosolize. Attempts to generate an atmosphere with standard dust generation equipment were unsuccessful. The test substance was ground in a ball mill for 24 hours. These attempts to grind the test substance resulted in the test substance adhering to the grinding media, rendering it useless. The test substance was then ground in a coffee mill, but again this resulted in an unsuccessful atmosphere generation. An effort was made to aerosolize the test material as a 33% w/w suspension in distilled water, but all attempts to generate the desired atmosphere with this suspension were unsuccessful. It was concluded that the overall results of the pre-test trials indicate that the physical properties of the test substance prevent the achievement of the required testing concentration and mass median aerodynamic diameter (Moore, 2005b).

(d) Dermal irritation

The potential of imazalil to cause primary irritation of the skin was studied in three New Zealand white rabbits that received a single application of technical-grade imazalil (purity 98.9%) to 6 cm^2 of skin for 4 hours at a total dose of 0.5 mL under a gauze patch. The animals were observed at 1 hour and then daily for 14 days, and irritation was scored according to the Draize method. Slight (grade 1) erythema was observed in one animal at 1 hour after treatment, but this animal scored zero at 24 hours and subsequently. Otherwise, all animals were free (all scores were zero) of dermal irritation for the entire 14-day observation period. Imazalil was considered to be non-irritating to the skin of rabbits (Teuns & Marsboom, 1987).

In a dermal irritation study, three New Zealand white rabbits were dermally exposed to 0.5 g imazalil technical moistened with 0.5 mL distilled water applied under a 2.5 cm × 2.5 cm gauze patch. After 4 hours, the patches and residual test material were removed, with irritation scoring at 1, 24, 48 and 72 hours. Erythema scores ranged from 0 to 2, and oedema scores ranged from 0 to 1. All scores

were zero at 72 hours. The primary dermal irritation score was 0.46, and the test material was designated as mildly irritating to the skin of rabbits (Dreher, 1990c).

The potential of imazalil to cause primary irritation of the skin was studied in three New Zealand white rabbits that were exposed to a single application of technical-grade imazalil (purity 97.46%) on 6 cm^2 of skin for 4 hours at a total dose of 0.5 g moistened with water. The animals were observed and irritation was scored (according to the Draize method) at 1, 24, 48 and 72 hours. At 1 and 24 hours after patch removal, all three treated sites exhibited well-defined (grade 2) erythema and very slight (grade 1) oedema. The overall incidence and severity of irritation decreased thereafter. All animals were free of dermal irritation by 72 hours. The primary dermal irritation index (mean score for 1, 24, 48 and 72 hours) was 1.8. Imazalil was mildly irritating to the skin of rabbits (Durando, 2006). This study was not previously evaluated by JMPR.

(e) Ocular irritation

In an eye irritation study with imazalil technical (purity not specified), 0.1 mL (approximately 96 mg) of test material was placed into the conjunctival sac of the right eye of each of three New Zealand white rabbits. All three eyes showed corneal opacity and iritis from 24 through 72 hours. All eyes had cleared (all scores zero) by day 7. These results indicate that imazalil is moderately irritating to the eyes of rabbits (Dreher, 1990d).

In an eye irritation study with imazalil (purity 97.6%), 0.1 g was instilled into the conjunctival sac of the left eye of each of three New Zealand white rabbits. All eyes showed corneal opacity from 1 hour through 14 days, and corneal opacity was still present in two eyes on day 21. Imazalil was severely irritating to the eyes of rabbits (Teuns et al., 1990b).

In an eye irritation study, 0.1 mL (65 mg) imazalil technical (purity not specified) was instilled in one eye of each of six rabbits. All six eyes showed corneal opacity from 1 to 72 hours after instillation, and one eye still showed corneal opacity on day 7, with clearing by day 14. These results indicate that imazalil is moderately irritating to the eyes of rabbits (Glaza, 1996).

(f) Dermal sensitization

In a dermal sensitization study, 20 female Dunkin-Hartley guinea-pigs were induced by intradermal injection of 1% imazalil technical (purity not stated) in arachis oil followed by epicutaneous induction with 10% w/w in arachis oil and epicutaneous challenge with 10% w/w in arachis oil. Groups of 10 controls given distilled water and vehicle were included. The animals were scored for sensitization 24 and 48 hours after challenge, according to the Magnusson and Kligman scale. No adverse reactions were noted at the test material sites of the test animals or the vehicle control sites of the control animals at 24 and 48 hours of observation. It was concluded that imazalil technical was non-sensitizing when tested according to the Magnusson and Kligman method (Dreher, 1990e).

In a dermal sensitization study, 20 male Pirbright guinea-pigs were induced by intradermal injection of 1% imazalil technical (purity 98.5%) in sesame seed oil followed by epicutaneous induction with 10% powder in petrolatum and epicutaneous challenge with 5% powder in petrolatum. Groups of 20 controls given the solvent and vehicle were included. The animals were scored for sensitization 48 and 72 hours after challenge according to the Magnusson and Kligman scale. The sensitization rate was 5% (one of 20 induced guinea-pigs showed a positive response at 48 hours following challenge; the same guinea-pig also had a positive response at 72 hours after challenge). It was concluded that imazalil technical had only a slight sensitizing potential when tested according to the Magnusson and Kligman method (Teuns et al., 1990c).

In a dermal sensitization study using the Buehler protocol, 0.4 g imazalil technical (purity 98.94%) was moistened with 95% ethanol and topically applied to the left side of each of 10 Hartley albino guinea-pigs. Exposure was for 6 hours, and this procedure was repeated on a once-a-week basis

for 3 consecutive weeks. Following a 2-week rest period, challenge doses of the test material moistened with acetone were applied to the right side of each of these 10 previously exposed guinea-pigs and an additional group of five previously unexposed guinea-pigs. None of the 15 guinea-pigs showed a positive response (Wnorowski, 1997).

(g) Single-dose toxicity study to derive an ARfD

In a newly submitted single-dose oral toxicity study conducted to facilitate the derivation of an ARfD, two parallel sets of Wistar rats (five of each sex per dose) were gavaged with a single dose of imazalil (purity 98.8%) at 0, 25, 100 or 400 mg/kg bw. The vehicle was polyethylene glycol 400. One set of animals (main group: control group and three treated dose groups) was terminated at 24 hours for examination of acute effects, whereas the parallel set (recovery group: control group and three treated dose groups) was subjected to termination at 7 days post-administration for examination of delayed effects or recovery. Mortality was assessed at least twice daily. Clinical signs were assessed on all animals at 1–2 and 4–5 hours after dosing and daily up to and including the day of necropsy. Necropsy was performed on day 2 for the main group animals and on day 8 for the recovery group animals. Body weights were recorded on days 1, 2, 4, 7 and 8. Feed consumption was recorded for days 1–2, 2–5 and 5–7. Neurobehavioural examinations were made on all recovery group males prior to treatment and at 4 hours, 24 hours and 7 days after treatment. Clinical laboratory investigations were made on day 2 for the main group animals and on day 8 for the recovery group animals. Haematology included assessment of white blood cells, differential leukocyte count, red blood cells, reticulocytes, red blood cell distribution width, haemoglobin, haematocrit, mean cell volume, mean cell haemoglobin, mean cell haemoglobin concentration and platelet count. Prothrombin time and activated partial thromboplastin time were determined. Clinical biochemistry included determination of liver enzymes (alanine aminotransferase [ALT], aspartate aminotransferase [AST], ALP), total protein, albumin, bilirubin, bile acids, urea, creatinine, glucose, cholesterol, triglycerides, phospholipids, electrolytes and inorganic phosphate. At necropsy, all animals were investigated for macroscopic abnormalities, and samples of all organs were subjected to histopathological examination. Organ weights were recorded for adrenal glands, ovaries, brain, prostate, epididymides, seminal vesicles, heart, spleen, kidneys, testes, liver, thymus, lungs and thyroid.

No preterminal mortalities were observed. No toxicologically relevant changes were noted in general clinical observations, body weights, feed consumption or macroscopic examination. Formulation analyses confirmed that formulations of test substance in polyethylene glycol 400 were prepared accurately and distributed homogeneously and were stable over at least 6 hours. At the high dose of 400 mg/kg bw, neurobehavioural observations revealed abnormal posture, piloerection, loss of righting reflex, hunched posture and slow breathing at 4 hours post-dosing. Body temperature was decreased by 1.1 °C compared with controls. At 100 mg/kg bw, one animal showed piloerection. No other signs were noted at any dose tested during later observations. Owing to the transient and incidental character of these findings, they were not considered to be toxicologically relevant. No toxicologically relevant effects on haematological parameters were observed that could be attributed to treatment. Decreased AST activity was noted at the end of the recovery period for both sexes at the high dose but was considered to be not relevant, as liver toxicity would be expected to have the opposite effect. At 400 mg/kg bw, clinical biochemistry changes consisting of lower glucose and urea levels and higher triglyceride and sodium levels in both sexes were seen 24 hours post-dosing. Cholesterol and potassium levels were decreased in males only. At the middle dose (100 mg/kg bw), changes at 24 hours post-dosing were confined to lower urea levels in females and decreased cholesterol and phospholipids in males. These alterations were not considered to be toxicologically relevant, as they were transient, not dose related or just naturally variable (as was apparent in the recovery groups). Statistically significant increases in absolute and relative liver weights were noted at the end of the entire study and were considered to be treatment related. Relative liver weight in high-dose males was increased by 10%. Absolute and relative liver weights in females were increased by 16% and 19% at 100 and 400 mg/kg bw, respectively. At the end of recovery, relative liver weight in high-dose females was still statistically significantly increased by 13%. Minimal or slight

hepatocellular vacuolation was found in most females at 100 and 400 mg/kg bw at both the 24-hour and 7-day terminations. Similar findings were made in high-dose males after 7 days. All other findings were considered to be within the normal background ranges encountered for Wistar-Han strain rats.

The NOAEL was 100 mg/kg bw, based on the increased incidence of slight hepatocellular vacuolation in males and females and increased liver weights in females at 400 mg/kg bw (Teunissen, 2013).

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

In a 90-day toxicity study not previously reported by JMPR, imazalil (purity not specified) was administered in the diet to groups of 10 albino Swiss mice of each sex per dose at a concentration of 0, 200, 400 or 800 parts per million (ppm) (equivalent to 0, 30, 60 and 120 mg/kg bw per day, respectively). General health and behaviour were checked daily, body weight was measured weekly and feed intake was measured weekly. Blood for haematological and clinical chemical investigations was collected at the end of the study. At termination of the study, the animals were examined postmortem, organ weights were recorded and gross and micropathological examinations were carried out on livers from all animals after dissection.

There were no treatment-related effects on mortality or clinical signs of toxicity. At termination, decreased body weights (by 12% compared with controls) and body weight gains (30% of controls) were observed in female mice at 800 ppm. Feed consumption was decreased in females at 800 ppm. Statistically significant changes in haematological parameters were observed in high-dose females, but these changes were minor and considered to be toxicologically not relevant. The following clinical chemistry parameters were statistically significantly different compared with controls: decreased cholesterol in females at 400 ppm and in both sexes at 800 ppm; decreased albumin and phospholipids in males at 400 ppm and in both sexes at 800 ppm; decreased bilirubin in both sexes at 400 and 800 ppm; and decreased AST in females at 800 ppm. Increases in relative liver weights were observed in males at 400 ppm ($P < 0.05$) and in females at 800 ppm ($P < 0.001$), and increases in absolute and relative liver weights were observed in males at 800 ppm. Vacuolar degeneration (small and large vacuolation) of hepatocytes was observed in male mice at 200 ppm and in both sexes at 400 and 800 ppm.

No NOAEL could be identified in this study, as hepatocytic vacuolation was seen at all doses in males (Verstraeten et al., 1993a).

Rats

In a 4-week range-finding toxicity study not previously evaluated by JMPR, imazalil (purity 98%) was administered via the diet to Fischer 344 rats (10 of each sex per dose) at a concentration of 0, 100, 1000, 2000 or 3000 ppm (equal to 0, 11.5, 116.7, 232.1 and 351.3 mg/kg bw per day for males and 0, 11.7, 124.0, 229.0 and 351.6 mg/kg bw per day for females, respectively). Haematological and clinical chemistry parameters were not evaluated. Urine analysis was not performed.

No clinical signs of toxicity were observed. No mortality occurred during the study. Reduced body weights were observed in males (10–12%, 14–19% and 22–28% at 1000, 2000 and 3000 ppm, respectively) and in females (8–10% and 15–20% at 2000 and 3000 ppm, respectively). Feed consumption was also reduced in males at 1000, 2000 and 3000 ppm and in females at 2000 and 3000 ppm. The absolute weights of the heart, spleen, kidneys and testes were lower in males at 1000, 2000 and 3000 ppm. In females, the absolute weights of all organs except the liver were lower at 3000 ppm, and the absolute weights of the spleen, kidneys, adrenals and ovaries were lower at 2000 ppm. The

absolute weights of the liver in females were increased at 1000, 2000 and 3000 ppm. At necropsy, reduced testes size was observed in males at 2000 and 3000 ppm.

The NOAEL was 100 ppm (equal to 11.5 mg/kg bw per day), based on decreased body weight, feed consumption and heart, spleen, kidneys and testes weights in males and increased liver weights in females at 1000 ppm (equal to 116.7 mg/kg bw per day) (Gur, Nyska & Crown, 1990).

In a 13-week dietary toxicity study, imazalil (purity 98%) was administered to Fischer 344 rats (20 of each sex per dose) at a concentration of 0, 40, 200 or 1000 ppm (equal to 0, 4.02, 18.78 and 94.58 mg/kg bw per day for males and 0, 4.05, 20.28 and 99.34 mg/kg bw per day for females, respectively). General health and behaviour were checked daily, and body weight and feed consumption were measured weekly. Water consumption was measured in weeks 1, 2, 7 and 10. Ophthalmology was performed on 10 rats of each sex per dose prior to commencement of treatment and then at 12 weeks. Blood for clinical chemistry and haematological investigations was collected in week 11 from 10 rats of each sex per dose, as was urine for urine analysis. At termination of the study, the animals were examined postmortem, and organs were processed and examined histologically.

No treatment-related mortality occurred. No clinical signs related to treatment were observed. Rats of the high-dose group (1000 ppm) showed a statistically significantly lower body weight than the controls from weeks 2 to 7 in the males (8–12%) and from weeks 2 to 5 in the females (6–7%) and consumed less feed than the control rats during weeks 1 and 8 in the males and sporadically in the females. Water intake was depressed in both sexes of the high-dose group. Ophthalmic examination showed no differences between the high-dose and control groups. The high-dose females excreted more urine and slightly alkaline urine compared with controls. No treatment-related effects on haematological parameters were observed. Among the females, declines in the activities of ALT (at 1000 ppm) and AST (at 200 and 1000 ppm) were detected. Gamma-glutamyl transferase (GGT) activity was increased in females at the high and intermediate doses, whereas bilirubin concentration was increased at the high dose only. Potassium levels were decreased in all treated groups of females. Among high-dose males, there were increases in GGT activity and urea concentration (Table 3).

Liver weights in both males and females at 1000 ppm were statistically significantly higher than in the controls (absolute: approximately 12% and 15% in males and females, respectively; relative: approximately 16% and 17% in males and females, respectively). The kidney weights (absolute and relative to body weight) were higher in females at 1000 ppm (6–8%), but there were no macroscopic or microscopic findings in the kidneys. No treatment-related lesions were detected at macroscopic examination. Hepatic dose-related lesions associated with treatment with imazalil were detected microscopically in males at 200 and 1000 ppm and in females at 1000 ppm. These lesions consisted of slight centrilobular hepatocytic hypertrophy associated with an increase in cytoplasmic eosinophilia (most probably representing an adaptive proliferative response of the smooth endoplasmic reticulum), focal or midzonal fine and large fatty vacuolation and a decrease in the severity of the cytoplasmic anoxic vacuolation. The maximum tolerated dose was not achieved in this study.

The NOAEL was 40 ppm (equal to 4.02 mg/kg bw per day), on the basis of liver histopathology and associated clinical chemistry changes at 200 ppm (equal to 18.78 mg/kg bw per day) (Gur, Nyska & Waner, 1991).

In a 14-week, non-GLP-compliant toxicity study, groups of Wistar rats (10 of each sex per group) were fed imazalil nitrate (purity not specified) at a concentration of 0, 5, 20 or 80 mg/100 g feed (equal to 0, 4.2, 16.0 and 64.0 mg/kg bw per day for males and 0, 4.4, 17.4 and 69.4 mg/kg bw per day for females, respectively).

Table 3. Changes in selected clinical chemistry parameters in rats following 13-week exposure to imazalil in the diet[a]

Dietary concentration (ppm)	ALP (IU/L)	ALT (IU/L)	AST (IU/L)	GGT (IU/L)	CPK (IU/L)	Urea (mmol/L)	Bilirubin (µmol/L)	Potassium (mmol/L)
Males								
0	141	29	52	0.28	295	7.25	3.48	3.4
	(11)	(3)	(6)	(0.39)	(109)	(0.83)	(0.72)	(0.2)
40	140	29	45	0.23	247	7.06	4.12	3.5
	(8)	(2)	(4)	(0.34)	(68)	(0.43)	(0.88)	(0.2)
200	135	27	51	0.54	279	7.20	3.37	3.5
	(4)	(3)	(9)	(0.60)	(98)	(0.59)	(1.05)	(0.40
1 000	134	27	45	1.78***	367	8.18***	3.19	3.3
	(15)	(3)	(8)	(0.76)	(77)	(0.76)	(0.85)	(0.2)
Females								
0	98	33	64	1.64	404	7.98	2.34	3.8
	(5)	(11)	(10)	(1.11)	(108)	(0.49)	(0.32	(0.3)
40	94	31	63	1.42	335	7.68	2.22	3.5***
	(7)	(8)	(12)	(1.08)	(43)	(0.89)	(0.54)	(0.2)
200	92	28	47***	3.41**	223***	7.81	2.23	3.6
	(8)	(8)	(6)	(0.94)	(49)	(0.69)	(0.47)	(0.2)
1 000	93	22*	47***	5.11***	430	8.22	3.35***	3.4**
	(6)	(2)	(6)	(1.16)	(101)	(0.68)	(0.77)	(0.2)

ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; CPK: creatine phosphokinase; GGT: gamma-glutamyl transferase; IU: international units; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

[a] Standard deviations provided in parentheses.

Source: Gur, Nyska & Waner (1991)

Appearance, behaviour, survival, feed consumption and urine analysis were not affected at any level. Body weights of the 80 mg/100 g feed group were slightly depressed (<5%). Haematological and blood chemistry values were within normal limits, except for an increase in bilirubin in both sexes in the 20 mg/100 g feed group ($P < 0.001$) and the 80 mg/100 g feed group ($P < 0.01$). The changes in bilirubin were minor and not considered to be toxicologically relevant. Decreases in specific gravity and creatine levels were observed in males at 80 mg/100 g feed. Organ weights were not affected by the treatment. Histological examination revealed no changes that could be attributed to imazalil in any organs or tissues except the livers of the 80 mg/100 g feed group, which showed a fatty reaction localized in the midzonal to centrilobular areas and vacuolation coincident with fatty spots in Scarlet red–stained sections.

The NOAEL was 20 mg/100 g feed (equal to 16.0 mg/kg bw per day), on the basis of liver toxicity manifested as a fatty reaction localized in the midzonal to centrilobular areas and vacuolation coincident with fatty spots in Scarlet red–stained sections at 80 mg/100 g feed (equal to 64.0 mg/kg bw per day) (Marsboom et al., 1972).

In a 90-day feeding study in rats, imazalil base was administered in the diet to groups of 10 male and 10 female Wistar rats at a concentration of 0, 800, 1600, 2400 or 3200 ppm. As a result of feed wastage, most likely resulting from poor palatability of the feed, it was not possible to accurately calculate the doses in units of milligrams per kilogram of body weight per day. Nevertheless, the

following doses were estimated by the study authors: 0, 64.4, 129, 181 and 252 mg/kg bw per day for males and 0, 78.7, 150, 236 and 333 mg/kg bw per day for females, respectively. The following parameters were evaluated: mortality, clinical signs, body weight, feed consumption, haematology, clinical chemistry, urine analysis, gross necropsy and organ weights. Livers and macroscopic lesions were examined for all animals in all treatment groups, but no other organs or tissues were histologically examined. Electron microscopy of liver sections was also conducted.

No treatment-related effects on mortality or clinical signs were observed. Compared with control values, dose-related decreased body weights and body weight gains were observed in treated animals of both sexes throughout the study at all doses. At 13 weeks for 3200 ppm animals, body weights were decreased by 19% in males and 11% in females, and body weight gains were decreased by 26% in males and 19% in females. In males, body weights were 9%, 11% and 19% lower than the control values at 800, 1600 and 2400 ppm, respectively. In females, decreased body weights were 4%, 8% and 10% lower than the control values at 800, 1600 and 2400 ppm, respectively. Decreases in feed consumption were noted at all doses, but the decrease in feed consumption was minor at 800 ppm in both sexes. Haematological parameters were not affected. The following minor changes in clinical chemistry parameters observed at 3 months were possibly related to the test material: decreased triglycerides in males at all treatment levels and in females at 3200 ppm; decreased phospholipids in males at all treatment levels; decreased blood urea nitrogen in males at 1600, 2400 and 3200 ppm; and decreased albumin in females at 2400 and 3200 ppm. Urine analysis was unremarkable. Gross necropsies at 3 months revealed increased incidences of dark-coloured livers and more pronounced lobulation in the liver, particularly in males at 2400 and 3200 ppm and in females at all doses. Relative liver weights were increased in males at all doses. Histopathological examination revealed treatment-related hepatocellular hypertrophy in nearly all treated animals at all doses. This hypertrophy was mild, and there was essentially no difference between treatment groups in incidence or severity. Mild fatty vacuolation was observed in males at doses of 1600 ppm and higher and in females at all doses.

No NOAEL could be identified. The lowest-observed-adverse-effect level (LOAEL) was 800 ppm (equal to 64.4 mg/kg bw per day), based on decreased body weights and body weight gains in males and females, decreased triglycerides and phospholipids in males, dark and more pronounced lobulation of livers in females, increased relative liver weights in males, mild hepatocellular hypertrophy in males and females, and mild fatty vacuolation in the livers of females (Van Deun et al., 1996a).

Imazalil base (purity 98.1%) was administered in the diet to groups of 10 Cpb:WU Wistar rats of each sex for 6 months, as part of a lifetime study summarized below (Lina et al., 1984; Til et al., 1985). Imazalil was admixed with the diet at a concentration of 0, 25, 100 or 400 ppm (equivalent to 0, 1.25, 5.0 and 20 mg/kg bw per day, respectively). General health and behaviour were checked daily, body weight was measured weekly for 12 weeks and then once every 2 weeks, and feed intake was measured weekly. Blood for haematological investigations was collected on day 174 from males and on day 175 from females. Urine was collected on day 178 for analysis. At termination of the study (on day 183 for males and on day 184 for females), the animals were examined postmortem, blood samples for clinical chemistry were taken, and organs were processed and examined histologically.

No treatment-related deaths or abnormal behaviours were noted. There were no treatment-related effects on body weight gain or feed intake. The leukocyte count was increased in males at 400 ppm in the absence of any statistically significant change in the differential leukocyte count; the effect is thus of dubious toxicological relevance. Lactate dehydrogenase activity was increased in females at the highest dose. Blood glucose concentration was increased in females at all doses, but without a dose–response relationship. No statistically significant differences between groups were seen in urinary parameters. Males at 400 ppm had increased relative kidney weights, and females had increased absolute and relative kidney and liver weights. Females at 400 ppm also showed increased

absolute thymus weight and relative lung weight. Gross and microscopic examination of the organs showed no effects that could be ascribed to treatment.

The NOAEL was 100 ppm (equivalent to 5.0 mg/kg bw per day), on the basis of changes in liver, kidney, lung and thymus weights in females and increased relative kidney weight in males at 400 ppm (equivalent to 20 mg/kg bw per day) (Lina et al., 1983).

Dogs

Groups of four male and four female beagles were given imazalil base (purity 98.8%) orally in gelatine capsules at a dose of 1.25, 2.5 or 20 mg/kg bw per day for 1 year, whereas four controls of each sex received the capsules alone. The animals were observed twice daily and were examined for clinical signs weekly. An ophthalmic examination was carried out prior to dosing, every 6 months and at the end of the study. An electrocardiogram and heart rate were recorded before the start of the study, at weeks 6, 14, 29 and 38, and at the end of the study. Body weights were recorded weekly. Haematological and clinical chemistry parameters were measured 2 weeks after the start of dosing and monthly thereafter. Urine was analysed 1 month after the start of dosing and then every 3 months. At the end of the study, all the animals were killed, selected organs were weighed, and gross and microscopic examinations were carried out. For statistical analysis of the histopathological findings, the two sexes were treated together.

All animals survived to termination. Clinical effects consisting of soft faeces, increased salivation, vomiting and decreased appetite were observed. No treatment-related ocular changes were observed at 20 mg/kg bw per day. No adverse effects of treatment on electrocardiogram values, heart rate, urinary parameters or haematological parameters were observed. Decreased weight gain and feed consumption were found only at the high dose. The only clinical chemical abnormalities observed were decreased serum calcium ($P < 0.05$) and substantially increased serum ALP activity ($P < 0.05$) at 20 mg/kg bw per day. Liver weights (absolute and relative to body weight) were increased only in the high-dose group. No treatment-related change was seen on macroscopic or microscopic examination; in particular, the histological appearance of the liver did not differ between treated and control groups.

The NOAEL was 2.5 mg/kg bw per day, on the basis of clinical signs, decreased body weight gain and feed consumption, decreased serum calcium concentration, increased serum ALP activity and increased liver weights at 20 mg/kg bw per day (Verstraeten et al., 1989).

Groups of three male and three female beagles were given imazalil base (purity 81.6–101.4%) orally in gelatine capsules at a dose of 1.25, 5 or 20 mg/kg bw per day for 24 months, and three controls of each sex received the capsules alone. The animals were observed daily. An ophthalmic examination was carried out prior to dosing and at 3, 6, 9, 12, 18 and 24 months after dosing. An electrocardiogram and heart rate were recorded before the start of the study and at weeks 4, 8, 12, 16, 20, 24, 36, 74 and 104. Body weights were recorded weekly. Haematological, clinical chemical and urinary parameters were measured prior to dosing and again after 2, 4, 8, 12, 16, 20, 24, 36, 48, 74 and 104 weeks of dosing. At the end of the study, all the animals were killed, selected organs were weighed, and gross and microscopic examinations were carried out.

There was no mortality in the study. At the high dose, decreased appetite in all animals and frequent and abundant salivation and sporadic emesis were observed. At 5 mg/kg bw per day, a slight decrease in appetite was seen during the first 2 months of the study. Feed consumption could not be measured accurately because of wastage. However, the feed consumption was definitely lower at the high dose. Controls and the 1.25 mg/kg bw per day dosed dogs showed a normal and comparable body weight gain during the study. At 5 mg/kg bw per day, weight gain was slightly lower than in controls ($P < 0.05$; 23.9% of control gain). The 20 mg/kg bw per day group body weight gain was also lower ($P < 0.01$; 11.6% of control gain) than in the controls at 24 months. No treatment-related ocular changes were observed. No adverse effects of treatment on electrocardiogram values or heart rate, urinary parameters, haematological parameters, gross pathology or organ weights were observed.

Examination of tissues revealed no compound- or dose-related effects except for slight ground glass aspect of the cytoplasm in the centrilobular areas of the liver in the dogs receiving 5 or 20 mg/kg bw per day. At 20 mg/kg bw per day, decreased calcium levels and increased ALP were observed; however, they were not considered toxicologically relevant because the changes were very minor and within the historical range of the laboratory.

The NOAEL was 1.25 mg/kg bw per day, based on decreased body weight gain and slight ground glass aspect of the cytoplasm in the centrilobular areas of the liver at 5 mg/kg bw per day (Marsboom et al., 1977).

(b) Dermal application

Rabbits

In a dermal toxicity study, groups of five male and five female New Zealand white rabbits received imazalil (purity 98.5%) in sesame oil on the shaved back under a porous gauze dressing at a dose of 0, 10, 40 or 160 mg/kg bw per day, 6 hours per day, 5 days per week, for 3 weeks. The animals were observed daily, body weights were determined initially and then weekly, and feed consumption was determined weekly. Haematological, clinical chemistry and urinary parameters were measured at the end of the study. Irritancy was scored according to the Draize method. The animals were killed at the end of the study; the organs were weighed and examined grossly and microscopically.

No adverse effects on behaviour were observed, and there was no irritation in the controls and little in the treated groups. Erythema was observed with both the vehicle (sesame oil) and the test compound; the severity was similar in all groups. Body weight gain and feed consumption were similar in all groups. Haematological and clinical chemistry parameters were not affected by the treatment. No gross or microscopic adverse effects on the skin, kidney, liver or lung were seen at any dose. Urinary changes (reduced creatinine concentration, reduced specific gravity and reduced urobilinogen concentration) were seen only in males at the highest dose.

The NOAEL was 40 mg/kg bw per day, based on urinary changes (reduced creatinine concentration, reduced specific gravity and reduced urobilinogen concentration) seen in males at 160 mg/kg bw per day (Teuns et al., 1991).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a study of carcinogenicity, groups of 50 male and 50 female albino Swiss mice received imazalil sulfate (purity 98.3%) via the drinking-water at a concentration of 0, 6.25, 25 or 100 ppm (equivalent to 0, 2.5, 10 and 40 mg/kg bw per day, respectively) for 18 months. All animals were examined daily for signs of waning health, abnormal behaviour, unusual appearance, occurrence of untoward clinical effects, manifestations of toxic and pharmacological responses and survival. At termination, the surviving mice were examined grossly, and the lungs, liver, pancreas, kidneys, spleen, testis, epididymides, ovary, mammary gland, adrenals, hypophysis, lymph nodes and any other organ or tissue suspected to present a neoplasia were examined microscopically.

No dose-related effects on overall survival rate or on the time at which mortalities occurred were observed. There were no dose-related effects on health, behaviour or appearance. There were no effects on gross pathology and no discernible treatment-related increases in tumour incidence. However, owing to several shortcomings in study design (e.g. overall survival <50% for most groups; fighting and cannibalism occurring during the first 8 months of the study, especially in male groups, when animals were held in groups of 25), this study is not considered to be acceptable for the current evaluation (Marsboom & Herin, 1979a).

In a study of carcinogenicity, groups of 50 male and 50 female SPF albino Swiss mice received diets containing imazalil (purity 96.9%) at a concentration of 0, 50, 200 or 600 ppm (equal to 0, 8.1, 33.4 and 105 mg/kg bw per day for males and 0, 9.9, 41.6 and 131 mg/kg bw per day for females, respectively) for approximately 23 months. The mice were observed daily, and those in extremis were killed and examined grossly and postmortem. The animals were weighed at the start of the study, weekly for the first 12 months, and then monthly until immediately before termination. Feed consumption was recorded weekly for the first 12 months and then monthly. Haematological examinations were carried out at 12 months on samples from the tail vein and in those animals killed terminally by carotid exsanguination; additional haematological examinations were undertaken, when possible, on animals killed in extremis. When the leukocyte count was above 25 000/mm^3, a differential leukocyte analysis was carried out. Clinical chemistry was undertaken on carotid blood at termination. At termination, the surviving mice were examined grossly, and selected organs were weighed and examined grossly and microscopically.

Appearance and behaviour were not affected by treatment. At the end of the study, the survival rates among controls were 30% of males and 54% of females, and no significant differences were seen among treated groups. Decreased body weight and body weight gain were seen in males at 600 ppm from week 2 to the end of the study (5–10% decrease in body weight and 15–20% decrease in body weight gain) and in males at 200 ppm from time to time early in the study (statistical significance was not achieved). Females at all doses showed decreased body weight and body weight gain from time to time, but the effects were slight, infrequent and probably biologically non-relevant. Weekly or monthly feed consumption was increased in all groups of treated males at many time intervals, and total feed consumption (throughout the study) was increased among those at 50 and 600 ppm. Females in all treated groups showed occasional increases in weekly or monthly feed consumption. There was considerable feed wastage and no clear dose–response relationship; the relevance of the findings on feed intake are thus dubious.

At termination, males at the high dose had a significant decrease in body weight, which was accompanied by increases in absolute and relative liver weights (Table 4). An increase in relative brain weight at 600 ppm in males was probably a reflection of the decrease in body weight. Other changes in organ weights observed in males (increased relative pancreatic weight at 50 ppm) and females (decreased absolute and relative lung weights at the high dose, relative lung weight at 50 ppm and absolute and relative splenic weights at 200 ppm) did not appear to be related to treatment. Increases in relative heart weight at 200 ppm, decreases in relative kidney weight at 600 ppm and decreases in absolute and relative brain weights at 50 ppm, all in females, likewise appeared to be without biological relevance.

Table 4. Mean liver weight data for mice ingesting imazalil in the diet for up to 23 months

	Mean liver weight data[a]			
Organ	**0 ppm**	**50 ppm**	**200 ppm**	**600 ppm**
Males				
Absolute weight (mg)	2 388 ± 878	2 082 ± 611 (87)	2 402 ± 712 (101)	2 812 ± 1 044* (118)
Relative weight (mg/100 g)	5 685 ± 1 826	5 112 ± 1 512 (90)	5 798 ± 1 768 (102)	7 060 ± 2 521** (124)
Females				
Absolute weight (mg)	2 143 ± 627	2 264 ± 392 (106)	2 088 ± 458 (97)	2 359 ± 1 541 (110)
Relative weight (mg/100 g)	5 509 ± 1 246	5 581 ± 664 (101)	5 580 ± 1 162 (101)	6 269 ± 3 745 (114)

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

[a] Values in parentheses are percentage of control values.

Source: Verstraeten et al. (1993b)

Some differences in haematological parameters were found between groups at 12 months. In males, the erythrocyte volume fraction was increased at 200 ppm, as was the haemoglobin at 200 and 600 ppm. In females at 200 and 600 ppm, the erythrocyte volume fraction, haemoglobin concentration and erythrocyte count were increased, and the platelet count was decreased. By 23 months, no treatment-related differences in haematological end-points were found between groups. No difference in leukocyte count was seen at any time. No differences in clinical chemistry parameters were seen that could be ascribed to the test material. Statistically non-significant gross morphological changes, including foci and nodules, were seen in the livers of males at the intermediate and high doses. No treatment-related non-neoplastic changes were observed at 50 ppm in mice of either sex or in females at 200 ppm. The livers of males at 200 and 600 ppm and those of females at 600 ppm showed focal cellular changes consisting of large and small vacuoles and pigmented, swollen sinusoidal cells. Increased incidences of males with hepatic neoplasms were found at 200 and 600 ppm, with increased incidences of neoplastic hepatic nodules; a similar increase in the incidence of females with hepatic neoplasms was found at the high dose (Table 5) (Verstraeten et al., 1993b).

Table 5. Incidence of liver tumours recorded by study pathologist

	0 ppm	50 ppm	200 ppm	600 ppm	*P*[a]
Males					
No. examined	*50*	*49*	*50*	*50*	–
Hepatocytic neoplasms	13	10	25*	25*	0.000 2
Hepatic neoplastic nodules	8	5	23**	17*	0.000 6
Hepatocytic carcinoma	5	7	6	11	0.068 5
Females					
No. examined	*50*	*50*	*50*	*50*	–
Hepatocytic neoplasms	4	6	2	11*	0.023 0
Hepatic neoplastic nodules	4	6	0	10	0.047 5
Hepatocytic carcinoma	0	1	2	2	0.100 0

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (chi-squared test, one-tailed)
[a] *P*-value computed for positive trend (Peto analysis).
Source: Verstraeten et al. (1993b)

The slides from this study were subjected to three evaluations: by the study pathologist, by Dr Stephen Sparrow, an external and qualified pathologist (Sparrow, 1993), and by a working group. The results of the first evaluation were reported by Verstraeten et al. (1993b), those of all three evaluations by Hess (1996) and those of the last two by Butler (1995).

In the first evaluation, the incidence of hepatocytic carcinoma was not found to be increased, but the incidences of hepatic neoplastic nodules and total hepatic neoplasms were increased in males at 200 and 600 ppm, with a dose-related trend. In females, the incidence of hepatic neoplasms was increased at the high dose, with a trend for neoplastic nodules and all hepatic neoplasms. The main differences between the first and second readings of the slides were in nomenclature, in particular for hepatic neoplastic nodules. Sparrow (1993) considered that the study pathologist had used the term "hepatic neoplastic nodule" to describe changes that he would have described as adenoma and some other non-neoplastic lesions. Sparrow (1993) found evidence for a trend in the incidence of hepatocellular carcinoma in males. A report on the relevance of the liver tumours prepared by Hess (1996) included a tabulation of Sparrow's (1993) findings. In a further report on the relevance of the liver tumours by Butler (1995), based on the findings of the working group, it was concluded that

there was evidence of an increased incidence of adenomas, but not carcinomas, in males at the two highest doses and that there was no statistically significant trend for carcinomas (Table 6).

Table 6. Classification of liver neoplasms reported by Verstraeten et al. (1993b) according to the findings of a working group

	Males			Females		
Dietary concentration (ppm)	**No. of animals examined**	**Adenoma**	**Carcinoma**	**No. of animals examined**	**Adenoma**	**Carcinoma**
0	50	5	5	50	4	0
50	49	3	5	50	5	1
200	50	14*	3	50	0	2
600	50	14*[a]	8	50	9	1

ppm: parts per million; *: $P < 0.05$ (pairwise comparison, one-tailed chi-squared test)
[a] $P < 0.05$ for trend (Peto) test.
Source: Butler (1995)

The NOAEL for toxicity was 50 ppm (equal to 8.1 mg/kg bw per day), on the basis of morphological changes (foci and nodules) in the livers of males at 200 ppm (equal to 33.4 mg/kg bw per day). Liver adenomas were observed in males at 200 and 600 ppm and in females at 600 ppm. The NOAEL for carcinogenicity was 50 ppm (equal to 8.1 mg/kg bw per day), based on an increased incidence of adenomas seen in males at 200 ppm (equal to 33.4 mg/kg bw per day) (Verstraeten et al., 1993b).

Rats

Groups of Wistar rats (10 of each sex per dose per termination time) were fed a 50% wettable powder of imazalil (50% imazalil, 25% aerosol and 25% starch) at 0, 5, 20 or 80 mg/100 g feed (active ingredient) for 6, 12 and 24 months. The exact doses (reported for the 6-, 12- and 24-month groups) were 0, 3.0–3.8, 12.1–14.7 and 48.0–61.7 mg/kg bw per day for males and 0, 3.8–3.9, 14.7–17.0 and 57.6–63.9 mg/kg bw per day for females, respectively.

No effects of treatment on mortality, clinical signs, body weights, feed consumption, haematology, urine analysis, clinical chemistry or gross pathology were observed. At 6 months, the livers of many 80 mg/100 g feed rats, particularly females, showed centrilobular swelling and numerous large vacuoles. No evident fatty degeneration was seen, but a tendency to some fatty surcharge was noted. At 12 months, these changes were still present. In addition, livers of the high-dose females exhibited a tendency to more concentrated glycogen in the centrilobular areas. No other effects were noted that could be attributed to the compound administration. Except for liver and kidneys, no consistent changes in absolute or relative organ weights were noted in any group at any termination. At 6 months, the absolute and relative liver weights of the 80 mg/100 g feed females were statistically significantly increased. At 12 and 24 months, the absolute liver weights of females were not statistically significantly different.

In males receiving 20 and 80 mg/100 g feed, absolute and relative liver weights were increased statistically significantly at the 12-month termination, but not at the 24-month termination. At 6 and 24 months, absolute and relative kidney weights were statistically significantly increased in the 80 mg/100 g feed female group. This trend was present at 12 months, but was not of statistical significance. Males receiving this dose exhibited, at 6 months, a trend towards increased absolute kidney weights and a statistically significant increase in relative kidney weights. At 12 months, male kidney weights were statistically significantly higher than their control values, but at 24 months, this

difference was not evident. Haematological and blood chemistry revealed no consistent changes, except that at 6 months, bilirubin values for the female 20 and 80 mg/100 g feed groups were statistically significantly elevated. This trend continued throughout the study in the 80 mg/100 g feed females, but was not statistically significant at the 12- and 24-month terminations. Males did not exhibit this trend at any level during the study.

A NOAEL was not identified in this study, because the results described are based on selected organs and various study durations (Marsboom at al., 1975).

In an 18-month study of toxicity, groups of 20 male and 20 female Wistar Cbp:WU rats were given diets containing imazalil (purity 98.1%) at a concentration of 0, 25, 100 or 400 ppm (equivalent to 0, 1.2, 5 and 20 mg/kg bw per day, respectively). The animals were observed daily and more thoroughly every 2 weeks. Body weight was recorded at the start of the study, weekly for 12 weeks, and every 2 weeks for the remainder of the study. Feed consumption was measured weekly. Blood was collected from the tail vein for haematological examination on days 538–539. Urine samples from all animals were analysed on day 541. At necropsy, blood was collected from 10 animals per group for clinical chemistry. At the end of the study, the animals were killed and examined postmortem. Selected organs were removed, weighed and examined grossly and microscopically.

General health, behaviour and survival were not affected by treatment. Females at the high dose had decreased weight, particularly in the second half of the study, and these animals showed reduced feed intake at a few times. One minor change was seen in haematological variables – namely, an increased thrombocyte count in females at the high dose. The plasma albumin concentration was decreased in males at the high dose, but no other treatment-related changes in clinical chemistry parameters were seen. Urinary parameters were unaffected. The absolute weight of the adrenals was increased in females at the intermediate and high doses, and the relative weights of the adrenals, kidneys, heart and brain were increased in females at the high dose. The relative adrenal weights were also increased in the other treated groups of females. Treatment-related pathological effects were seen in the livers of males at the high dose, involving an increase in the lobular pattern and periportal cytoplasmic vacuolation of hepatocytes. Multivacuolar hepatocytes were seen more frequently in groups at the high dose than at other doses. Intracytoplasmic inclusion bodies were seen. There was no evidence of treatment-related neoplasia. Staining with periodic acid–Schiff and oil red O showed that the vacuoles contained neither starch nor fat.

The NOAEL was 100 ppm (equivalent to 5 mg/kg bw per day), on the basis of decreased weight gain in females, decreased plasma albumin concentration in males and pathological changes in the livers of males at 400 ppm (equivalent to 20 mg/kg bw per day) (Lina et al., 1984). Although there was no evidence that imazalil was carcinogenic, the duration of the study and the number of animals used were insufficient to exclude that possibility.

Groups of Wistar rats (50 of each sex per group) were administered imazalil (purity 101.2%) in the diet at a concentration of 0, 2.5, 10 or 40 mg/100 g feed (equivalent to 0, 2.5, 10 and 40 mg/kg bw per day) for 24 months. The animals were 3–4 months of age at the initiation of the carcinogenicity study. Animals were examined daily for behavioural changes and toxicity. At the conclusion of the study, gross and microscopic examinations were conducted on all surviving animals. During the course of the study, examinations were performed on animals that had died or were terminated in extremis. Microscopic examinations were conducted on the major tissues and organs and on all lesions that were noted during the course of the gross examinations.

There was substantial mortality over the course of the study, and very few animals survived the full 24 months. It was considered that this was due to the fact that the animals were 3–4 months old at the start of the study and that they were not maintained under SPF conditions. Evaluation of the data relating to the time on study at which animals died did not suggest that imazalil contributed to the increased mortality or the accelerated death rate. There were no dose-related events noted on gross pathology.

There were no significant differences with respect to the overall tumour rate or the individual types of tumours reported in any of the dose groups. There is no evidence that imazalil was responsible for tumour formation in any organ or tissue examined in the study.

A NOAEL was not identified in the study because of the high mortality rate and accelerated death rate, unlikely due to treatment with imazalil (Marsboom & Herin, 1979b).

In a 24-month study, groups of 50 SPF Wistar (Hannover) rats of each sex received diets containing a preparation called "imazalil 50 premix", which nominally contained 50% imazalil (in fact, 49–50% imazalil, 25% aerosol and 25% corn starch) at a concentration of 0, 50, 200, 1200 or 2400 ppm (equal to 0, 2.4, 9.7, 58 and 120 mg/kg bw per day for males and 0, 3.4, 13.5, 79 and 157 mg/kg bw per day for females, respectively). The animals were observed at least daily. Ophthalmic examinations were carried out in controls and in animals at the highest dose before dosing and towards the end of the treatment. Body weight was measured on the first day of dosing, then weekly and at the end of dosing and additionally in any animal killed in extremis. Feed consumption was measured weekly. Haematological and clinical chemistry parameters were measured in blood samples from all groups at 6, 12 and 18 months and near the end of dosing. At the same time, urine was analysed. Immediately before the terminal kill at 24 months, each rat was examined. A full necropsy was undertaken on all animals, and macroscopic abnormalities were noted. Selected organs were removed and weighed, and samples of tissue were taken and processed for histopathological examination. Blood samples were taken at necropsy for measurement of testosterone, luteinizing hormone, thyroid stimulating hormone (TSH), thyroxine (T_4) and triiodothyronine (T_3) by enzyme immunoassay, the assays for TSH and luteinizing hormone being rat specific. Toxicokinetics was determined for the first four rats at each dietary concentration other than the controls in blood samples taken on days 180, 362 and 726 of the study. Imazalil was measured in these blood samples.

The mortality rate was not affected by the treatment. Increased feed wastage was observed for males at the highest dietary concentration and for females at the two highest concentrations. Females at the highest concentration showed a decrease in subcutaneous tissue mass and alopecia. No statistically significant difference was seen between animals at the highest concentration and controls in the ophthalmic examination. The body weights of males at 50 ppm were lower than those of controls at weeks 96, 99, 101 and 102, and their body weight gain was decreased during weeks 90–94 and weeks 96–104. An increase in body weight gain was seen in males at 200 ppm in week 1, but no other effect on body weight or body weight gain was seen. Decreased body weight and body weight gain were seen in males at 1200 ppm, consistently from week 1 to week 26 and less so thereafter. In males at 2400 ppm, body weight and body weight gain were decreased throughout the study (by 15–25%). The body weights of females at 50 ppm were lower than those of controls at weeks 82, 84, 85, 87, 88, 100 and 101, and body weight gain was decreased in weeks 54, 73, 78, 81–89 and 100–104. A decrease in body weight gain was seen in females at 200 ppm in week 34, with no other statistically significant change in body weight or body weight gain at any other time. Decreases in body weight were seen in females at 1200 ppm from the third week of the study and in body weight gain from the first week. In females at 2400 ppm, body weight and body weight gain were decreased throughout the study (by 25–35%) (Table 7). Feed consumption was decreased in weeks 23, 52, 93 and 96 for males at 50 ppm and in weeks 53, 90, 93 and 95 for males at 200 ppm. Decreased feed consumption was seen at 1200 ppm intermittently throughout the study and at 2400 almost throughout the study. Total feed intake of males throughout the study was decreased at the two highest dietary concentrations. Females at 50 ppm had increased feed consumption in week 27 and decreased feed consumption in weeks 52, 83 and 88. In females at 200 ppm, increased feed consumption was seen in weeks 10, 11 and 35–37, and decreased feed consumption in weeks 51, 83 and 93. The feed consumption of females at 1200 and 2400 ppm was decreased almost throughout the study. As for males, total feed intake was decreased at the two highest dietary concentrations, and feed conversion was increased by 10% and 12% at 1200 ppm and 2400 ppm, respectively.

Table 7. Mean body weight of rats in a 24-month study

	Mean body weight (% of vehicle control)									
	Males					Females				
Week	0 ppm	50 ppm	200 ppm	1 200 ppm	2 400 ppm	0 ppm	50 ppm	200 ppm	1 200 ppm	2 400 ppm
4	100	98.9	99.6	93.8***	85.1***	100	100	98.3	94.9***	90.4***
13	100	97.7	98.5	93.7***	85.9***	100	99.6	99.6	94.2***	90.7***
26	100	98.2	99.1	95.5**	87.5***	100	99.6	98.0	93.5***	88.2***
52	100	98.8	99.4	97.2	89.6***	100	97.1	96.4	89.9***	86.0***
78	100	99.0	99.2	97.3	88.0***	100	94.6	95.9	82.2***	77.1***
104	100	96.0	97.1	96.0*	85.3***	100	94.3	95.5	81.5***	75.2***

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Mann-Whitney *U*-test, two-tailed)
Source: Van Deun (1999a)

In males at the two highest dietary concentrations, decreased erythrocyte volume fraction, mean cell volume and mean cell haemoglobin were found consistently, with decreased haemoglobin concentration at weeks 78–79 and 104–105. Minor changes, including decreased mean cell volumes at weeks 25 and 52, were seen in males at 200 ppm. The haematological picture in females was more complex: increases in haemoglobin concentration at weeks 25–26 and 78–79 and increases in erythrocyte count at weeks 25–26, 52–53 and 78–79 were observed in animals at dietary concentrations of 200, 1200 and 2400 ppm, respectively. Additionally, an increase in erythrocyte count was seen at weeks 104–105 in rats at the highest dietary concentration. Decreased mean cell volume and mean cell haemoglobin concentration were seen at all times in females at 1200 and 2400 ppm and at weeks 25–26 and 52–53 in females at 200 ppm. Thus, imazalil at a dietary concentration of 50 ppm did not affect haematological parameters, minor changes were seen at 200 ppm, and definite changes were seen at 1200 and 2400 ppm.

A number of differences between groups were observed in clinical chemistry parameters, many of which were transient or inconsistent. The more consistent changes included decreased activity of ALP and AST in animals of both sexes at all dietary concentrations. Of greater clinical relevance were decreases in calcium and total protein concentrations observed at most times in animals at 1200 and 2400 ppm. Increased glucose concentrations were observed at all times in males and females at the highest dietary concentration; at weeks 52–53 and 104–105 in males and at weeks 52–53, 78–79 and 104–105 in females at 1200 ppm; and at weeks 25–26, 52–53 and 78–79 and 104–105 in females at 200 ppm. Triglyceride and phospholipid concentrations were decreased in males at 2400 ppm and in females at the two highest dietary concentrations at all times. The blood urea nitrogen concentration was decreased in males at the two highest dietary concentrations at weeks 25–26, 52–53 and 78–79 and 104–105 and in females at concentrations above 200 ppm. Decreases in ALP, AST and ALT activities and blood urea nitrogen concentration are generally considered not to be adverse. However, the changes in calcium and total protein, which were observed at most times in animals at 1200 and 2400 ppm, may be clinically relevant. Furthermore, the increases in blood glucose concentration seen at 2400 ppm in both sexes, at 200 ppm in females at all times, and from time to time in both sexes at 1200 ppm are likely to be clinically relevant.

The urine of females showed decreased specific gravity at some times, with increased pH and increased urinary volume at the two highest dietary concentrations.

The testosterone and luteinizing hormone concentrations were little altered in male rats, a significant increase in testosterone being observed only at 1200 ppm. The TSH concentrations tended to be higher at 200 and 1200 ppm, the T_4 concentrations were definitely lower in males at 1200 and 2400 ppm, and the T_3 concentrations were similar in all groups. In females, the T_3 concentration was decreased at the two highest dietary concentrations, T_4 was decreased at 1200 ppm only, whereas TSH

was not consistently affected. Despite these inconsistent results, it seems likely that imazalil affected the pituitary–thyroid axis when given at a dietary concentration of 1200 or 2400 ppm.

The organ weights of animals killed at term after receiving the highest dietary concentration showed some differences from controls, but these were considered to be a reflection of changes in total body weight, with the following exceptions. In males, relative liver weight was increased at the two highest dietary concentrations, with no effect on absolute liver weight. In females, absolute liver weight was decreased at 2400 ppm, and relative liver weight was increased at 200 ppm and above. At termination, in males, absolute thyroid weight was increased at 1200 and 2400 ppm, and relative thyroid weight was increased at all dietary concentrations, whereas in females, absolute thyroid weight was decreased at 1200 and 2400 ppm, and relative thyroid weight was unaffected (Table 8). The sponsor argued that it was appropriate to include decedents in the analysis of organ weights, as the effects on the thyroid were unlikely to be fatal (Tables 9 and 10).

Table 8. Thyroid weights of rats killed at termination after receiving diets containing imazalil for 2 years

Thyroid weight	0 ppm	50 ppm	200 ppm	1 200 ppm	2 400 ppm
Males					
Absolute (mg)	44	51	101	76*	67*
Relative (mg/kg bw)	84	103*	202*	154*	145***
Terminal body weight (g)	*522*	*499*	*509*	*503*	*449****
Females					
Absolute (mg)	42	35	46	31**	26***
Relative (mg/kg bw)	128	112	153	112	104
Terminal body weight (g)	*328*	*310*	*315*	*273****	*251****

bw: body weight; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Mann-Whitney *U*-test, two-tailed)
Source: Van Deun (1999a)

Table 9. Thyroid weights of rats killed before the end of the study after receiving diets containing imazalil for 2 years

Thyroid weight	0 ppm	50 ppm	200 ppm	1 200 ppm	2 400 ppm
Males					
Number examined	*8*	*12*	*9*	*11*	*5*
Absolute (mg)	42	34	38	53	39
Relative (mg/kg bw)	107	85	92	130	112
Females					
Number examined	*18*	*14*	*15*	*10*	*13*
Absolute (mg)	32	28	34	70	26
Relative (mg/kg bw)	125	109	138	322	128

bw: body weight; ppm: parts per million
Source: Van Deun (1999a)

The study author concluded that the increased relative liver weight observed at 200 ppm in females, unaccompanied as it was by a decrease in absolute body weight, was an incidental finding.

Further, the increase in relative thyroid weight seen at 50 and 200 ppm in males, in the absence of absolute thyroid weight changes, is not likely to be biologically relevant.

Table 10. Thyroid weights of rats killed before and at the end of the study after receiving diets containing imazalil for 2 years

Thyroid weight	0 ppm	50 ppm	200 ppm	1 200 ppm	2 400 ppm
Males					
Number examined	*40*	*35*	*38*	*34*	*41*
Absolute (mg)	44	47	89	71	64*
Relative (mg/kg bw)	88	98	181*	148*	142***
Females					
Number examined	*30*	*31*	*35*	*40*	*34*
Absolute (mg)	38	33	43	38	26***
Relative (mg/kg bw)	127	111	148	154	111

bw: body weight; ppm: parts per million; *: $P < 0.05$; ***: $P < 0.001$ (Mann-Whitney *U*-test, two-tailed)
Source: Van Deun (1999a)

The pathological changes seen grossly at termination included an increased frequency of liver foci and swollen thyroid glands in males at 1200 and 2400 ppm and decreased adiposity in males at 2400 ppm. The changes seen in females included decreased adiposity and decreased inspissated secretions in the mammary glands at 1200 and 2400 ppm.

An increased prevalence of hepatocellular adenoma was seen in males at the highest dietary concentration, in those killed at termination of the study and when all animals were included in the analysis (Table 11).

Table 11. Incidence of liver adenoma and carcinoma in rats after receiving diets containing imazalil for 2 years

	Males					Females				
	0 ppm	50 ppm	200 ppm	1 200 ppm	2 400 ppm	0 ppm	50 ppm	200 ppm	1 200 ppm	2 400 ppm
No. of livers examined	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*
Hepatocellular adenoma	4	2	3	4	13*	2	1	2	1	2
Hepatocellular carcinoma	1	0	0	0	1	0	0	0	0	0

ppm: parts per million; *: $P < 0.05$
Source: Van Deun (1999a)

Males at 1200 and 2400 ppm also had an increased incidence of follicular cell neoplasia of the thyroid. In males at 2400 ppm that were killed at termination, the incidence of follicular cell adenoma was also increased (Table 12). Addition of animals that died before the end of the study had little effect on the total incidence of the tumours (Table 13). As these tumours are likely to be incidental findings and not the cause of death, the most appropriate tabulation is probably that in Table 14, showing a clear increase in follicular cell neoplasia in males at 1200 and 2400 ppm and in

females at 2400 ppm. The incidences of thyroid follicular cell adenomas and carcinomas in males at 1200 and 2400 ppm exceeded the historical control values of the laboratory (Table 15). The decreased incidences of pituitary and mammary gland hyperplasia were probably related to the reduced body weights of animals at the highest concentration in the diet. No differences in neoplastic findings were found between groups.

Table 12. Incidences of thyroid follicular cell neoplasia in animals killed at termination of the study after receiving diets containing imazalil for 2 years

	Incidence of thyroid follicular cell neoplasia				
	0 ppm	**50 ppm**	**200 ppm**	**1 200 ppm**	**2 400 ppm**
Males					
Total	3/40	7/35	6/38	9*/34	12*/41
Adenoma	3/40	7/35	5/38	7/34	10*/41
Carcinoma	0/40	0/35	2/38	2/34	2/41
Females					
Total	3/30	3/31	4/35	3/40	1/34
Adenoma	3/30	3/31	3/35	3/40	1/34
Carcinoma	0/30	0/31	1/35	0/40	0/34

ppm: parts per million; *: $P < 0.05$
Source: Van Deun (1999a)

Table 13. Incidences of thyroid follicular cell neoplasia in animals killed or dying before termination of the study after receiving diets containing imazalil for 2 years

	Incidence of thyroid follicular cell neoplasia				
	0 ppm	**50 ppm**	**200 ppm**	**1 200 ppm**	**2 400 ppm**
Males					
Total	1/10	1/15	0/12	2/16	0/9
Adenoma	1/10	1/15	0/12	2/16	0/9
Carcinoma	0/10	0/15	0/12	0/16	0/9
Females					
Total	4/20	2/19	0/15	1/10	0/16
Adenoma	4/20	2/19	0/15	0/10	0/16
Carcinoma	0/20	0/19	0/15	1/10	0/16

ppm: parts per million
Source: Van Deun (1999a)

Table 14. Numbers of thyroid follicular cell tumours in all animals killed or dying after receiving diets containing imazalil for 2 years

	Number of thyroid follicular cell tumours				
	0 ppm	**50 ppm**	**200 ppm**	**1 200 ppm**	**2 400 ppm**
Males					
Total	4	8	6	11*	12*

	Number of thyroid follicular cell tumours				
	0 ppm	**50 ppm**	**200 ppm**	**1 200 ppm**	**2 400 ppm**
Adenoma	4	8	5	9	10
Carcinoma	0	0	2	2	2
Females					
Total	7	5	4	4	1*
Adenoma	7	5	3	3	1
Carcinoma	0	0	1	1	0

ppm: parts per million; *: $P < 0.05$
Source: Van Deun (1999a)

Table 15. Historical control data on liver and thyroid tumours for the 24-month rat carcinogenicity study

End-point	**Group 1**	**Group 2**
Males		
Hepatocellular adenoma	3/50 (6%)	4/50 (8%)
Hepatocellular carcinoma	0/50 (0%)	2/50 (4%)
Thyroid follicular cell adenoma	3/50 (6%)	5/50 (10%)
Thyroid follicular cell carcinoma	0/50 (0%)	1/50 (2%)
Females		
Hepatocellular adenoma	1/50 (2%)	3/50 (6%)
Hepatocellular carcinoma	1/50 (2%)	2/50 (4%)
Thyroid follicular cell adenoma	0/50 (0%)	3/50 (6%)
Thyroid follicular cell carcinoma	0/50 (0%)	0/50 (0%)

Source: Van Deun (1999b)

The non-neoplastic findings included decreased incidences of haemorrhagic degeneration of the adrenals in female rats at the highest dietary concentration, in all animals and in the survivors to termination when the results for these animals were analysed separately. The study pathologist suggested that this lesion is commonly seen in female rats of this age. The incidence of transitional cell hyperplasia was increased in all treated males, but with no dose–response relationship; furthermore, this finding was significant only in rats at 50, 200 and 1200 ppm when the decedents were included in the analysis. Females at the highest dietary concentration that survived to termination and survivors and decedents taken together had an increased frequency of mineralization of the transitional epithelium of the renal pelvis. In the livers of animals that survived to termination and of all animals, an increased frequency of centriacinar hepatocyte hypertrophy was seen at the two highest concentrations. An increase in the frequency of periacinar hepatocyte hypertrophy was seen in females at the two highest dietary concentrations when the results for all animals were analysed together and at 1200 ppm in survivors only. Changes in the portal tract were discerned in males at the highest concentration, only when all animals were included in the analysis. Clear cell foci were observed in females at the highest concentration that survived to termination. Decreased frequencies of basophilic foci and foci of hepatocellular alteration were observed in the livers of surviving females at 200 and 2400 ppm and in survivors and decedents together at 2400 ppm. Eosinophilic foci and foci of hepatocellular alteration were seen in surviving males at the two highest concentrations and in all animals at the highest dietary concentration when the results for all animals were analysed together. Fatty vacuolation was found in the livers of surviving males and in survivors and decedents taken

together at the two highest concentrations and in female survivors at 1200 ppm at termination. Focal cystic degeneration of the liver was seen in males at 2400 ppm (both survivors separately and all animals), whereas pigment-laden hepatocytes were seen in females at the three highest dietary concentrations, whether or not the decedents were included in the analysis. In the thyroid gland, cystic focal follicular hyperplasia was seen in male survivors at the two highest concentrations and in those at the highest concentration only when all animals were included in the analysis. Few differences were found between groups with respect to pathological changes in decedents, when the findings were analysed separately; none appeared to be clearly treatment related, with the possible exception of an increased incidence of transitional cell hyperplasia in the kidney in males and periacinar hepatocyte hypertrophy and pigment-laden hepatocytes in females at the highest dietary concentration.

It might be argued that no NOAEL can be identified in this study, as effects on body weight, body weight gain and feed consumption were seen at some times in males and females at 50 ppm. However, the findings at 200 ppm suggest that the first two effects were not dose related. In females, increased feed consumption was observed at some times at 50 ppm, whereas in males, the situation was less clear, the decreases observed were small and the decreases occurred at only a few times.

The NOAEL for toxicity was therefore 50 ppm (equal to 2.4 mg/kg bw per day), on the basis of minor haematological changes in animals of both sexes and increased blood glucose concentration and liver changes (increases in relative liver weight and an increased incidence of pigmented hepatocytes) in females at 200 ppm (equal to 9.7 mg/kg bw per day). The NOAEL for carcinogenicity was 200 ppm (equal to 9.7 mg/kg bw per day), based on an increased incidence of follicular cell neoplasia of the thyroid in males at 1200 ppm (equal to 58 mg/kg bw per day) (Van Deun, 1999a).

In a 30-month study, four groups of Wistar Cbp:WU rats (50 of each sex per group) were administered imazalil base (purity 98.1%) in the diet at a concentration of 0, 25, 100 or 400 ppm (equal to 0, 1.0, 3.6 and 15.0 mg/kg bw per day for males and 0, 1.2, 4.7 and 19.7 mg/kg bw per day for females, respectively). Animals were 4–5 weeks old at the start of the study and were housed five of each sex per cage. Animals were observed daily for general condition and behaviour and examined periodically for visible/palpable tumours or signs of illness and weekly for body weight and feed consumption (per cage measurements). Ophthalmoscopic examinations were performed on control and high-dose animals initially and at weeks 52 and 104. Plasma albumin measurements were determined in 10 male and 10 female rats in each of the control and high-dose groups from blood collected, after overnight fasting, from the aorta. Macroscopic examinations were performed on all animals. Organ weights were determined on all surviving animals and included adrenals, brain, heart, kidneys, liver, lungs, ovaries, pancreas, pituitary, spleen, testes, thymus and thyroid. Histopathological examinations were performed on all control and high-dose animals for the presence of hyperplastic, preneoplastic and neoplastic changes. The same evaluations were conducted on all low- and mid-dose animals, but included only the liver, spleen, pituitary, thyroid, adrenals, brain and testes. Similarly, microscopic examinations for non-neoplastic changes in the liver and kidney were performed in control and high-dose animals only.

General cage-side observations were unremarkable, and there were no compound-related effects on mortality, with 50% mortality occurring approximately 812 days into the study for both sexes. Body weight of high-dose males was reduced for the first 13 weeks and remained lower than in controls throughout the study, but this did not adversely affect their lifespan. There were no effects on body weight in females. Mean weekly feed consumption and plasma albumin were unaffected by treatment. Absolute and relative organ weights were variable in males among all groups, with only decreased absolute brain weight in 100 and 400 ppm males being statistically significant. There were no statistically significant differences between controls and treated females regarding organ weight changes. Ophthalmoscopic examinations were unremarkable between treated and control groups, except for a marginally increased incidence of cataracts among high-dose males (i.e. 37 controls, 43 high dose).

Macroscopic examinations were limited and unremarkable. There were also no compound-related effects detected upon microscopic examination. Although imazalil was not oncogenic in this

study, there were several tumours reported among all groups, including controls, which are indicative of geriatric changes (e.g. benign pituitary tumours, fibroadenomas of the mammary gland, benign fibromatous polyp in the uterus, as well as phaeochromocytoma of the adrenal, the latter predominantly in males) (Table 16).

Table 16. Selected neoplasms in rats fed imazalil for 30 months[a]

	Males				Females			
Tumours	**0 ppm**	**25 ppm**	**100 ppm**	**400 ppm**	**0 ppm**	**25 ppm**	**100 ppm**	**400 ppm**
Pituitary	(47)	(49)	(48)	(47)	(48)	(49)	(50)	(46)
Solid tumour	2	0	0	1	0	0	2	1
Spongiocytic tumour	1	0	0	0	0	0	1	1
Haemorrhagic tumour	6	2	10	4	7	10	7	4
Pleomorphic haemorrhagic tumour	4	7	5	2	4	4	6	4
Para intermedia tumour	0	0	0	0	0	0	1	0
Uterus	–	–	–	–	(50)	(23)	(24)	(50)
Multiple polyps	–	–	–	–	13	12	12	18
Adenocarcinoma	–	–	–	–	1	4	5	3
Epidermoid carcinoma	–	–	–	–	0	1	0	1
Mammary glands	(46)	(1)	(2)	(45)	(49)	(27)	(23)	(49)
(Multiple) fibroma	–	–	–	–	2	0	1	0
Adenoma	–	–	–	–	0	1	0	1
(Multiple) fibroadenoma	1	0	1	0	22	16	16	13
Papillary carcinoma	–	–	–	–	2	2	0	1
Carcinoma	0	0	0	0	0	0	0	1
Adrenals	(30)	(49)	(48)	(50)	(50)	(48)	(50)	(49)
Phaeochromocytoma	15	7	8	9	0	2	0	3

[a] Figures in parentheses represent the number of animals from which this tissue was examined microscopically. In this table, a benign tumour is ignored if a malignant tumour of the same histogenetic origin is also present in the same tissue. Absence of a numeral (marked with a dash) indicates that the lesion specified was not identified or that the organ is not relevant to the sex of the animal examined.

Source: Til et al. (1985)

The NOAEL for toxicity was 100 ppm (equal to 3.6 mg/kg bw per day), on the basis of decreased weight gain in males at 400 ppm (equal to 15.0 mg/kg bw per day). Imazalil was not carcinogenic at doses up to and including 400 ppm (equal to 15.0 mg/kg bw per day) (Til et al., 1985).

2.4 Genotoxicity

The results of studies of genotoxicity of imazalil are summarized in Table 17.

Table 17. Results of assays for the genotoxicity of imazalil

End-point	Test object	Concentration/dose	Purity (%)	Results	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0.5–30 µg/plate in DMSO (±S9)	Not reported	Negative	Steelman & Schreiner (1977)
Reverse mutation	*S. typhimurium* TA97, TA98, TA1535, TA1538	5–500 µg/plate in DMSO (±S9)	98.7	Negative	Vanparys & Marsboom (1988a)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537; *Escherichia coli* WP2*uvr*A	9.8–313 µg/plate in DMSO (±S9)	>98	Negative	Watabe (1992)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	31.25–500 µg/plate in DMSO (±S9)	96.4	Negative	Jenkinson (1990a)
Chromosomal aberration	Human lymphocytes	9–145 µg/mL in DMSO (±S9)	99.7	Negative	Vanparys et al. (1990)
Unscheduled DNA synthesis	Male Wistar (WU) rat primary hepatocytes	0.9–30 µg/mL in DMSO (±S9)	97.6	Negative	Fautz (1990)
Forward mutation	Chinese hamster lung V79 cells; *Hprt* locus	10–100 µg/mL in DMSO (±S9)	98.3	Negative	Van Gompel, Vanparys & Van Cauteren (1995)
In vivo					
Micronucleus formation	Male Wistar rats	0, 10, 40, 160 mg/kg bw (ip)	Not reported	Negative	Vanparys & Marsboom (1979)
Micronucleus formation	Male and female albino Swiss mice	0, 20, 80, 320 mg/kg bw (orally)	98.7	Negative	Vanparys & Marsboom (1988b)
Micronucleus formation	Male and female albino BKW mice	100 mg/kg bw (orally)	96.4	Negative	Jenkinson (1990b)
Unscheduled DNA synthesis	CD-1 mouse hepatocytes ex vivo	125, 250 mg/kg bw (gavage)	97–98.1	Negative	Clare (1996)
Sex-linked recessive lethal test	*Drosophila melanogaster* Berlin K males	250, 1 000 ppm	Not reported	Negative	Vanparys & Marsboom (1982)
Dominant lethal test	Male and female albino Swiss mice	Single oral dose 0, 10, 40, 160 mg/kg bw	Not reported	Negative	Vanparys & Marsboom (1987a,b)

bw: body weight; DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; *Hprt*: hypoxanthine–guanine phosphoribosyltransferase; ip: intraperitoneally; ppm: parts per million: S9: 9000 × *g* supernatant fraction from rat liver homogenate

In an in vivo comet assay not previously evaluated by JMPR, groups of five Swiss mice of each sex were given daily oral doses of technical-grade imazalil (purity 95%) at 0 or 10 mg/kg bw for 28 days. Imazalil was suspended in 0.2 mL corn oil. Tail moment was calculated from measurements of comet tail length and tail intensity; long tail nuclei were also analysed. A statistically significantly increased tail moment was apparent in the liver cells of treated animals of both sexes compared with

controls (accompanied by statistically significant increases in tail length, long tail nuclei and tail intensity). Statistically significant sex differences in all measured parameters, with the exception of tail length, were also noted. The study authors concluded that, over time, the low concentrations of imazalil present in the environment and cypermethrin in food, and especially their mixtures with carbendazim, have genotoxic properties that could be particularly dangerous in mammalian organisms (Đikić et al., 2012).

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a non-GLP-compliant fertility study, Wistar rats (20 of each sex per dose) received imazalil (purity 98.8%) at 0, 5, 20 or 80 mg/100 g feed (equivalent to 0, 7.5, 30 and 120 mg/kg bw per day, respectively) as follows: males for 60 days prior to pairing with females and through copulation; females for 14 days prior to pairing and throughout pregnancy.

Pregnancy rate was not affected at any level, regardless of treatment of either sex. Test group data for all other measured or observed parameters were comparable with the control data (Marsboom, 1977).

In a two-generation study, groups of 24 male and 24 female Wistar rats were given diets containing imazalil (purity 98.0%) at a nominal dose of 0, 5, 20 or 80 mg/kg bw per day for 60 days before mating (both sexes), during a 3-week mating period (both sexes), during gestation and until weaning of the first generation. The F_0 males were killed after mating. The first generation (F_1) received imazalil during growth, mating, gestation and weaning of the second generation (F_2). The F_1 males were killed after mating. The doses during the premating period were equal to 0, 4.2, 17.6 and 70.5 mg/kg bw per day for males and 0, 5.0, 21.5 and 104.3 mg/kg bw per day for females, respectively. During the F_0 gestation, the intakes were 0, 4.0, 16.3 and 86.3 mg/kg bw per day, respectively, and those during the F_1 gestation were 0, 4.3, 18.6 and 87.8 mg/kg bw per day, respectively. Thus, the intakes tended to be slightly lower than the nominal doses. The animals were observed daily for general health, appearance and behaviour. Body weights were recorded weekly before mating and, for females, on days 1 and 22 after copulation. Body weights were also recorded during the 3-week lactation period. Feed consumption was recorded weekly before mating for both sexes and during gestation and lactation for females. The body weights of both F_1 and F_2 pups in each litter were recorded 8–12 hours after parturition and on days 4, 14 and 21. The kidneys, liver and reproductive organs of the F_0 and F_1 generations were examined histologically.

Two F_1 females at the high dose were killed in extremis, and one F_2 female at the high dose and one in the control group died soon after parturition or during lactation. F_0 males at the high dose showed decreased body weight gain before mating; a similar but less pronounced effect was seen in females before mating, but an effect was also seen in F_1 and F_2 dams during gestation, at parturition and at days 4, 14 and 21 of lactation. No effects on body weight gain were seen in other treated groups, and there was no clear effect on feed consumption in any group. Histopathological changes (liver vacuoles) were seen in F_0 males at the high dose, but no lesions were seen in the male reproductive system at any dose. Decreased numbers of corpora lutea were seen in F_0 females at the high dose. At the high dose, both F_1 and F_2 generations had considerably reduced numbers of live pups and larger numbers of stillborn pups, and the F_1 generation had fewer implantations; the duration of gestation was increased in both generations, by approximately 1 day, with an associated increase in the incidence of dystocia. The survival rates of pups during lactation were decreased in all F_1 treated groups on days 4, 14 and 21; in the F_2 generation, survival was decreased at all times at the high dose and on days 14 and 21 at the low dose; at the intermediate dose, the survival was comparable with that of controls, so that there was no dose–response relationship. No differences in body weight were seen between F_1 and F_2 pups. No teratogenic effect was observed.

As noted above, the survival rates of F_1 pups during lactation were decreased in all treated groups on days 4, 14 and 21 of lactation and in the F_2 generation at 5 mg/kg bw per day (days 14 and 21) and 80 mg/kg bw per day (days 4, 14 and 21); moreover, there was no dose–response relationship for the effect in the F_1 generation at day 4, although there was at days 14 and 21. It is arguable that the data, especially for days 14 and 21, indicate a dose-related effect, but there was clearly no dose-related effect in F_2 pups. There is no biologically plausible explanation for the difference between the two generations, and a statistical re-examination on a litter basis showed that the mortality rate was statistically significantly increased only in the group at the high dose.

The NOAEL for parental toxicity was 20 mg/kg bw per day, on the basis of reduced maternal body weight gain and hepatotoxicity (vacuoles) in males at 80 mg/kg bw per day. The NOAEL for offspring toxicity was 20 mg/kg bw per day, on the basis of decreased numbers of live pups, decreased survival rate of pups and increased numbers of stillbirths at 80 mg/kg bw per day. The NOAEL for reproductive toxicity was 20 mg/kg bw per day, on the basis of increased duration of gestation for the F_0 and F_1 females and decreased gestation rate in F_1 females at 80 mg/kg bw per day (Dirkx et al., 1992b).

In a three-generation study, groups of 20 female Wistar rats received imazalil (purity not reported) on a non-continuous basis for three generations. Imazalil was administered as 50% wettable powder at a dietary concentration of 0, 5, 20 or 80 mg/100 g feed (or 0, 50, 200 and 800 ppm) (equivalent to 0, 7.5, 30 and 120 mg/kg bw per day, respectively). Prior to and after the period of organogenesis and throughout lactation, the females of the first and second generations received only the basal laboratory diet. In each of these generations, the dams received the various levels from day 6 through day 15 of pregnancy. In the third generation, the dams received the various levels from 3 weeks until 3 months of age and further from days 1 to 21 after mating.

There was a trend towards a lower number of live births at the 80 mg/100 g feed level in all generations. No other effects were noted at any other level. Waved ribs and absence of metacarpal and metatarsal bones were seen in both control and treated groups. No abnormalities were noted in any fetuses in any generation that were not within the normal range for the rats used (Marsboom, 1975a).

The submitted report did not include a GLP statement or any individual animal data, and therefore this study is of limited value.

In another three-generation study, groups of Wistar rats (10 of each sex per dose) were fed imazalil (purity 98.8%) in the diet at a concentration of 0, 50, 200 or 800 mg/100 g feed (equivalent to 0, 7.5, 30 and 120 mg/kg bw per day, respectively) and subjected to a standard three-generation, two litter per generation, reproduction study. Observations included growth, feed consumption, mortality and the standard indices of reproduction (mating, fertility, gestation, viability and lactation).

The reproductive performance of rats was unaffected by imazalil at any dose. There was no maternal mortality, and the parental generations were unaffected by imazalil. No fetal abnormalities or embryonic effects were noted. Litter size, weight and survival were normal.

This study is of limited value because all parameters were not measured and individual animal data were not provided (Marsboom, 1978).

(b) Developmental toxicity

Mice

Twenty-four female COBS mice received imazalil sulfate (purity 98.7%) orally at a dose of 0, 2.5, 10 or 40 mg/kg bw per day from days 6 through 16 of gestation. All animals were killed on day 19 of gestation.

No effects on mortality, body weight, feed consumption, pregnancy rate, number of live, dead or resorbed fetuses, pup weight or mean litter size were found. During gross observations, talipes valgus (club foot) was observed in all groups, the incidence not being statistically significantly different in any dose group when compared with the control group. No additional abnormalities were seen after radiographic examinations and Lapras' sectioning technique (Marsboom, Gillardin & Sanz, 1985).

The developmental toxicity of imazalil sulfate (purity 99.7%) was studied in groups of 30 inseminated COBS CD-1 mice given a dose of 0, 40, 80 or 120 mg/kg bw per day by gavage on days 6–16 of gestation. All animals were killed on day 19 of gestation.

Treatment did not affect the maternal mortality rate, but maternal body weight gain and feed consumption were statistically significantly decreased at the intermediate and high doses. Litter size and the number of live pups were reduced in all treated groups. The number of resorptions was increased at the high dose. No teratogenic effects were seen, and there were no differences between groups in mean pup weights.

The NOAEL for maternal toxicity was 40 mg/kg bw per day, based on decreased body weight gain and feed consumption at 80 mg/kg bw per day. A NOAEL for embryo and fetal toxicity could not be established in view of the decreases in litter size and number of live pups at all doses (Gillardin, Sanz & Marsboom, 1987).

In another study of developmental toxicity, groups of 30 inseminated COBS CD-1 mice were given imazalil (purity 99.3–99.5%) at a dose of 0, 10, 40, 80 or 120 mg/kg bw per day by gavage on days 6–16 of gestation. Deaths were recorded during the study, and the animals were examined daily for abnormal clinical signs. Body weights were determined on day 1, daily on days 6–17 and on day 19 after copulation. Feed consumption was recorded over days 1–5, days 6–16 and days 17–18. On day 19 after copulation and before killing, all survivors underwent a complete physical examination. The surviving dams were then killed, and macroscopic changes were recorded; the weights of the uteri were recorded after removal of that organ in toto. The dams were examined for numbers of live and dead fetuses, implantation sites and embryos undergoing resorption. All live fetuses were weighed, and live and dead fetuses were examined for external abnormalities. The pups were sexed, and the fetuses were examined radiographically. The fetuses were then dissected, and bones were stained with alizarin.

Fourteen mice died during the study: one control, four at 80 mg/kg bw per day and nine at 120 mg/kg bw per day. At the highest dose, piloerection, excitability, convulsions, hypothermia and prostration were observed. Tremor was observed in one female at 120 mg/kg bw per day. Body weights were decreased on days 17 and 19 in mice at the two highest doses in comparison with concurrent controls; moreover, the body weight gain of these animals was decreased during days 6–17 after copulation, and that of animals at 40 and 120 mg/kg bw per day was decreased during days 17–19 after copulation. The total body weight gain (uterine weight subtracted) over the whole period of 1–19 days was decreased at doses of 40 mg/kg bw per day and above. Feed consumption was decreased during dosing on days 6–16 in mice at 80 and 120 mg/kg bw per day and after dosing in those at 40 mg/kg bw per day and above. The pregnancy rates and number of implantations were comparable among groups, whereas fewer live fetuses were found in animals at the highest dose, and the number of resorptions was increased. The body weight of pups was decreased at the highest dose, but the sex ratio was similar in all groups. The frequency of minor changes in ribs was increased at 40 and 80 mg/kg bw per day, but not at the highest dose; there was thus no clear dose–response relationship. The number of major abnormalities was comparable in all groups.

The NOAEL for maternal toxicity was 10 mg/kg bw per day, on the basis of decreased body weight gain and reduced feed consumption after treatment at 40 mg/kg bw per day. In addition, deaths occurred at doses of 80 mg/kg bw per day and above. The NOAEL for embryo and fetal toxicity was 80 mg/kg bw per day, as the number of live fetuses was reduced, the number of resorptions was

increased and the body weights of the pups were decreased at the highest dose. There was no evidence of teratogenicity (Levron et al., 1991).

Rats

In a developmental toxicity study (non-GLP-compliant), Wistar rats (20 of each sex per dose) received imazalil (purity not specified) at 0, 5, 20 or 80 mg/kg bw per day in the diet during gestation days 6–15. Fetuses were delivered by caesarean section on day 21.

Body weights, body weight gains and feed consumption were comparable among the treated and control groups. No mortality occurred during the study. No differences in percentage of pregnancies or average duration of gestation were noted between the test and control groups. There was no difference between the control and treated groups with respect to the average number of implantations, litter size, number of live and dead fetuses, number of resorptions or fetal weights. No abnormalities were noted among the fetuses in the control and various test groups, with waved ribs in one litter of the group recorded at 80 mg/kg bw per day (Marsboom, 1970).

This study is of limited value because all parameters were not measured and individual animal data were not provided.

The developmental toxicity of imazalil sulfate (purity 99.9%) was studied in groups of 24 pregnant Sprague Dawley OFA.SD (IOPS Caw) rats treated by gavage at a dose of 0, 40, 80 or 120 mg/kg bw per day on days 6–16 of gestation. The dams were examined daily and weighed 1, 6, 17 and 22 days after copulation. Feed consumption was recorded during days 1–5 (before dosing), 6–16 (during dosing) and 17–21 (after dosing). At the end of the study, on day 22 of gestation, the dams were killed and examined for live and dead fetuses, implantation sites and resorptions. Live fetuses were weighed, and live and dead ones were examined for anomalies radiographically and by alizarin staining to visualize skeletal anomalies.

One animal at 40 mg/kg bw per day died, but this death was considered to be unrelated to treatment, as no others occurred during the study. Maternal body weight was reduced at the end of dosing at all three doses on day 17 and at the high dose on day 22, in comparison with concurrent controls. Decreased body weight gain was seen at the high dose. Decreased feed consumption was seen at all three doses throughout treatment. The rates of pregnancy and numbers of corpora lutea and implantations were similar in all groups. The number of live fetuses was decreased and the number of resorptions was increased at 120 mg/kg bw per day. At the intermediate and high doses, the birth weights of the pups were decreased. No teratogenic effects were seen.

The NOAEL for embryo and fetal toxicity was 40 mg/kg bw per day, on the basis of reduced pup weight at 80 mg/kg bw per day. No NOAEL for maternal toxicity could be identified because of decreased maternal body weight at all doses (Gillardin et al., 1988).

In an embryotoxicity and teratogenicity study, 20 female Wistar rats per group received various levels of imazalil nitrate (purity not provided) from day 16 of pregnancy through a 3-week lactation period. The doses used in this study were 0, 5, 20 and 80 mg/100 g feed.

No differences in percentage of pregnancies or average duration of gestation were noted between test and control groups. Average litter size was slightly decreased at the high dose of 80 mg/100 g feed (9) compared with the controls (11). The percentage of dead fetuses was higher in the 80 mg/100 g feed group (27%) when compared with the controls (4%), 5 mg/100 g feed (0%) and 20 mg/100 g feed (1%) groups. No abnormalities were observed in offspring from the control or test groups. No effects on pup body weight or body weight gain were observed (Marsboom, 1975b).

Rabbits

Groups of 20 female New Zealand white rabbits received imazalil nitrate (purity 99.2%) in water by gavage at 0, 0.63 or 2.5 mg/kg bw per day on days 6–18 of pregnancy. All surviving animals were killed on day 28 of pregnancy.

Four females died during the test (three in the control group and one in the 0.63 mg/kg bw per day group). Body weight gain of the dams was decreased in both dose groups, as well as average litter size, the percentages of live fetuses and 24-hour survival rates. The percentages of resorbed fetuses increased with increasing dose. Retrospective statistical analysis showed that the effects on maternal body weight, litter size and number of resorptions were not statistically significant. A small number of anomalies in all groups (hydrocephaly, fused ribs and deformed legs) were considered not to be dose related. Individual data were not available to the Meeting. In this study, embryotoxic and maternally toxic effects were observed in both dose groups. Therefore, this study was repeated at lower doses, as described below (Marsboom, 1974).

Groups of 15 female New Zealand white rabbits received imazalil nitrate (purity 97.8%) in water by gavage at 0, 0.16 or 0.63 mg/kg bw per day from days 6 to 18 of pregnancy. The surviving animals were killed on day 28 of pregnancy.

Mortality (three dams in the 0.16 mg/kg bw per day group died during the study), body weight and pregnancy rate were not statistically significantly different among the groups. Mean litter size was normal in all groups, and no statistically significant differences were seen with regard to the number of live, dead or resorbed fetuses, birth weight or 24-hour survival rate. Fetal skeletal examination and fetal sectioning revealed no compound-related abnormalities (Marsboom & Dirkx, 1981).

Groups of 15 female New Zealand white rabbits received technical-grade imazalil nitrate (purity 99%) in water by gavage at 0, 1.25, 2.5 or 5 mg/kg bw per day on days 6–18 of pregnancy. All animals were killed on day 28 of pregnancy. The dams were examined for mortality and body weight. The number of live, dead and resorbed fetuses, litter size, survival rate and pup weight were recorded, and all live and dead fetuses were examined for gross pathology (fetal skeletal examination and fetal sectioning).

One rabbit at the lowest dose and one at the highest dose died. There was no clear effect of treatment on body weight gain or pregnancy rate. No differences were seen between groups in the numbers of live or dead fetuses or resorptions, birth weights of pups or 24-hour survival rate.

The NOAEL for both maternal and embryo/fetal toxicity was 5 mg/kg bw per day, the highest dose tested (Dirkx & Marsboom, 1985).

In a developmental toxicity study, pregnant albino rabbits (15 per dose) were given imazalil sulfate (purity 98.2–100%) by gavage at a dose of 0, 5, 10 or 20 mg/kg bw per day on days 6–18 of gestation. The animals were observed daily for ill-health, abnormal behaviour or other clinical effects. Body weights were recorded on the morning of day 0 and on days 6, 19 and 28 of gestation. Feed consumption was recorded during days 0–5, 6–19 and 19–28. On day 28 of gestation, the surviving animals were killed and necropsied, the uterus was removed and the weight recorded, and the dams were examined for the numbers of dead and live fetuses and resorptions. The numbers of corpora lutea were determined, and the pups were sexed. Live fetuses were weighed, and both live and dead fetuses were examined externally by radiographic examination and alizarin staining. The fetuses were then dissected.

Eight out of 15 animals at 20 mg/kg bw per day and one out of 15 animals at 10 mg/kg bw per day died during the experiment. These deaths occurred late during the study and were not due to a single-dose effect. Emphysema and/or pneumonia were present in the majority of these animals. Five

of those at the high dose that died showed weight loss. No statistically significant differences in clinical end-points were found in the survivors. On day 19 of gestation, the body weight of animals at 20 mg/kg bw per day was decreased in comparison with that of concurrent controls. Feed consumption was reduced in animals at 10 mg/kg bw per day in comparison with concurrent controls during days 0–5 and in animals at 10 and 20 mg/kg bw per day during days 6–18. The pregnancy rates and numbers of corpora lutea were comparable between groups. Animals at 20 mg/kg bw per day had a decreased number of live pups, and those at 10 and 20 mg/kg bw per day had increased numbers of resorptions, although these changes were not statistically significant. The increase in resorptions at 10 mg/kg bw per day was within the historical control range; however, the current Meeting concluded that there was a trend towards an increase in resorptions with increasing dose and that the increase in resorptions was therefore treatment related (Table 18).

Table 18. Selected parameters in a developmental toxicity study in rabbits

Parameters	Control	5 mg/kg bw per day	10 mg/kg bw per day	20 mg/kg bw per day
No. of dead or sacrificed females	0/15	0/15	1/15	8/15**
No. of pregnant females	15/15	11/15	14/15	13/15
Body weight (g)				
Day 0	3 622	3 633	3 571	3 625
Day 6	3 774	3 774	3 711	3 757
Day 19	4 055	4 005	3 841	3 770*
Day 28	4 136	4 173	3 993	4 036
Weight of gravid uterus (g)	420	384	322	218
Weight change of pregnant females (g)	93	155	100	194
Feed consumption (g)				
Days 0–5	1 298	1 250	1 160*	1 155
Days 6–18	2 726	2 635	2 227*	2 096*
Days 19–27	1 651	1 484	1 495	1 748
Litter data				
No. of live fetuses/female	6.4	6.2	5.0	3.6
No. of dead fetuses/female	0.47	0.00	0.00	0.00
Mean litter size	6.9	6.2	5.0	3.6
No. of resorptions/female	0.67	0.64	1.46	3.71
No. of implantations/female	7.5	6.8	6.5	7.3
No. of corpora lutea/female	8.9	8.6	9.2	7.7
Mean weight of live fetuses (g)	41.3	42.4	45.2	39.0
Sex ratio (%)	46.8	34.6*	54.1	59.7
Number of malformed pups	1	2	0	0

bw: body weight; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$
Source: Dirkx et al. (1992a)

Developmental toxicity was observed at 20 mg/kg bw per day, as evidenced by a 5% decrease in fetal weight. There was a statistically significant decrease in the number of male fetuses at 5 mg/kg bw per day, but not at higher doses. Fetuses of does at 20 mg/kg bw per day had an increased

incidence of thirteenth rib and missing sternum in comparison with concurrent controls, but, especially in the latter case, there was no clear dose–response relationship, and the 2000 Meeting considered these findings to be coincidental.

The NOAEL for maternal toxicity was 5 mg/kg bw per day, on the basis of reduced feed consumption at 10 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 5 mg/kg bw per day, on the basis of an increased incidence of resorptions and a decreased number of live pups at 10 mg/kg bw per day (Dirkx et al., 1992a).

The Meeting considered the proposal newly submitted by Martens (2015) with respect to establishing an ARfD. In 2005, JMPR reviewed the available toxicological data for imazalil, and an ARfD of 0.05 mg/kg bw was established on the basis of maternal and fetal toxicity observed in the above-described study of developmental toxicity in rabbits (Dirkx et al., 1992a). Since the last review by JMPR, a single-dose toxicity study in rats and an acute neurotoxicity study in rats became available. The Meeting considered these studies for setting an ARfD for imazalil and concluded that the selected developmental toxicity study is still the most appropriate for setting the ARfD. The study suggests a trend towards increased incidence of resorptions and decreased number of live pups at 10 mg/kg bw per day, which cannot be discounted.

2.6 Special studies

(a) Neurotoxicity

Acute neurotoxicity

In a range-finding acute oral neurotoxicity study not previously evaluated by JMPR, imazalil (purity 95.5%) in polyethylene glycol (PEG) 400 was administered to groups of three Crl:CD®(SD) rats of each sex per dose by oral gavage at a dose of 40, 65, 110, 180 or 300 mg/kg bw. Owing to insufficient toxicity at the high dose, a sixth group of three rats of each sex was administered a single oral dose of 600 mg/kg bw. All animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily. Body weight was recorded daily from the first day of dosing. A modified functional observational battery (FOB) was conducted on all animals at 1, 3, 4.5, 6 and 24 hours after dose administration. All animals were euthanized following the last FOB assessment (day 1) and discarded without macroscopic examination.

There were no unscheduled deaths at any dose. Clinical observations, including piloerection, hypoactivity, unkempt appearance, red material around the nose, yellow material on the ventral abdominal area and rocking, lurching or swaying while ambulating, were noted in a single female in the 600 mg/kg bw group the day following dose administration. Yellow material on the ventral abdomen was also noted in another female at 600 mg/kg bw on the day following dosing. Body weight loss was noted for a single female in the 300 mg/kg bw group (10 g) and for all females in the 600 mg/kg bw group (8–13 g). During the FOB on the day of dosing, treatment-related findings were observed in 1–3 animals of each sex in the 300 and 600 mg/kg bw groups. The onset of these findings (soiled fur, low arousal and decreased rearing counts in males and females and piloerection in females) occurred at 3–4.5 hours following dosing; however, the incidences were highest at 4.5–6 hours following dosing. Mean rearing counts were maximally reduced in males in the 600 mg/kg bw group at 4.5 hours post-dosing. Mean rearing counts in females at 600 mg/kg bw began decreasing at 3 hours post-dosing and remained decreased for the remaining assessments. Low arousal was noted in one or two males or females at 65, 110 and/or 180 mg/kg bw; however, this finding has been reported in the historical control animals (14/208 on study day 0).

The NOAEL for systemic toxicity in this preliminary acute neurotoxicity study was 180 mg/kg bw, based on FOB findings at 300 mg/kg bw.

The time of peak effect was found to be 6 hours post-dosing, based on the increased incidence of FOB findings. Based on the results from this preliminary study, the doses selected for the main study were 0, 60, 180 and 600 mg/kg bw (Beck, 2006a).

In the main acute neurotoxicity study, which was not previously evaluated by JMPR, groups of Crl:CD®(SD) rats (12 of each sex per dose) were given imazalil (purity 95.5%) in PEG 400 orally by gavage at a dose of 0, 60, 180 or 600 mg/kg bw and observed for 14 days. Neurobehavioural assessment (FOB and motor activity testing) was performed on all animals predosing and on days 0, 7 and 14. Body weight and feed consumption were measured weekly throughout the study. At study termination, six animals of each sex per group were euthanized and perfused in situ for neuropathological examination; brain weight was recorded for these animals. Of the perfused animals, six animals of each sex from the control and high-dose groups were subjected to histopathological evaluation of brain and peripheral nervous system tissues.

At 600 mg/kg bw, four females were found dead or euthanized in extremis on study day 1 or 2. Prior to and on the day of death, clinical signs, including cool limbs, yellow material on the abdomen, hypoactivity, tremors, hunched posture, prostration and/or increased respiration, were observed in these animals. No treatment-related effects on body weight were observed in females; however, males at 600 mg/kg bw had decreased body weight gain (16%) and feed consumption (10%) in the first week post-dosing.

No changes in FOB parameters were observed in males at any time or in females on days 7 and 14. During the FOB testing, alterations observed in females at 600 mg/kg bw on day 0 at the time of peak effect included an increased incidence of the following: slightly soiled fur (5/12), crusty deposits around nose (7/12), low arousal (5/12), gait changes (hunched body [4/12] and walking on tiptoes [3/12]), no touch response (8/12), no olfactory response (5/12) and no approach response (7/12). These findings were noted in 0–2 control animals. The mean rearing count (1.7) and body temperature (35.8 °C) were decreased in females at 600 mg/kg bw on day 0 at the time of peak effect, compared with those values in controls (4.4 and 37.6 °C, respectively). During the motor activity testing, significant decreases in total and ambulatory counts (63% and 68%, respectively) compared with the control values were observed during the first 15-minute interval at the time of peak effect on day 0 in females at 600 mg/kg bw. Overall counts for total and ambulatory activity were significantly reduced on study days 0 (34–47%) and 7 (29–40%) in females at 600 mg/kg bw. At necropsy, no treatment-related effects on brain weight or measurements (length and width) were observed. The Meeting noted that the highest dose used was above the LD_{50}, and therefore the FOB results are due to general toxicity. On microscopic examination of the central and peripheral nervous systems, minimal axonal degeneration in the peripheral and spinal nerves and spinal nerve roots was observed in both treated and control groups of both sexes, with no consistent increase in the treated groups.

The NOAEL for systemic toxicity of imazalil in rats was 180 mg/kg bw, based on deaths in females, decreased body weight gain in males and FOB and motor activity alterations at the time of peak effect on day 0 in females at 600 mg/kg bw. The NOAEL for acute neurotoxicity was 600 mg/kg bw, the highest dose tested (Beck, 2006b).

Developmental neurotoxicity

A published study of reproductive toxicity with measurement of neurobehavioural end-points was reported in which imazalil (purity >99%) was administered in the diet to groups of 10 Crj:CD-1 mice of each sex from 5 weeks of age in the F_0 generation to 9 weeks of age in the F_1 generation. The compound was given at a concentration of 0%, 0.012%, 0.024% or 0.048% (equal to 0, 120, 240 and 480 ppm), resulting in respective intakes for the F_0 generation of 0, 19, 39 and 79 mg/kg bw per day for males and 0, 26, 45 and 102 mg/kg bw per day for females before conception; 0, 18, 34 and 74 mg/kg bw per day during mating; 0, 21, 38 and 83 mg/kg bw per day during gestation; and 0, 65, 152 and 262 mg/kg bw per day during lactation. The respective intakes in the F_1 generation were 0, 21, 41 and 87 mg/kg bw per day for males and 0, 24, 49 and 93 mg/kg bw per day for females.

In the F_0 generation, there was no effect on the body weight of either sex before conception or on the body weights of dams during gestation or lactation. Feed intake was increased in females at the low and high doses before conception, but not in those at the intermediate dose. A number of indicators of exploratory behaviour were increased in males at the high dose. In the F_1 generation, litter size and weight and sex ratio at birth were unaffected by treatment. The mean body weights of animals at the intermediate and high doses were reduced in early lactation. Indicators of behavioural development were affected, as follows. In males, surface righting was affected at the intermediate and high doses on postnatal day 4 and at the intermediate dose on postnatal day 7. Swimming head angle was affected in males at the high dose on postnatal day 4. In females, surface righting was affected in all treated F_1 offspring on postnatal day 7, without a clear dose–response relationship, and swimming head angle was affected in females at the high dose on postnatal day 4. No convincing dose–response relationships were seen for other observations, such as on multiple water T-maze performance in week 7. By week 8, there were no effects on the exploratory behaviour of either sex.

The doses used in this study were higher than those used in other studies reviewed in this monograph. Furthermore, although the results suggest that neurobehavioural end-points can be adversely affected in mice exposed to imazalil in their diet during development, comparison with the study of Levron et al. (1991), described in section 2.5(b) above, suggests that the lowest dose was maternally toxic. The NOAEL for developmental neurotoxicity was 120 ppm (equal to 19 mg/kg bw per day), based on effects on surface righting in males at 240 ppm (equal to 39 mg/kg bw per day) (Tanaka, 1995).

In another published study, which was not previously evaluated by JMPR, designed to investigate the effects of imazalil on birth outcome and neurobehavioural effects in offspring, groups of 10 pregnant Crlj:CD1 mice were given diets containing 6, 18 or 54 ppm imazalil (purity >99%) throughout gestation and lactation. This resulted in respective intakes of about 0, 0.85, 2.49 and 7.87 mg/kg bw per day during gestation and of 0, 3.2, 8.4 and 26.8 mg/kg bw per day during lactation.

No overt effects were noted in the dams. In offspring, there were no convincing test substance–related effects on behaviour, other than a transient and inconsistent, but dose-related, delay in surface righting ability in males on postnatal day 4, but not on postnatal day 7.

Regarding exploratory behaviour in the F_1 generation, there was no treatment-related difference ($P < 0.05$) in movement time at 3 weeks of age, but movement time was statistically significantly longer ($P = 0.0206$) in the low-dose group of males at 8 weeks of age. Spontaneous behaviour examination indicated that movement time increased in males, but decreased in females, in the low-dose groups in the F_1 generation.

The results of the study showed that some neurobehavioural parameters were inconsistently affected at all doses (Tanaka et al., 2013).

(b) Mechanistic studies on liver and thyroid toxicity

Liver pathology: mice

The effects of imazalil on liver morphology, enzymes and metabolism were studied in SPF albino Swiss mice. Imazalil (purity 98.5%) was administered in the diet to groups of 40 male and 40 female mice at a concentration of 0, 50, 200 or 600 ppm (equal to 0, 13, 53 and 160 mg/kg bw per day for males and 0, 16, 64 and 200 mg/kg bw per day for females for the first month and 0, 12, 46 and 140 mg/kg bw per day for males and 0, 14, 55 and 170 mg/kg bw per day for females for 3 months) for 1 or 3 months. An interim termination of 15 mice of each sex per group was carried out at 1 month, with 10 being assigned for toxicological and toxicokinetics evaluation and five for biochemistry. The remaining animals were killed at 3 months, 20 being used for toxicological and toxicokinetics evaluation and five for biochemistry. The mice were examined daily, and body weight and feed consumption were measured weekly. ALT, AST and ALP activities were measured in serum at both termination times, and selected organs were weighed and examined grossly. Only the livers

were examined, but they were examined microscopically, and the livers of one control mouse and one mouse of each sex at the high dose at the interim kill were examined ultramicroscopically.

No deaths were observed during the study, and imazalil had no effects on appearance or behaviour. No difference in body weight was observed between groups. Feed consumption was increased in males at 200 and 600 ppm over weeks 1–4 and in all treated females, probably due to feed wastage. The activity of ALT was increased in animals of each sex at the high dose at 1 month, whereas the activity of ALP was increased only in males at this dose. At 3 months, ALT activity was increased in males at 600 ppm, but the level was within the normal range seen in other studies. At 1 month, the absolute weight of the liver was increased in males at 200 ppm, and absolute and relative liver weights were increased in males and females at 600 ppm. At this time, males at the intermediate dose had increased absolute kidney weights, females at the high dose had decreased absolute and relative pancreatic weights, and females at the intermediate dose had increased absolute and relative adrenal weights. At 3 months, males at the high dose had increased absolute and relative liver weights, and males at 200 ppm had increased relative heart and kidney weights. Also in males, a decrease in absolute and relative thymus weights was seen at 200 ppm, and a decrease in absolute thymus weight was observed at 600 ppm. In females at 3 months, absolute and relative liver weights were increased and relative pancreatic weight was decreased at 600 ppm. In both males and females, the increases in liver weight appeared to be dose related. The changes in liver weights were accompanied by an increased incidence of dark livers at the high dose.

Histological examination at 1 month showed an increase in the numbers of small and large vacuoles, especially in the periportal area, in males and females at the high dose and in males at the intermediate dose. Similar findings were made at 3 months, with the addition of vacuolation in females at the intermediate dose. In mice at 600 ppm, ultramicroscopic examination of the liver showed an increased number of lipid droplets in hepatocytes, mainly in the periportal area. Moreover, the rough endoplasmic reticulum of the affected hepatocytes differed morphologically from that of hepatocytes from control animals: the rough endoplasmic reticulum in hepatocytes from treated animals was not arranged in parallel stacks of cisternae, but was diffused through the cytoplasm (Van Deun et al., 1994).

Liver pathology: rats

In a published study, addition of imazalil to suspensions of isolated rat hepatocytes at a concentration of 0.75 mmol/L caused acute cell death accompanied by depletion of intracellular glutathione and protein thiols, with accumulation of intracellular malondialdehyde (Nakagawa & Moore, 1995).

The hepatotoxic potential of imazalil (purity not reported), with and without L-buthionine-*S*,*R*-sulfoximine, a glutathione synthesis inhibitor, was examined by administering the inhibitor at 900 mg/kg bw to groups of four male Fischer 344/DuCrj rats by intraperitoneal injection, followed 1 hour later by imazalil by stomach tube at a single dose of 0, 170, 255 or 340 mg/kg bw (the last dose being approximately equal to the LD_{50}). Another group received imazalil without L-buthionine-*S*,*R*-sulfoximine, and two groups received the vehicle or L-buthionine-*S*,*R*-sulfoximine. The rats were killed 24 hours after administration of imazalil. Serum was taken for measurements of ALT and AST activity and cholesterol, urea, phospholipid and triglyceride concentrations. The kidneys and livers were removed and weighed; the livers were homogenized for measurement of malondialdehyde, and sections of the two organs were stained for histopathological examination.

Imazalil, with or without L-buthionine-*S*,*R*-sulfoximine, did not affect body weight, liver weight or kidney weight. When given alone, it caused a dose-dependent decrease in ASP activity but not ALT activity. Only the high dose of imazalil alone decreased the serum concentration of triglycerides, whereas those of cholesterol and phospholipids were decreased at the intermediate and high doses. The serum concentrations of triglycerides, cholesterol and phospholipids were decreased at the intermediate and high doses when L-buthionine-*S*,*R*-sulfoximine had been given, and there was

some indication of a greater effect in these animals compared with those given imazalil alone. L-Buthionine-*S,R*-sulfoximine and imazalil induced fatty infiltration of the liver, the distribution of which was predominantly midzonal or periportal, but there was no evidence of hepatocellular necrosis. Malondialdehyde, an index of lipid peroxidation, was found at increased concentrations in animals given imazalil at the high dose, whether or not they also received L-buthionine-*S,R*-sulfoximine, but the concentration of malondialdehyde was higher when both compounds were given. No histopathological changes were observed in the kidneys, nor were urea concentrations in urine increased (Nakagawa & Tayama, 1997).

Liver enzyme induction and liver cell proliferation: mice

In the 3-month oral mechanistic toxicity study with 1-month interim termination described above (Van Deun et al., 1994), liver microsomes were prepared from 10 mice of each sex after 1 and 3 months of treatment with imazalil at 0, 50, 200 or 600 ppm. The purpose of this study was to investigate the mechanism of liver changes seen with imazalil in the mouse. The microsomal protein and cytochrome P450 (CYP) contents of the mouse livers, the uridine diphosphate–glucuronosyltransferase (UGT) activity and several monooxygenase activities were determined to detect induction and/or inhibition of cytochrome P450. Spectrophotometric or fluorimetric determinations were performed employing standard procedures for measuring cytochrome P450 activities.

The administration of imazalil through the feed at a concentration of 50, 200 or 600 ppm for 1 month caused statistically significant increases in relative liver weight and in the hepatic microsomal protein and cytochrome P450 contents of both male and female SPF albino Swiss mice. Treatment with imazalil for 1 month also induced the *N*-ethylmorphine *N*-demethylation (EMD) and 7-pentoxyresorufin *O*-dealkylase (PROD) activities, whereas aniline hydroxylation and 7-ethoxyresorufin *O*-deethylation (EROD) were decreased in both male and female mice. After dosing for 3 months, EMD was virtually unaffected, and aniline hydroxylation, PROD, lauric acid hydroxylation (LAH) and especially EROD were decreased for both sexes. 7-Ethoxycoumarin *O*-deethylation (ECOD) was induced in males after 1 month of dosing and in both males and females after 3 months of dosing. The UGT activity was not affected in male mice and was slightly induced after 1 month of treatment and inhibited after 3 months of treatment in female mice.

Imazalil was a mixed inducer and inhibitor of cytochrome P450 in the mouse (Vermeir, Lavrijsen & Van Leemput, 1994).

In the 3-month oral mechanistic toxicity study with 1-month interim termination described above (Van Deun et al., 1994), liver samples were taken to investigate cell proliferation in livers of mice treated with imazalil via the diet for up to 3 months. For each dose group, formalin-fixed liver samples embedded in paraffin wax from 20 animals were analysed. Liver sections were microwaved in a 0.1 mol/L ethylenediaminetetraacetic acid (EDTA) solution and stained using an anti-proliferating cell nuclear antigen (PCNA) antibody in conjunction with an avidin/biotin peroxidase–conjugated detection system. Approximately 1000 hepatocytes were evaluated from each liver section employing microscope-assisted cell counting. The total cell count and the PCNA-positive cell count were scored by the study pathologist. For both sexes, Bartlett's test was statistically significant at the 1% level (both cases: $P < 0.001$), using the untransformed data as well as the logarithmically transformed data. This indicated that there was evidence of heterogeneity of variance between the treatment groups. Hence, each group was compared with the vehicle control group using Wilcoxon's rank sum test. For both sexes, no statistically significant results between dosed groups and vehicle groups were found.

There was no induction of hepatic cell proliferation by imazalil administered orally to albino Swiss mice for 3 months at concentrations up to 600 ppm (Lawrence, 2001). This study was not previously evaluated by JMPR.

Imazalil (purity > 98%) administered orally to mice at 1 or 10 mg/kg bw per day for 3 days enhanced EROD and PROD activities in the microsomes of small intestinal mucosa and liver, indicating induction of CYP1A and CYP2B. Additionally, immunochemical analyses indicated induction of CYP2B, CYP2C and CYP3A (benzyloxyquinoline *O*-debenzylation [BQ]) (Muto et al., 1997).

In a more recently completed study, imazalil (purity 97.5%) was investigated for its potential to induce mouse CYP2B10, CYP3A11 and cell proliferation as measured by replicative DNA synthesis during cell cycle S-phase in isolated primary mouse hepatocytes. Following a range-finding test, freshly isolated female CD-1 mouse hepatocytes were cultivated as monolayer cultures in appropriate culture flasks and 96-well plates. Hepatocytes were exposed to imazalil at a concentration of 3, 10, 30 or 100 µmol/L or to vehicle alone (0.5% volume per volume [v/v] dimethyl sulfoxide [DMSO]). Three to six replicates, depending on the assay, were performed. Cytotoxicity was measured as change in intracellular ATP concentration. Bioluminescent determination of released ATP was carried out using the assay kit Cell-Titre Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer's instructions. Levels of murine cytochrome P450 activity were determined spectrofluorometrically by formation of resorufin or 7-hydroxyquinoline from CYP2B10 or CYP3A11 activity, respectively. The activity of CYP2B in cultured hepatocytes was assessed spectrofluorometrically by the formation of resorufin from pentoxyresorufin. The activity of CYP3A in cultured hepatocytes was assessed spectrofluorometrically by the formation of 7-hydroxyquinoline. The number of hepatocytes undergoing replicative DNA synthesis was measured by incorporation of 5-bromo-2′-deoxyuridine (BrdU) in DNA and subsequent immunochemical detection; epidermal growth factor served as the positive control. Approximately 1500 hepatocytes per replicate were counted.

Treatment with imazalil at 30 or 100 µmol/L resulted in statistically significantly lower intracellular ATP, with levels being reduced to 84% and 2% of control values, respectively. The cytotoxicity observed following treatment with imazalil at 100 µmol/L was considered excessive, and therefore replicative DNA synthesis and CYP2B10 (PROD) and CYP3A11 (BQ) data either could not be measured at this concentration or were excluded from data analysis. In addition, the cytotoxicity observed following treatment with imazalil at 30 µmol/L was considered to be biologically relevant and sufficient to demonstrate that imazalil had been tested to an appropriately high concentration. Treatment with imazalil at 10 or 30 µmol/L resulted in statistically significant increases in replicative DNA synthesis of 1.8- and 1.5-fold relative to the controls, respectively. Treatment with imazalil at 3 µmol/L resulted in a statistically significant increase in CYP2B10 (PROD) activity to 117% of the control value, whereas at 30 µmol/L, a statistically significant decrease in CYP2B10 activity to 15% of the control value was observed. The decrease in cytochrome P450–mediated enzyme activity (PROD) observed in the mouse hepatocyte cultures is probably due to the inhibition of CYP2B10, in addition to the cytotoxicity described above. Treatment with imazalil at 3, 10 or 30 µmol/L exhibited a dose-dependent increase in CYP3A11 (BQ) activity of 1.3-, 2- and 2.1-fold compared with control values, respectively.

In conclusion, treatment of mouse hepatocytes with imazalil resulted in the limited induction of CYP3A11, followed by dose-dependent inhibition of CYP2B10 and increased cell proliferation (Elcombe, 2012b). This study was not previously evaluated by JMPR.

In order to investigate alterations in hepatocytes and potential proliferation induced by imazalil and, if observed, reversibility of the effect, groups of 10 male Crl:CD-1®(ICR) BR mice were treated for 2 or 13 weeks with imazalil (purity 96.2%) at a concenteration of 0, 100, 200, 300, 600 or 1200 ppm in the feed. Additional groups (10 animals) at 0 or 600 ppm were necropsied after 2- or 4-week recovery periods. Osmotic minipumps with BrdU were implanted 7 days prior to necropsy. Liver samples were analysed for cell proliferation. Slices of liver and duodenum were fixed in buffered formalin. Labelling index was determined as the percentage of cells in the DNA replicative phase or S-phase that had incorporated BrdU using immunohistochemical staining, counting at least

2000 liver cells per animal. In addition, PCNA immunostaining of hepatocytes was performed for control and high-dose mice.

Statistically significant increases in sorbitol dehydrogenase levels were noted at 600 and 1200 ppm, and a statistically significant increase in ALT was noted at 1200 ppm only. Relative or absolute and relative liver weights were increased at 600 and 1200 ppm. Increased incidences of centrilobular hypertrophy were noted at 200 ppm and above. A low incidence of minimal hepatocellular necrosis and increasing incidence and severity of hepatocellular vacuolation at weeks 2 and 13 were noted at 400 ppm and above. Cell proliferation in the liver, determined by BrdU and PCNA labelling, appeared not to be increased upon imazalil treatment.

These results indicate that there is no induction of hepatic cell proliferation following oral administration of imazalil to Crl:CD-1®(ICR) BR mice at concentrations up to 1200 ppm for 2 or 13 weeks (O'Neill, 2002). This study was not previously evaluated by JMPR.

In a similar study on the potential liver cell proliferation induced by imazalil, groups of six male Crl:CD-1®(ICR) BR mice were treated for 4 days with imazalil (purity 96.2%) at a concentration of 1200 ppm in the feed. A concurrent control received untreated diet. Osmotic minipumps with BrdU were implanted 1 day prior to commencement of treatment. Animals were observed for survival and clinical signs; body weights and feed consumption were measured weekly. At termination, blood chemistry, gross necropsy, liver weights and hepatic histopathological examinations were conducted, and slices of liver and duodenum were fixed in buffered formalin. Liver samples were analysed for cell proliferation. Labelling index was determined as the percentage of cells in the DNA replicative phase or S-phase that had incorporated BrdU using immunohistochemical staining, counting at least 2000 liver cells per animal. Quantitative data were tested for statistical significance (Student's *t*-test). Labelling index data were evaluated for normality using Shapiro-Wilk's test and then subjected to Dunnett's test.

There were no clinical findings, and all animals survived the study. For the treated group, decreased feed consumption was noted on day 1, and body weight gain and terminal body weight were reduced compared with control mice. ALT values and both absolute and relative liver weights were increased. Histopathology of the liver revealed minimal centrilobular hypertrophy, with minimal hepatocyte necrosis also observed in five out of the six treated mice. Cell proliferation in the liver determined by BrdU labelling was statistically significantly increased upon imazalil treatment.

These results indicate that there is a strong mitogenic response in the liver following acute oral administration of imazalil to CD-1 mice at a concentration of 1200 ppm (Elmore, 2004a). This study was not previously evaluated by JMPR.

Liver enzyme induction and liver cell proliferation: rats

Groups of 20 SPF Wistar rats were given diets containing imazalil (purity not specified) at a concentration of 0, 200, 400 or 800 ppm for 3 months. Over the first month, these dietary concentrations resulted in intakes of 0, 21, 42 and 82 mg/kg bw per day for males and 0, 22, 45 and 90 mg/kg bw per day for females, respectively; however, over the entire 3 months of the study, the intakes of imazalil were lower, at 0, 16, 32 and 64 mg/kg bw per day for males and 0, 19, 38 and 76 mg/kg bw per day for females, respectively. Ten animals of each sex at each dietary concentration were killed after 1 month. The rats were observed clinically and for morbidity and mortality at least daily. Ophthalmological examinations were performed at the start of the study and before the interim and terminal sacrifices in the first 10 animals in the control group and those at the high dietary concentration. Body weight was determined weekly, as was feed consumption. Blood was taken at the interim and terminal sacrifices for haematological and clinical chemical investigations; urine was analysed at the same times. Necropsies were performed at both the interim and terminal sacrifices. Selected organs were weighed in each case, and any gross changes were noted. Pieces of liver from four rats of each sex per dietary concentration were taken at both the 1-month and 3-month sacrifices.

Liver microsomes were assayed for protein and cytochrome P450 content and for certain microsomal enzyme activities – namely, aniline hydroxylation, EMD, EROD, PROD, LAH, ECOD and UGT activity towards 4-nitrophenol. The plasma concentrations of imazalil were measured.

No deaths were seen. The clinical effects were similar in the various groups, and no ophthalmological abnormalities were found. Body weight was reduced in females at 800 ppm in comparison with concurrent controls at weeks 7 and 9. Body weight gain was reduced in males at 800 ppm at weeks 1–3 and in females at 400 and 800 ppm in week 1 and at 800 ppm in week 9. No effect was observed on feed consumption, except in week 1, when there was decreased consumption by rats of each sex at the high dietary concentration. At the 1-month termination, total leukocyte and lymphocyte counts were decreased in both sexes at dietary concentrations of 400 and 800 ppm, and neutrophil and basophil counts were decreased in males at 800 ppm. In females, total leukocyte and lymphocyte counts were decreased at 200, 400 and 800 ppm, monocyte count was decreased at 800 ppm and the mean cell haemoglobin concentration was decreased at 200 ppm. At 3 months, males showed a decrease in basophil count at 400 ppm and in erythrocyte volume fraction at 200, 400 and 800 ppm. Also in males, the haemoglobin concentration was decreased at 400 ppm, the mean cell volume at 800 ppm and the mean cell haemoglobin concentration at 200 and 800 ppm. Females had a decrease in mean cell volume at 800 ppm and an increase in mean cell haemoglobin concentration at 200 and 800 ppm. The decrease in mean cell volume in animals of both sexes at 800 ppm at 3 months may have been treatment related. Otherwise, these changes were considered minor and were mostly not dose related or fell within the reference range for the strain of rats.

Some minor changes in some clinical chemistry parameters were seen at the low and intermediate doses (200 and 400 ppm), but no treatment-related effects on clinical chemistry or urine were seen at concentrations below 800 ppm. At that concentration, males had increased serum calcium, phosphate, albumin, cholesterol, triglyceride and blood urea nitrogen concentrations at 1 month, whereas females had decreased calcium, glucose and triglyceride concentrations; the total protein level was increased in both sexes. At 3 months, the serum calcium concentration was decreased in males and females. The decreases in bilirubin concentration and ALT and AST activities that were observed in the study were considered not adverse, and the inconsistent nature of the other clinical chemical changes casts doubt on their clinical relevance. Treatment-related changes in urinary parameters were not seen (Van Deun et al., 1996b).

The tissue samples from Van Deun et al. (1996b) were further evaluated for cytochrome P450 content and other enzyme activities. After 1 month, the liver microsomal protein content was increased in males at all concentrations and in females at the high concentration. The liver cytochrome P450 content was increased in animals of both sexes at the high concentration. The activities of EROD and PROD were increased in males at the intermediate and high dietary concentrations, whereas the activity of ECOD was increased at 200 and 800 ppm; UGT activity was increased at the high concentration. At 1 month in females, increased activity was seen for EMD, EROD, PROD and ECOD at all dietary concentrations and for UGT activity at the high concentration. Additional induction was not observed in males at 3 months, and increased activity of EROD was observed at dietary concentrations of 200 and 800 ppm and of PROD and ECOD only at the high concentration. However, in females, the cytochrome P450 content was further increased at 800 ppm. Increased aniline hydroxylation was seen at all dietary concentrations, EMD activity at the intermediate and high concentrations, EROD and PROD activities at all dietary concentrations and LAH at 200 and 800 ppm. Females at 3 months also showed increased activity of ECOD at 400 and 800 ppm and of UGT at 800 ppm. After 1 month, the plasma concentrations of imazalil ranged from less than 2 to 4.4 ng/mL in males and from less than 2 to 3.8 ng/mL in females at the three dietary concentrations. At 3 months, the corresponding concentrations were 1.1–2.2 ng/mL in males and 1.0–4.0 ng/mL in females.

Increased absolute and relative liver weights were observed after 1 month in males and females at 400 and 800 ppm. Increased relative liver weights were seen in males and increased absolute liver weights in females at 200 ppm. Increased relative thyroid weights were observed in males and increased absolute adrenal weights in females at 800 ppm. Decreased thymus weights, both absolute and relative, were seen in males at the high dietary concentration. At 3 months, males at 800

ppm had increased relative liver and kidney weights, whereas females had increased relative kidney weights at 800 ppm and increased absolute and relative weights of the adrenals at 400 and 800 ppm. No gross pathological changes were observed that appeared to be related to treatment. Hepatic changes were seen histologically at dietary concentrations of 400 ppm and above at 1 month; these comprised hepatocyte swelling in males and large vacuoles in females. Adrenal changes (cortical cell swelling) were observed at 3 months in 2/10 females at 800 ppm and 1/10 females at 400 ppm; the study authors considered that this finding corresponded to the increase in adrenal weights observed. No histopathological differences between groups were seen in the liver at 3 months. No histopathological differences were observed in the thyroid at 1 or 3 months (Vermeir, Lavrijsen & Meuldermans, 1995).

Groups of 10 male and 10 female SPF Wistar Hannover rats were given diets containing technical-grade imazalil (purity not specified) at a concentration of 0, 800, 1600, 2400 or 3200 ppm (equal to 0, 64, 129, 181 and 252 mg/kg bw per day for males and 0, 79, 150, 236 and 333 mg/kg bw per day for females, respectively). The rats were observed daily, and body weights and feed consumption were measured weekly. Deaths were recorded. Haematological and clinical chemical measurements were performed on blood samples from all surviving animals before the terminal sacrifice, and the concentration of imazalil was determined by gas chromatography with mass spectrometry by Sterkins (1996). Urine was analysed before terminal sacrifice. TSH, T_3, T_4 and testosterone concentrations were measured in serum from blood taken at termination. At necropsy, the animals were examined and weighed, and any gross pathological changes were noted. The liver was examined histopathologically, as were other organs judged to be grossly abnormal. Electron microscopy was carried out on the livers of one male and one female control animal and one male and one female animal fed the highest dietary concentration of imazalil. Liver microsomes from four rats of each sex per group were assayed for protein and cytochrome P450 content, and the activities of UGT and several monooxygenases were determined by Vermeir & Lavrijsen (1996).

A statistically significantly lower body weight compared with concurrent controls was found from week 2 of the study in males at the two highest concentrations and from time to time in males at 1600 ppm. The females showed decreased body weight from week 1 at the two highest concentrations and from week 2 at 1600 ppm. The findings for body weight gain were similar, except that decreased body weight gain in comparison with controls was found in animals of both sexes at 2400 and 3200 ppm throughout the study. Sporadic decreases in feed consumption were seen in all groups of treated males, particularly frequently at 2400 ppm. Among females, decreased feed consumption was observed at concentrations of 1600 ppm and above from time to time.

Sporadic changes in haematological parameters were seen, most of which were not dose related and did not therefore appear to be related to treatment. However, a decrease in monocyte count was seen in males at dietary concentrations of 1600 ppm and above. Furthermore, increased erythrocyte volume fraction, haemoglobin and red cell count were seen, with decreases in mean cell volume and increases in mean cell haemoglobin concentration in females at most concentrations, including the lowest. Decreased AST activity was seen at all doses in both sexes, and decreased ALT activity and blood urea nitrogen concentration were seen at concentrations of 1600 ppm and above in males; such changes are not generally considered to be adverse. Decreased serum albumin was seen in females at the two highest dietary concentrations. A decrease in serum calcium and an increase in phosphate concentration were seen in females at 1600, 2400 and 3200 ppm. Decreased triglyceride and phospholipid concentrations were observed in males at all dietary concentrations, and a decreased triglyceride concentration was seen in females at the highest dietary concentration. No treatment-related intergroup differences in urinary end-points were seen. The TSH, T_3, T_4 and testosterone concentrations in serum showed no clear differences between groups.

At termination, the body weights of males were decreased at concentrations of 1600 ppm and above. Also in males, decreased absolute lung weights were seen at 2400 and 3200 ppm, and increases in relative liver weight were seen at all concentrations. Other changes in organ weights of males either were not dose related or reflected changes in total body weight. Similar findings were

made in females, which showed a decrease in body weight at dietary concentrations of 1600 ppm and above. A decrease in absolute lung weight was also seen in females at these concentrations. At postmortem examination, gross changes that appeared to be related to treatment were seen only in the liver. The livers of males at 2400 and 3200 ppm were dark, and more pronounced lobulation was seen at dietary concentrations of 1600 ppm and above. Dark livers were also found in females at 2400 and 3200 ppm, and more pronounced lobulation was seen only at 3200 ppm. Histological examination revealed hepatocellular hypertrophy in males at all dietary concentrations, which was particularly pronounced in centrilobular zones. Also in males, an increased frequency of hepatocytes with small vacuoles was seen at 2400 and 3200 ppm, and an increased frequency of fine oil red O–positive material was seen at all concentrations. Similar changes were seen in females: they had hepatocellular hypertrophy, which was particularly pronounced in centrilobular zones, at all dietary concentrations. Further, they showed an increased frequency of hepatocytes with small vacuoles at all concentrations and large vacuoles at and above 2400 ppm. An increased frequency of fine oil red O–positive material was seen in females at concentrations of 1600 ppm and higher. Histological examination of the thyroid gland revealed no significant intergroup differences. On electron microscopy, the livers of all animals at all four dietary concentrations, but not the controls, showed small and/or large lipid droplets in both centrilobular and perilobular areas. Additionally, whorls of smooth endoplasmic reticulum were seen, this change not being present in controls.

The protein content of microsomes was increased in males at concentrations of 1600 ppm and above and in females at and above 2400 ppm. The activities of EMD, EROD, PROD and ECOD were statistically significantly induced in animals of both sexes at nearly all dietary concentrations. An increase in lauric acid hydroxylase activity was seen more frequently in the livers of males than in those of females, but was statistically significant only in males at 2400 ppm. Aniline hydroxylase activity was induced in females at all dietary concentrations. UGT activity was induced at the two highest dietary concentrations in females. At 800 ppm, the plasma concentration of imazalil was at or below the limit of detection of the assay, which was 1 ng/mL, in males and slightly above the limit of detection in females, at 1.3 ng/mL. In males, the concentrations of imazalil were 3.4. 4.4 and 11 ng/mL at 1600, 2400 and 3200 ppm, respectively, and the equivalent figures for females were 9.4, 6.8 and 17 ng/mL. These values suggest that detoxification pathways may have been saturated at dietary concentrations of 2400 ppm and higher.

It was concluded that imazalil is a nonspecific inducer of hepatic enzymes and that it caused morphological changes in the livers of rats. No effect was seen at 3 months in either the concentration of thyroid hormones or the histological appearance of the thyroid (Sterkins, 1996; Van Deun et al., 1996a, 1999; Vermeir & Lavrijsen, 1996).

Male Wistar rats were dosed orally by gavage at a dose of 10 or 40 mg/kg bw per day for 7 days. Hepatic microsomes were prepared by differential centrifugation, about 24 hours after the last dose for half of the rats and after a drug-free period of 7 days for the remaining animals. The concentrations of microsomal protein, cytochrome P450, NADPH–cytochrome *c* reductase, as well as the magnitude of the activities of the phase I enzymes aniline hydroxylase, ECOD and EROD, were determined.

The results of the study demonstrate that imazalil only slightly increased some cytochrome P450–dependent enzyme activities at 40 mg/kg bw per day. Relative liver weights, NADPH–cytrochrome *c* reductase activity and phase I enzyme aniline hydroxylase were not affected by the treatment. There was only a weak and dose-dependent increase in ECOD and EROD activities, but this was fully reversible, as these enzyme activities returned to the control values after a drug-free period of 7 days (Lavrijsen et al., 1987).

Twenty-one paraffin blocks of rat liver from a previously conducted 24-month control carcinogenicity study in rats (Van Deun, 1999b) were analysed in this study. Treatment was for 7

days with imazalil and phenobarbital at unknown levels or routes of administration. Animals were dosed intraperitoneally with BrdU (100 mg/kg bw) 6 hours prior to necropsy.

Cell proliferation in the liver determined by BrdU labelling was not increased upon imazalil treatment or upon phenobarbital treatment. The results indicate that there is apparently no mitogenic response in the liver following 7 days of administration of imazalil to rats. However, the positive control also resulted in no increase of hepatic cell proliferation; thus, the results of this study are unconvincing (Elmore, 2004b). This study was not previously evaluated by JMPR.

In a more recently completed study not previously evaluated by JMPR, imazalil (purity 97.46%) was administered in the diet to groups of five male Crl:WI(Han) rats for 1, 2, 7, 14 or 28 consecutive days at a concentration of 0, 200, 1200 or 2400 ppm. A positive control group was fed 1200 ppm phenobarbital on a similar regimen. All animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily, and detailed physical examinations were performed at least weekly. Individual body weights and feed consumption were recorded at least weekly. Blood samples collected at necropsy were analysed for levels of ALT and sorbitol dehydrogenase. Osmotic minipumps with a 30 mg/mL solution of BrdU were implanted 4 days prior to necropsy. The liver was removed, and slices of liver and duodenum were fixed in buffered formalin. Weights of liver and thyroids with parathyroids were determined for all animals. Microscopic examination was performed on the liver, thyroid and duodenum from all animals at each scheduled necropsy. Liver sections (right portion of the median lobe, right and left lobes), thyroid and duodenum were stained for BrdU, caspase and 4-hydoxy-2-nonenal. For each dose group, the remaining section of the median lobe of five animals was collected, subjected to preparation of microsomes and assayed for CYP2B1/2 activity (PROD) and UGT (4-methylumbelliferone assay). In addition, portions (approximately 50–100 mg) of the median liver lobe collected at the end of the 28-day exposure were analysed for mRNA expression using reverse transcriptase polymerase chain reaction for the following: constitutive androstane receptor (CAR), *Cyp2b1/2*, *Cyp3a1*, *Cyp3a2* and *Gadd45β* (using cyclophilin as reference gene).

There were no test substance–related effects on clinical observations, serum chemistry parameters or macroscopic examinations. Test substance–related lower mean body weights and body weight changes compared with the control group were observed at 1200 and 2400 ppm during multiple intervals throughout the study; at necropsy days 14 and 28, mean body weights of animals treated at 2400 ppm were reduced by more than 10%. The 1200 and 2400 ppm groups consumed less feed overall than the control group throughout the study. There were test substance–related higher mean absolute and/or relative liver weights on study days 14 and 28 in the 1200 and 2400 ppm groups. In comparison, the phenobarbital-treated group had higher mean absolute and relative liver and thyroid/parathyroid weights on study days 2, 7, 14 and 28. Dietary administration of imazalil was associated with alterations in hepatocellular staining affinity (increased cytoplasmic homogeneity), which was equivocally detectable as early as study day 1, more readily apparent at study day 2 and clearly visible at study days 7–28. The cytoplasmic alteration was interpreted as a metabolic response to imazalil. There was no histopathological indication of morphological injury to the liver in response to imazalil, and there were no imazalil-related alterations in serum ALT or sorbitol dehydrogenase. Phenobarbital-related hepatocellular changes were more pronounced than imazalil-related changes at later time points of the study and included overt centrilobular hepatocellular hypertrophy at study days 14 and 28. In addition, higher serum ALT and sorbitol dehydrogenase levels in phenobarbital-treated rats at study days 2, 7 and 14 indicated functional as well as morphological hepatocellular alterations. Results of BrdU immunohistochemical staining of liver and thyroid gland revealed an increased level of BrdU incorporation in the liver of phenobarbital-treated rats at study day 7, indicating hepatocellular proliferative activity, with no similar effect noted in imazalil-treated rats. An increased rate of BrdU incorporation was noted in the thyroid gland at study day 14 in rats receiving 2400 ppm imazalil and in phenobarbital-treated rats at study days 7, 14 and 28. Additionally, neither phenobarbital nor imazalil showed any increases in apoptosis (as measured by caspase immunohistochemistry) or oxidative stress (as measured by 4-hydroxy-2-nonenal immunohistochemistry). Significant increases in CYP2B1/2 and UGT1A activities were observed

following treatment with 1200 or 2400 ppm imazalil, with these increases appearing to be both dose and time dependent. Although a plateau effect in CYP2B1/2 activity was present at study day 7 with the 1200 ppm imazalil treatment level, exposure to 2400 ppm caused the largest changes in both CYP2B1/2 and UGT1A activities following a 28-day dosing regimen. The enzyme induction in the phenobarbital group was statistically significantly higher than that of the 2400 ppm imazalil group. The induction in the latter was, on average, 9% (PROD) and 39% (4-methylumbelliferone glucuronidation) as effective as that in the 1200 ppm phenobarbital group. Similarly, mRNA levels of *Cyp2b1* showed an induction by imazalil and a larger induction by phenobarbital. A striking similarity between both treatments was also noted for the induction at the mRNA level of CAR (NR1I3), *Cyp3a1*, *Cyp3a2* and *Gadd45*. Levels of CAR (NR1I3) did not show induction at the mRNA level when compared with animals on the basal diet. Levels of mRNA for *Cyp3a1, Cyp3a2* and *Gadd45β* were all increased in both the imazalil-exposed rats and the phenobarbital-exposed rats.

The results indicated that both imazalil and phenobarbital showed parallel effects, with phenobarbital being the more potent agent (Mertens, 2011). This study was not previously evaluated by JMPR.

Liver enzyme induction and liver cell proliferation: humans

The aim of a study by Elcombe (2012c), which was not previously evaluated by JMPR, was to investigate whether imazalil (purity 97.46%) is able to induce CYP2B6, CYP3A4 and cell proliferation (as measured by replicative DNA synthesis during cell cycle S-phase and using epidermal growth factor as the positive control) in isolated primary human hepatocytes. Following a range-finding test, freshly isolated female human hepatocytes were cultivated as monolayer cultures in appropriate culture flasks and well plates. Hepatocytes were exposed to imazalil at a concentration of 1, 3, 10 or 30 µmol/L or to vehicle alone (0.5% v/v DMSO) for 96 hours. Three to six replicates, depending on the assay, were performed. Cytotoxicity was measured as the change in intracellular ATP concentration. Bioluminescent determination of released ATP was carried out using the assay kit Cell-Titre Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer's instructions. Levels of human cytochrome P450 activities were determined spectrofluorometrically by formation of resorufin (according to Burke et al., 1985) or 7-hydroxyquinoline from pentoxyresorufin (PROD – CYP2B6) or benzyloxyquinoline (BQ – CYP3A4), respectively. The number of hepatocytes undergoing replicative DNA synthesis was measured by incorporation of BrdU in DNA and subsequent immunochemical detection.

Treatment with imazalil at 3, 10 or 30 µmol/L resulted in statistically significantly lower intracellular ATP, with levels being reduced to 94%, 77% and 12% of control values, respectively. The cytotoxicity observed following treatment with 30 µmol/L was considered excessive, and therefore replicative DNA synthesis and CYP2B6 (PROD) data either could not be measured at this concentration or were excluded from data analysis. In addition, the cytotoxicity observed following treatment with 10 µmol/L was considered biologically relevant and sufficient to demonstrate that imazalil had been tested to a suitably high concentration. No concentration of imazalil induced increases in replicative DNA synthesis (S-phase of the cell cycle), indicating that it did not induce cell proliferation. Treatment with imazalil at 10 µmol/L resulted in a statistically significant decrease in CYP2B6 activity (PROD), whereas data could not be measured at 30 µmol/L. Treatment with imazalil at 10 or 30 µmol/L resulted in statistically significant decreases in CYP3A4 activity (BQ). The decreased enzyme activities are probably due to inhibition by imazalil. The viability of the human hepatocytes and hence the validity of the test were verified by the marked stimulation of S-phase by epidermal growth factor.

Imazalil-induced liver enzyme activity was not seen in this cultured human hepatocyte study (Elcombe, 2012c). In contrast, enzyme induction was seen both in a companion mouse hepatocyte study (Elcombe, 2012b) and in a study in humanized mice in vivo (Elcombe, 2012a). The inhibition of cytochrome P450 by residual imazalil explains the poor induction of enzyme activities. However, increased expression of mRNA and protein for CYP2B and CYP3A was seen in imazalil-treated hPXR/hCAR mice and to a much greater degree in imazalil-treated wild-type C57Bl/6 mice in

previous tests. This suggests that although imazalil is a CAR activator, human CAR is less responsive than mouse CAR to imazalil. In the in vivo study in C57B/6 mice and hPAXR/hCAR mice (Elcombe, 2012a), imazalil stimulated S-phase. However, imazalil did not stimulate S-phase in human hepatocytes in vitro. It should be noted that hPXR/hCAR mice are humanized only for two genes (CAR and pregnane X receptor [PXR]), but all other genes involved in pathways of indirect activation and downstream signalling leading to cell proliferation remain murine. No increase in cell proliferation was observed in human hepatocytes exposed to imazalil at concentrations up to and beyond the toxicity threshold. Moreover, treatment with imazalil did not induce CYP2B6 or CYP3A4. These data demonstrate that human hepatocytes, unlike mouse hepatocytes, exhibit no cell proliferation in response to imazalil (Elcombe, 2012c). This study was not previously evaluated by JMPR.

CAR/PXR receptor agonism: mice

The capacity of imazalil to act as a CAR/PXR agonist in wild-type mice and mice humanized for CAR and PXR was investigated in a recently completed study not previously evaluated by JMPR. Phenobarbital, a well-studied CAR/PXR activator, was included as a positive control. The strains used in this study were wild-type C57Bl/6NTac mice and mice humanized for PXR and CAR (hPXR/hCAR). Each control or treatment group consisted of 20 male wild-type and 20 male hPXR/hCAR mice. Animals were exposed to imazalil (purity 97.46%) at a dietary concentration of 0, 50, 200 or 600 ppm or to phenobarbital at a dietary concentration of 1000 ppm for either 7 or 28 days. All diets were found to be homogeneous, and achieved concentrations of test items were between 89% and 102% of the target concentrations. The mean achieved intakes of imazalil at 0, 50, 200 and 600 ppm, calculated for the duration of the study, were 0, 6.7, 30 and 96 mg/kg bw per day, respectively, for wild-type mice and 0, 7.2, 26 and 78 mg/kg bw per day, respectively, for hPXR/hCAR mice. The mean achieved intakes of phenobarbital at 1000 ppm were 155 mg/kg bw per day for wild-type mice and 128 mg/kg bw per day for hPXR/hCAR mice.

Seven days before termination, animals were subcutaneously implanted with osmotic minipumps containing approximately 200 µL BrdU solution. The animals were terminated in a staggered fashion on consecutive days, such that five animals from each group (replicates I and III) were terminated on day 8 or day 29 and five animals from each group (replicates II and IV) were terminated on day 9 or day 30. Only animals treated for 7 days were analysed for the study-specific end-points, whereas samples from the animals treated for 28 days were stored for potential later analysis. Animals were checked for clinical signs and mortality once a day. Body weights were determined on day 3 and at termination (7-day exposure) or weekly (28-day exposure). Feed consumption was determined weekly. Terminal plasma was collected by cardiac puncture; ALT, AST, ALP, cholesterol and triglycerides were determined. Liver weight was determined, and liver and duodenum samples were fixed and evaluated histopathologically. Liver samples were processed, and subcellular fractions were assayed for caspase-3-activity, lipid peroxidation and cytochrome P450 activities; peroxisome β-oxidation (cyanide-insensitive palmitoyl-coenzyme A oxidation) was also assessed in liver fractions (heavy pellets). CYP3A and CYP3B were determined by western blot. A TaqMan® gene expression analysis assay was performed to determine *Cyp2b10*, *Cyp3a11* and *Gadd45β* (murine β-actin was used as the internal standard) in hepatic mRNA.

There were no clinical signs or mortalities, and body weights and liver weights were unaffected. Imazalil had no effect on ALT, AST or ALP in either strain of mouse. Administration of phenobarbital at 1000 ppm increased ALT and ALP in both wild-type and hPXR/hCAR mice, whereas AST was unaffected. Plasma cholesterol levels were significantly reduced following 7 days of administration of imazalil at 600 ppm and of phenobarbital at 1000 ppm in both wild-type and hPXR/hCAR mice. At histopathological examination, a few microscopic findings were recorded in the liver. Minimal to moderate diffuse, periportal or centrilobular hepatocellular vacuolation was observed in the animals of both mouse lines treated with both imazalil and phenobarbital. In addition, focal or multifocal inflammatory cell infiltration was noted in almost all mice of all groups. Portal mononuclear cell infiltrates also occurred in individual animals of all groups. Hepatocellular

diffuse/centrilobular hypertrophy was noted in phenobarbital-treated animals only. Minimal to slight hypertrophy was also noted in hPXR/hCAR mice administered 600 ppm imazalil. Hepatocellular hypertrophy was not observed in any wild-type mice administered imazalil. An increase in total cytochrome P450 was observed at 200 and 600 ppm imazalil and with phenobarbital in wild-type mice (1.5-, 2.5- and 3.7-fold compared with control values, respectively) and at 600 ppm and with phenobarbital in humanized mice (1.8- and 3.8-fold compared with control values, respectively). Imazalil induced *Cyp2b10* mRNA and protein expression and enzyme activity in a dose-dependent manner in both wild-type and hPXR/hCAR mice, but to a lesser extent compared with phenobarbital, and markedly less in hPXR/hCAR mice than in wild-type mice. Imazalil induced *Cyp3a11* mRNA expression in a dose-dependent manner in both wild-type and hPXR/hCAR mice. *Cyp3a* protein expression and enzyme activity were induced at 600 ppm and with phenobarbital. Imazalil and phenobarbital induced hepatic apoptosis markers *Gadd45β* mRNA expression and Caspase-3 cleavage products in both wild-type and hPXR/hCAR mice. Dietary administration of phenobarbital or imazalil for 7 days had no effect on lipid peroxidation in the livers of wild-type or humanized mice. Administration of imazalil at all concentrations to wild-type mice increased S-phase in liver by up to 6-fold relative to control values, whereas administration of phenobarbital at 1000 ppm increased S-phase by 31.2-fold. Administration of imazalil at all concentrations to hPXR/hCAR mice increased S-phase by up to 3.4-fold relative to control values, whereas phenobarbital at 1000 ppm increased S-phase by 24.5-fold. The imazalil-mediated induction of *Cyp2b10* and, to a lesser degree, *Cyp3a11* in the presence of increased hepatocellular S-phase strongly suggests that imazalil is an activator of the xenosensing nuclear receptors CAR and (probably) PXR.

Imazalil appeared to be a more potent activator of mouse CAR than human CAR, as noted by generally smaller responses in humanized CAR mice when compared with the wild-type mice at comparable doses. It should be noted that hPXR/hCAR mice are humanized only for two genes, but all other genes involved in pathways of indirect activation and downstream signalling leading to cell proliferation remain murine. Phenobarbital, a known non-genotoxic rodent liver carcinogen and CAR activator, was evaluated alongside imazalil and induced similar, albeit more marked, findings to those seen at high imazalil doses. Overall, it is considered that the liver effects seen with imazalil are CAR dependent.

The CAR/PXR activation with dose-responsive changes were seen at imazalil dietary concentrations of 200 ppm and above (Elcombe, 2012a). This study was not previously evaluated by JMPR.

Effects on the thyroid gland, liver enzyme induction and proliferation in thyroid, liver and jejunum: rats

In a study to investigate the mechanisms underlying the effects of imazalil on the thyroid gland, groups of 50 SPF male Wistar (Hannover) rats were given diets containing imazalil (purity 98.8%) at a concentration of 0, 400, 1200 or 3200 ppm (equal to 0, 41, 123 and 328 mg/kg bw per day, respectively) for 4 weeks; a further group received a diet containing phenobarbital at 1200 ppm (equal to 126 mg base equivalent/kg bw per day). Interim kills were carried out after 1, 2 and 4 weeks of treatment and after a 4-week (week 8) or 9-week (week 13) recovery period, so that the size of the group at each dietary concentration at the time of termination was 10. The rats were observed daily, and body weights and feed consumption were measured weekly. Blood was taken at termination for measurement of TSH, T_3, T_4 and other clinical chemical end-points, including bilirubin, total protein and ALP, ALT and AST activities. The animals were killed at the times described above, five animals from each group being injected 6 hours before death with BrdU. At termination, a full postmortem examination was carried out, and macroscopic abnormalities were noted. The thyroid (including parathyroids) and liver were weighed. The thyroid and liver were examined histologically after haematoxylin and eosin staining, and the thyroid, liver and sections of the jejunum from BrdU-treated rats were stained immunohistochemically for BrdU-labelled cells. Liver and thyroid microsomes were prepared from liver pieces and the left thyroid glands (Vermeir, Lavrijsen & Meuldermans, 2001). Induction or inhibition of the hepatic microsomal cytochrome P450 enzymes aniline hydroxylase,

EMD, PROD, 5′-monodeiodinase and T_4 glucuronosyltransferase was assessed in tissue from the five animals from each group not injected with BrdU; microsomal protein and thyroid peroxidase activity in the thyroid were also measured.

No treatment-related deaths were seen. There was significant feed wastage at the high dose of 3200 ppm. Sedation was noted in the phenobarbital-treated animals during week 1 and week 2. Mid- and high-dose male rats, respectively, weighed up to 8% ($P < 0.01$) and 19% ($P < 0.01$) less than vehicle controls, gained 15% ($P < 0.01$) and 36% ($P < 0.01$) less weight and consumed 7% ($P < 0.01$) and 15% ($P < 0.01$) less feed during the 4-week treatment period. The positive control group had mean body weights similar to those of controls during the 4-week treatment period, but weighed 6–7% ($P < 0.01$) less than controls from weeks 5 to 8 of the recovery period. Feed consumption was decreased sporadically in animals at 400 ppm, but this was considered not biologically relevant. At 1200 ppm, feed consumption was decreased during dosing and for the first 3 weeks of the recovery period, whereas at 3200 ppm, feed consumption was lower than in the controls during treatment and for 1 week afterwards. In the group given phenobarbital, feed consumption was increased during week 2 of dosing. At 400 ppm, no difference in clinical chemical end-points relative to controls was seen.

At 1200 ppm, ALP activity was decreased by 22% ($P < 0.05$) in mid-dose rats treated for 2 weeks. Serum AST activity showed dose-related decreases during weeks 1 and 2; the decreases were 17–30% ($P < 0.05$) for the mid-dose group and 41–45% ($P < 0.01$) for the high-dose group. Data for the positive control showed statistically significant increases for serum total protein at weeks 1, 2 and 4 (7–13%, $P < 0.01$ or < 0.05), an increase in serum albumin at week 8 (6%, $P < 0.05$), a decrease in ALP activity at week 2 (31%, $P < 0.05$) and a small increase in ALT activity at week 2 (28%, $P < 0.05$). TSH concentrations were increased in the phenobarbital-treated animals and in all imazalil-treated animals at 8 weeks. No statistically significant increases in TSH concentration were seen at other times; nevertheless, the increases at 1 and 2 weeks may have been biologically relevant. The T_4 concentrations were lower in all imazalil-treated groups after 1 week. After 2 weeks, no differences in T_4 concentration were seen, whereas at 4 weeks, the concentrations were increased in rats at 1200 and 3200 ppm and in those given phenobarbital. At week 8 (i.e. during the recovery period), an increased T_4 concentration was seen in the group at 1200 ppm and in the group given phenobarbital. An increase was also seen in the group given 3200 ppm at week 13. The T_3 concentration was decreased in the group given 3200 ppm at week 4 and in the group given phenobarbital at weeks 1, 2 and 4.

Increased hepatic activities of cytochrome P450, aniline hydroxylase, EMD and PROD were seen in both the imazalil-treated and the phenobarbital-treated groups at weeks 1, 2 and 4. Induction was often found at the lowest dietary concentration. Enzyme induction was largely absent during week 8, indicating that it was a reversible phenomenon. The activity of 5′-monodeiodinase was decreased in the group given 3200 ppm imazalil, and T_4 glucuronosyltransferase activity was induced in all imazalil-treated groups after 1 week of treatment and also at week 4 in the group given 3200 ppm. Thyroid peroxidase activity was increased in the group at 3200 ppm after weeks 1 and 4 and reduced after 2 weeks of treatment; however, the changes were neither statistically significant nor clearly dose related.

Increases in the relative weight of the thyroid of animals at 3200 ppm were seen at week 2 only, whereas phenobarbital increased both the relative and absolute thyroid weights at this time. At week 2, a positive trend in the relative thyroid weight with dose of imazalil was found. At week 1, the relative and absolute weights of the liver were increased at 400 and 3200 ppm, and only the relative weight was increased at 1200 ppm. At week 2, the relative liver weight was increased at 1200 and 3200 ppm, whereas at week 4, the relative and absolute liver weights were increased at 400 and 1200 ppm. The relative liver weight was increased at week 4 at the highest dietary concentration. At 8 weeks, a decrease in absolute but not relative liver weight was observed at the intermediate and high dietary concentrations of imazalil. Phenobarbital increased the absolute and relative weights of the liver at 1, 2 and 4 weeks, but this effect had disappeared by 8 weeks. At the terminal kill at 13 weeks, the highest concentration of imazalil had produced a decrease in the absolute weight but not in the relative weight of the liver, whereas phenobarbital had no effect.

The only gross findings of interest were in the liver and consisted of swelling in a few animals at 400 and 1200 ppm at weeks 2 and 4, respectively, and swelling and increased lobulation at 3200 ppm from week 1 onwards. Histologically, hepatic centrilobular hypertrophy was found at concentrations of 400 ppm and above, and the occurrence of this change appeared to be dose related. Periportal hypertrophy was found at 1200 and 3200 ppm, whereas hepatic vacuolation was seen at the highest dietary concentration. All three changes in the liver were also seen with phenobarbital. These changes were not observed 8 and 13 weeks after the start of the study (i.e. during the recovery period). Significant increases in the frequency of thyroid follicular cell hypertrophy were seen only with phenobarbital. This lesion disappeared during the recovery period. Immunohistochemistry for BrdU-labelled cells did not reveal intergroup differences. The authors concluded that imazalil alters thyroid status by affecting hepatic and thyroid enzymes involved in the synthesis, metabolism and excretion of T_4 (Piccirillo, 2000; Verbeek et al., 2000; Vermeir, 2000). These studies were not previously evaluated by JMPR.

Conclusion

Qualitatively, these studies show that imazalil causes hypertrophy of the liver and, at high doses, some interference with thyroid function. The quantitative differences between the studies may be related to their duration. The results for induction of specific hepatic enzymes were not easy to interpret, as they tended to be inconsistent, and dose–effect relationships were not always clear. However, the 2001 Meeting concluded that imazalil is a relatively nonspecific enzyme inducer in the liver and that its enzyme-inducing effects are reversible (Annex 1, reference *94*).

(c) Endocrine disruption

Imazalil was tested for its ability to activate the estrogen receptor in vitro in a proliferation assay with MCF7 cells and a screening test for yeast estrogen. Statistically significant agonism was not observed (Vinggaard, Breinholt & Larsen, 1999).

11β-Hydroxylation and 19-hydroxylation in the mitochondria of gerbils (*Meriones unguiculatus*) in vitro were suppressed in parallel by about 75% by imazalil (purity not reported) at 1 µmol/L (Drummond, Mason & McCarthy, 1988).

In a study of the effect of imazalil (purity 97–99.9%) on CYP19 aromatase activity in human placental microsomes, the median inhibitory concentration was 0.34 µmol/L (Vinggaard et al., 2000).

(d) Studies on metabolites

The acute oral toxicity and/or the genotoxic potential of the metabolites R043449 (FK284), R061000 (FK772) and R014821 were evaluated in recently completed studies that have not previously been evaluated by JMPR. In addition, a study designed for setting an ARfD, also not previously evaluated by JMPR, was carried out with R14821.

R043449 (FK284) (3-[2-(2,4-dichlorophenyl)-2-hydroxymethyl]-2,4-imidazolidinedione)

In an acute oral toxicity study, six female Sprague Dawley rats received a single gavage dose of R043449 (purity 101.5%) in 10 mL/kg bw 0.8% aqueous hydroxypropyl methylcellulose at 2000 mg/kg bw. Animals were observed for clinical signs and mortality before and immediately after dosing, at 5, 15, 30 and 60 minutes after dosing, as well as at 3, 6 and 24 hours after administration. Thereafter, all animals were observed daily for up to 14 days. Observations for changes to skin and fur, eyes and mucous membranes, respiratory and circulatory systems, autonomic and central nervous systems, somatomotor activity as well as behaviour patterns were made at least once a day. Attention was also paid to possible tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. Individual body weights were recorded before administration of the test item and thereafter in weekly

intervals up to the end of the study. Changes in weight were calculated and recorded. At the end of the experiments, all animals were terminated, dissected and inspected macroscopically. All gross pathological changes were recorded. Necropsy and macroscopic inspection of animals that died prematurely were carried out as soon as possible after death.

There were no clinical signs or mortalities during the study. All animals gained weight normally during the study. There were no findings upon necropsy.

The LD_{50} was greater than 2000 mg/kg bw (Haferkorn, 2010a).

The mutagenicity of R043449 (purity 101.5%) was examined in a bacterial reverse mutation assay using five *Salmonella typhimurium* strains (TA98, TA100, TA102, TA1535 and TA1537). Two independent experiments were carried out, each with and without metabolic activation (S9 liver fraction derived from Aroclor 1254–induced rats). Ten concentrations ranging from 0.316 to 5000 µg/plate were tested. Pronounced cytotoxicity (scarce background lawn and reduction of the number of revertants by more than 50%) was noted at the top concentration of 5000 µg/plate.

No mutagenic effect (no increase in revertant colony numbers compared with control counts) was observed for R043449 tested up to a cytotoxic concentration of 5000 µg/plate in any of the five test strains in two independent experiments (plate incorporation and preincubation test), each without and with metabolic activation (Flügge, 2010).

In an in vitro chromosomal aberration test, R043449 (purity 100%) was tested in Chinese hamster V79 lung cells up to cytotoxic doses for 3 hours with and without S9 mix and for 20 hours without S9 mix. DMSO was used as the vehicle. In assay 1, insolubility was detected at the end of treatment in the 500–1000 µg/mL concentration range with and without S9 mix. Cytotoxicity was observed at 1000 µg/mL. Without S9 mix (20-hour treatment, harvesting at 28 hours), cytotoxicity was observed at 300 µg/mL. No statistically significant increases in mean percentage of aberrant cells (excluding gaps) were observed at any of the concentrations used in any of the experiments. Polyploid metaphases (1–4) were found in some cases in the negative (vehicle) control, test item–treated or positive control samples in both assays, whereas no endoreduplicated metaphases were found in any samples.

In conclusion, no statistically significant increases in mean percentage of aberrant cells (excluding gaps) were noted with and without S9 mix. Therefore, R043449 is considered to be not clastogenic under the conditions of this study (Hargitai, 2017a).

In an in vitro mammalian cell gene mutation test, R043449 (purity 100%) was tested in Chinese hamster ovary (CHO K1) cells at the *Hprt* locus. Two assays were carried out; in assay 1, treatments were carried out for 5 hours with and without S9 mix, and in assay 2, treatments were carried out for 5 hours with S9 mix and for 24 hours without S9 mix. DMSO was used as the vehicle. In both assays, minimal to full insolubility was detected in the 600–800 µg/mL range at the end of treatment with or without S9 mix. Cytotoxicity was observed at the top doses in all assays.

No statistically significant or biologically relevant increase in mutant frequency was observed at any concentration tested, and no dose–response relationship was evident after trend analysis both with and without S9 mix. Therefore, it is concluded that R043449 (FK284) is not mutagenic in mammalian cells in either the presence or absence of S9 mix under the conditions of this assay (Hargitai, 2017b).

R061000 (FK772) ((±)-3-[2-(2,4-dichlorophenyl)-2-(2,3-dihydroxypropoxy)ethyl]-2,4-imidazolidinedione)

In an acute oral toxicity study, six female Sprague Dawley rats received a single gavage dose of R061000 (purity ~97.3%) at 2000 mg/kg bw in 10 mL/kg bw 0.8% aqueous hydroxypropyl methylcellulose. Animals were observed for clinical signs and mortality before and immediately after dosing, at 5, 15, 30 and 60 minutes after dosing, as well as at 3, 6 and 24 hours after administration. Thereafter, all animals were observed daily for up to 14 days. Observations for changes to skin and fur, eyes and mucous membranes, respiratory and circulatory systems, autonomic and central nervous systems, somatomotor activity as well as behaviour patterns were made at least once a day. Attention was also paid to possible tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. Individual body weights were recorded before administration of the test item and thereafter in weekly intervals up to the end of the study. Changes in weight were calculated and recorded. At the end of the experiments, all animals were terminated, dissected and inspected macroscopically. All gross pathological changes were recorded. Necropsy and macroscopic inspection of animals that died prematurely were carried out as soon as possible after death.

There were no mortalities during the study. Piloerection was observed in all animals 15 minutes to 3 hours after administration. There were no other clinical signs. All animals gained weight normally during the study. There were no findings upon necropsy.

The LD_{50} was greater than 2000 mg/kg bw (Haferkorn, 2010b).

The mutagenicity of R061000 (purity ~97.3%) was examined in a bacterial reverse mutation assay using five *S. typhimurium* strains (TA98, TA100, TA102, TA1535 and TA1537) in the presence and absence of metabolic activation (S9 liver fraction derived from Aroclor 1254–induced rats). Six concentrations of R061000 ranging from 31.6 to 5000 µg/plate were employed in the plate incorporation test and in the preincubation test, each carried out without and with metabolic activation.

No signs of cytotoxicity were noted at the top concentration of 5000 µg/plate. No mutagenic effect (no increase in revertant colony numbers compared with control counts) was observed for R061000 tested up to 5000 µg/plate in any of the five test strains in two independent experiments (plate incorporation and preincubation test), each without and with metabolic activation.

R061000 (FK772) was not mutagenic up to the limit concentration of 5000 µg/plate in this bacterial reverse mutagenicity assay (Flügge, 2012).

In an in vitro chromosomal aberration test, R061000 (purity 97.7%) was tested in Chinese hamster V79 lung cells up to cytotoxic doses for 3 hours with and without S9 mix and for 20 hours without S9 mix. DMSO was used as the vehicle. In both assays, no insolubility and no large changes in pH or osmolality were detected at the end of treatment at concentrations up to 2000 µg/mL with and without S9 mix. In assay 1, no statistically significant increases in mean percentage of aberrant cells (excluding gaps) were observed at any of the concentrations used both with and without S9 mix. In assay 2, with S9 mix, no statistically significant increases in mean percentage of aberrant cells (excluding gaps) were observed at any of the concentrations used. Without S9 mix, statistically significant increases in mean percentage of aberrant cells (excluding gaps) were observed at 750 and 1000 µg/mL. The mean percentage of aberrant cells (excluding gaps) of 4.0% was at the upper limit of the historical control range of the laboratory (0–4%), and the response at 1000 µg/mL of 5.3% was obtained at a cytotoxic level (relative increase in cell count of 35%). However, based on the very weak response (20% aberration level is 4320 µg/mL, which is above the maximum concentration that is appropriate for testing), lack of repeatability and lack of biological relevance, the observed results were not considered indicative of clear and significant clastogenic activity under the conditions of this test system. The occurrence of polyploid and endoreduplicated metaphases was recorded. Polyploid metaphases (1–6) were found in some cases in the negative (vehicle) control, test item–treated or

positive control samples in both assays. No endoreduplicated metaphases were found in assay 1, whereas an endoreduplicated metaphase was recorded in assay 2 for the negative (vehicle) control sample without S9 mix and one test item–treated sample with S9 mix. The negative (vehicle) control data were within the acceptable range for the spontaneous chromosomal aberration frequency, and the positive control substances demonstrated the sensitivity of the test system.

Therefore, R061000 is considered not clastogenic under the conditions of this study (Hargitai, 2017c).

In an in vitro mammalian cell gene mutation test, R061000 (purity 97.7%) was tested in Chinese hamster ovary (CHO K1) cells at the *Hprt* locus in the presence and absence of metabolic activation. DMSO was used as the vehicle. Minimal to full insolubility was detected at the end of the treatment in assay 1 at 1000–1750 µg/mL with S9 mix and in assay 2 at 1900–2000 µg/mL without S9 mix and at 1000–2000 µg/mL with S9 mix.

No statistically significant increase in the mutant frequency value was observed in this experiment at any examined concentration. No dose–response relationship was observed (a trend analysis showed no effect of treatment). Therefore, it is concluded that R061000 (FK772) is not mutagenic in mammalian cells in either the presence or absence of S9 mix under the conditions of this assay (Hargitai, 2017d).

R014821 ((RS)-1-(2,4-dichlorophenyl)-2-imidazol-1-yl-ethanol)

In a single-dose oral toxicity study, two parallel sets of Wistar rats (five of each sex per dose per set) were gavaged with a single dose of R014821 (batch no. M12GB4078; purity 98.0%), at 0, 125, 500 or 2000 mg/kg bw. The vehicle was PEG 400. One set of animals (control group and three treated dose groups) was terminated at 24 hours for examination of acute effects, whereas the parallel set was subjected to termination at 7 days post-administration for delayed effects or recovery. Mortality was assessed at least twice daily. Clinical signs were assessed on all animals daily up to and including the day of necropsy. Necropsy was performed on day 2 for main group animals and on day 8 for recovery groups. Body weights were recorded on days 1, 2, 4, 7 and 8. Feed consumption was recorded for days 1–2, 2–5 and 5–7. Neurobehavioural examinations were made on all recovery group males prior to treatment and at 4 and 24 hours and 7 days after treatment. Clinical laboratory investigations were made on day 2 for main group animals and on day 8 for recovery group animals. Haematological and clinical chemistry parameters were evaluated. Prothrombin clotting time was determined. At necropsy, all animals were investigated for macroscopic abnormalities, and samples of all organs (according to guidelines) were subjected to histopathological examination. Organ weights were recorded for selected organs. Formulation analyses confirmed that formulations of test substance in PEG 400 were prepared accurately and homogeneously distributed and were stable over at least 6 hours.

No preterminal mortalities were observed. Lethargy was noted in one male at the top dose. Body weight generally remained static at 2000 mg/kg bw in males over the entire study; cumulative total feed consumption up to the end of week 1 was reduced by 20% in this group. In females, body weight gain at 500 and 2000 mg/kg bw was about half those of the controls and the 125 mg/kg bw group; cumulative total feed consumption up to the end of week 1 was likewise 12% lower in these groups. Neurobehavioural observations at 2000 mg/kg bw comprised lethargy in two animals at 4 hours post-dosing, abnormal posture in one animal at 4 hours post-dosing, both being reversible within 24 hours, hunched posture after 7 days in one animal and loss of righting reflex in three animals after 24 hours, fully reversible within 7 days. Owing to the transient and incidental nature of these findings, they were not considered to be toxicologically relevant. Rectal temperature was not affected. No toxicologically relevant effects on haematological parameters were observed that could be attributed to treatment. No microscopic correlate for the significant prolongation of prothrombin clotting was seen in males at 2000 mg/kg bw. Various changes in clinical biochemistry parameters were noted that were essentially reversible within 7 days, except for decreased bilirubin in males and

decreased urea levels in females, which were considered to be within the normal range for rats of this age and strain. At 125 and 500 mg/kg bw, these findings were not accompanied by clear histopathological lesions. These findings comprised lower ALP, lower total protein in males and higher total protein in females. Albumin, bilirubin, urea, phospholipids, potassium and calcium were reduced, and sodium levels were increased. At the top dose (2000 mg/kg bw), liver, adrenal and spleen weights (absolute and relative to body weight) were increased, without macroscopic pathological findings. At the end of recovery, absolute and relative liver weights remained increased in both sexes at the top dose. Increased adrenal weights in all male dose groups did not show any dose–effect relationship and were considered to be caused by relatively low control values and hence not of toxicological relevance. Microscopic examination revealed a minimal increase in myelopoiesis of the sternal bone marrow in males treated with 2000 mg/kg bw and terminated at 24 hours. After recovery of 7 days, hepatocellular vacuolation of liver in both sexes at the middle and top doses was found. Hypertrophy of the follicular epithelium of the thyroid gland in males and cortical hypertrophy of adrenal glands in females were seen at 2000 mg/kg bw. All other findings were considered to be within the normal background ranges encountered for Wistar-Han strain rats.

The NOAEL was 125 mg/kg bw, based on reduced feed consumption and body weight gain at 500 mg/kg bw (Beerens-Heijnen, 2013).

In an in vitro chromosomal aberration test, R014821 (purity 99.4%) was tested in Chinese hamster V79 lung cells up to cytotoxic doses for 3 hours with and without S9 mix and for 20 hours without S9 mix. DMSO was used as the vehicle. Cytotoxicity was observed at 400 µg/mL. No statistically significant increases in the mean percentage of aberrant cells (excluding gaps) were observed at any of the concentrations used both with and without S9 mix.

Therefore, R014821 is considered to be not clastogenic under the conditions of this study (Hargitai, 2017f).

In an in vitro mammalian cell gene mutation test, R014821 (purity 99.4%) was tested in Chinese hamster ovary (CHO K1) cells at the *Hprt* locus in the presence and absence of metabolic activation. DMSO was used as the vehicle. Excessive cytotoxicity was observed at 400–500 µg/mL. The mutation frequency of the vehicle control was within the historical control range of the laboratory in all assays. The positive controls gave the anticipated increases in mutation frequency over the controls and were in good concordance with historical data in all assays.

It can be concluded that R14821 is not mutagenic in mammalian cells in either the presence or absence of S9 mix under the conditions of this assay (Hargitai, 2017e).

3. Observations in humans

3.1 Medical surveillance on manufacturing plant personnel

A statement was received from the occupational medical advisor for one of the imazalil manufacturing plants. Up to 2016, there was no evidence or indication of negative effects in workers as a result of active participation in imazalil processing (Wiener, 2016).

3.2 Case reports

A 43-year-old female veterinary technician developed contact dermatitis following exposure to the veterinary antimycotic Imaverol, which contains imazalil (enilconazole). Patch testing showed sensitivity to several imidazoles, including imazalil (Van Hecke & de Vos, 1983).

A case of palatal and nasal infection with *Alternaria* was treated with imazalil at doses progressing from 50 to 1200 mg administered topically and orally. The drug was tolerated, but it was

found to be bitter and unpleasant, with an aftertaste lasting for hours, and nausea occurred at high doses (≥800 mg). Very limited studies of pharmacokinetics were carried out: at a dose of 400 mg/day, the maximum serum concentration was about 2 µg/mL, whereas at a dose of 1200 mg/day, the maximum serum concentration was about 4 µg/mL. The half-life was about 2 hours, and administration of 1200 mg daily for 1 month did not result in accumulation. No changes in clinical chemical parameters were seen (Stiller & Stevens, 1986).

3.3 Experimental studies

A study in volunteers (19 males and one female) was undertaken to examine the local effects of imazalil on the skin. Imazalil base (0.2% w/w), imazalil base (0.2% w/w) ointment, imazalil sulfate (526 mg base/L), Fungaflor (68% w/w imazalil) and two control formulations not containing imazalil were applied under occlusive adhesive strips for 48 hours. The skin was then examined for redness, oedema, irritation or inflammation. There were no complaints of burning, itching or pain, and the skin at the sites of application appeared normal in all cases (Desplenter & Verhamme, 1979).

Comments

Biochemical aspects

After administration of [^{14}C]imazalil to rats by gavage at a single dose of 1.25 or 20 mg/kg bw or a repeated dose of 1.25 mg/kg bw of non-radiolabelled material for 14 days followed by a single oral dose of radiolabelled test compound, [^{14}C]imazalil was rapidly and nearly completely absorbed. Most of the label was excreted (approximately 90%) within 24 hours. More imazalil appeared in the urine (49–60% of the administered dose) than in the faeces (36–48% of the administered dose) with all dosing regimens. At 96 hours after oral administration of [^{14}C]imazalil, tissue concentrations (including carcass) of radioactivity were about 1% of the administered dose. Nearly 50% of the radiolabel retained in the body was found in the liver (Mannens, Van Leemput & Heykants, 1993).

Very little imazalil was excreted unchanged, and the compound was metabolized to at least 25 metabolites. The metabolic profiles in the urine and faeces were largely comparable. Moreover, identical metabolites were recovered in the excreta regardless of sex, dose or dosing regimen. The main routes of metabolism were epoxidation, epoxide hydration, oxidative *O*-dealkylation, imidazole oxidation and scission, and oxidative *N*-dealkylation. The metabolic pattern was similar for both sexes after both oral and intravenous administration (Mannens, Van Leemput & Heykants, 1993).

Toxicological data

In rats, the lowest acute oral LD_{50} for imazalil was 227 mg/kg bw (Niemegeers, 1979), the acute dermal LD_{50} was greater than 2000 mg/kg bw (Dreher, 1990b) and the acute inhalation LC_{50} was 1.84 mg/L (Blagden, 1990). Imazalil was mildly irritating to the skin of rabbits (Dreher, 1990c) and severely irritating to their eyes (Teuns et al., 1990b). It was not sensitizing to the skin of guinea-pigs, as determined by the Magnusson and Kligman test (Teuns et al., 1990c) and the Buehler test (Wnorowski, 1997).

In a single-dose oral toxicity study, rats were administered imazalil via gavage at a dose of 0, 25, 100 or 400 mg/kg bw. The NOAEL was 100 mg/kg bw, based on an increased incidence of slight hepatocellular vacuolation in both sexes and increased liver weights in females at 400 mg/kg bw (Teunissen, 2013).

Short- and long-term studies in mice, rats and dogs showed that the main target organ of toxicity was the liver.

In a 3-month toxicity study in mice, imazalil was administered in the diet at a concentration of 0, 200, 400 or 800 ppm (equivalent to 0, 30, 60 and 120 mg/kg bw per day, respectively). A NOAEL

could not be identified in this study, as hepatocytic vacuolation was seen at all doses in males (Verstraeten et al., 1993a).

In a 4-week range-finding toxicity study in rats, imazalil was administered at a dietary concentration of 0, 100, 1000, 2000 or 3000 ppm (equal to 0, 11.5, 116.7, 232.1 and 351.3 mg/kg bw per day for males and 0, 11.7, 124.0, 229.0 and 351.6 mg/kg bw per day for females, respectively). The NOAEL was 100 ppm (equal to 11.5 mg/kg bw per day), based on decreased body weight, feed consumption and heart, spleen, kidney and testis weights in males and increased liver weights in females at 1000 ppm (equivalent to 116.7 mg/kg bw per day) (Gur, Nyska & Crown, 1990).

In a 13-week study in rats, imazalil was administered at a dietary concentration of 0, 40, 200 or 1000 ppm (equal to 0, 4.02, 18.78 and 94.58 mg/kg bw per day for males and 0, 4.05, 20.28 and 99.34 mg/kg bw per day for females, respectively). The NOAEL was 40 ppm (equal to 4.02 mg/kg bw per day), on the basis of liver histopathology and associated clinical chemistry changes at 200 ppm (equal to 18.78 mg/kg bw per day) (Gur, Nyska & Waner, 1991).

Imazalil was administered to rats for 6 months at a dietary concentration of 0, 25, 100 or 400 ppm (equivalent to 0, 1.25, 5.0 and 20 mg/kg bw per day, respectively). The NOAEL was 100 ppm (equivalent to 5.0 mg/kg bw per day), on the basis of changes in liver, kidney, lung and thymus weights in females and increased relative kidney weights in males at 400 ppm (equivalent to 20 mg/kg bw per day) (Lina et al., 1983).

In a 1-year study, dogs received imazalil at a dose of 0, 1.25, 2.5 or 20 mg/kg bw per day orally in gelatine capsules. The NOAEL was 2.5 mg/kg bw per day, on the basis of clinical signs, decreased body weight gain and feed consumption, decreased serum calcium concentration, increased serum ALP activity and increased liver weights at 20 mg/kg bw per day (Verstraeten et al., 1989).

In a 2-year study, dogs received imazalil at a dose of 0, 1.25, 5 or 20 mg/kg bw per day by capsule. The NOAEL was 1.25 mg/kg bw per day, based on decreased body weight gain and slight ground glass aspect of the cytoplasm in centrilobular hepatocytes at 5 mg/kg bw per day (Marsboom et al., 1977).

The overall NOAEL for imazalil in dogs was 2.5 mg/kg bw per day, on the basis of decreased body weight, decreased body weight gain and liver toxicity seen at 5 mg/kg bw per day.

In a study of carcinogenicity, mice received imazalil sulfate via the drinking-water at a concentration of 0, 6.25, 25 or 100 ppm (equivalent to 0, 2.5, 10 and 40 mg/kg bw per day, respectively) for 18 months (Marsboom & Herin, 1979a). This study was not considered to be acceptable for the evaluation because of several shortcomings in its design and conduct.

In a 23-month study of carcinogenicity, mice received imazalil at a dietary concentration of 0, 50, 200 or 600 ppm (equal to 0, 8.1, 33.4 and 105 mg/kg bw per day for males and 0, 9.9, 41.6 and 131 mg/kg bw per day for females, respectively). The NOAEL for toxicity was 50 ppm (equal to 8.1 mg/kg bw per day), on the basis of morphological changes (foci and nodules) in the livers of males at 200 ppm (equal to 33.4 mg/kg bw per day). Liver adenomas were observed in males at 200 and 600 ppm and in females at 600 ppm. The NOAEL for carcinogenicity was 50 ppm (equal to 8.1 mg/kg bw per day), based on an increased incidence of adenomas seen in males at 200 ppm (equal to 33.4 mg/kg bw per day) (Verstraeten et al., 1993b).

In an 18-month study of toxicity in rats, imazalil was administered at a dietary concentration of 0, 25, 100 or 400 ppm (equivalent to 0, 1.2, 5 and 20 mg/kg bw per day, respectively). The NOAEL was 100 ppm (equivalent to 5 mg/kg bw per day), on the basis of decreased body weight gain in females, decreased plasma albumin concentration in males and pathological changes in the livers of males at 400 ppm (equivalent to 20 mg/kg bw per day) (Lina et al., 1984). Although there was no evidence that imazalil was carcinogenic, the duration of the study and the number of animals used were insufficient to exclude that possibility.

In a 24-month study of toxicity and carcinogenicity, rats received diets containing imazalil at a concentration of 0, 50, 200, 1200 or 2400 ppm (equal to 0, 2.4, 9.7, 58 and 120 mg/kg bw per day for males and 0, 3.4, 13.5, 79 and 157 mg/kg bw per day for females, respectively). The NOAEL for

toxicity was 50 ppm (equal to 2.4 mg/kg bw per day), on the basis of minor haematological changes in both sexes and increased blood glucose concentrations and hepatic changes (increased relative liver weight and an increased frequency of pigment-laden hepatocytes) in females at 200 ppm (equal to 9.7 mg/kg bw per day). The NOAEL for carcinogenicity was 200 ppm (equal to 9.7 mg/kg bw per day), based on an increased incidence of follicular cell neoplasia (adenoma and carcinoma combined) of the thyroid in males at 1200 ppm (equal to 58 mg/kg bw per day) (Van Deun, 1999a).

In a 30-month study of carcinogenicity, imazalil was administered to rats at a dietary concentration of 0, 25, 100 or 400 ppm (equal to 0, 1.0, 3.6 and 15.0 mg/kg bw per day for males and 0, 1.2, 4.7 and 19.7 mg/kg bw per day for females, respectively). The NOAEL for toxicity was 100 ppm (equal to 3.6 mg/kg bw per day), on the basis of decreased body weight gain in males at 400 ppm (equal to 15.0 mg/kg bw per day). No treatment-related histopathological effects were observed in the liver, and there was no treatment-related increase in the incidence of tumours (Til et al., 1985). The Meeting noted that the highest dose tested was lower than the LOAEL for carcinogenicity in the 24-month study.

The Meeting concluded that imazalil is carcinogenic in mice and rats.

Imazalil was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found (Steelman & Schreiner, 1977; Vanparys & Marsboom, 1979, 1982, 1987a,b, 1988a,b; Fautz, 1990; Jenkinson, 1990a,b; Vanparys et al., 1990; Watabe, 1992; Van Gompel, Vanparys & Van Cauteren, 1995; Clare, 1996).

The Meeting concluded that imazalil is unlikely to be genotoxic.

The results of several mechanistic studies of the liver effects of imazalil in mice and rats and thyroid effects in rats indicate that imazalil has a phenobarbital-like mode of action in the induction of liver and thyroid tumours in rodents. The modes of action for these tumours were assessed using the International Programme on Chemical Safety (IPCS) human relevance framework (see Appendix 1). It was concluded that these carcinogenic responses are not relevant to humans.

In view of the lack of genotoxicity and the lack of human relevance of the tumours observed in mice and rats, the Meeting concluded that imazalil is unlikely to pose a carcinogenic risk to humans.

A two-generation study of reproductive toxicity was conducted in rats, in which imazalil was administered in the diet at a nominal dose of 0, 5, 20 or 80 mg/kg bw per day. The NOAEL for parental toxicity was 20 mg/kg bw per day, on the basis of reduced maternal weight gain and hepatotoxicity (vacuoles) in males at 80 mg/kg bw per day. The NOAEL for offspring toxicity was 20 mg/kg bw per day, on the basis of decreased numbers of live pups, decreased survival rate of pups and increased numbers of stillbirths at 80 mg/kg bw per day. The NOAEL for reproductive toxicity was 20 mg/kg bw per day, on the basis of increased duration of gestation for the F_0 and F_1 females and decreased gestation rate in F_1 females at 80 mg/kg bw per day (Dirkx et al., 1992b).

In a developmental toxicity study in mice, imazalil was administered by gavage during gestation days 6 through 16 at a dose of 0, 40, 80 or 120 mg/kg bw per day. The NOAEL for maternal toxicity was 40 mg/kg bw per day, on the basis of reduced body weight gain and feed consumption at 80 mg/kg bw per day. No NOAEL was identified for embryo and fetal toxicity, as litter size and the number of live pups were decreased in all dose groups (Gillardin, Sanz & Marsboom, 1987).

In a second study in mice, imazalil was administered by gavage on gestation days 6 through 16 at a dose of 0, 10, 40, 80 or 120 mg/kg bw per day. The NOAEL for maternal toxicity was 10 mg/kg bw per day, on the basis of decreased body weight gain and reduced feed consumption at 40 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 80 mg/kg bw per day, on the basis of reduced number of live fetuses, increased number of resorptions, and decreased pup body weights at 120 mg/kg bw per day. There was no evidence of teratogenicity (Levron et al., 1991).

In a study of developmental toxicity in rats, imazalil was administered by gavage on gestation days 6 through 16 at a dose of 0, 40, 80 or 120 mg/kg bw per day. No teratogenic effects were seen. The NOAEL for embryo and fetal toxicity was 40 mg/kg bw per day, on the basis of reduced pup

body weight at 80 mg/kg bw per day. A NOAEL for maternal toxicity could not be identified because of lower maternal body weight in all the groups when compared with controls (Gillardin et al., 1988).

The developmental toxicity of imazalil in rabbits was studied at gavage doses of 0, 1.25, 2.5 and 5 mg/kg bw per day administered on gestation days 6–18. The NOAEL for both maternal and embryo/fetal toxicity was 5 mg/kg bw per day, the highest dose tested (Dirkx & Marsboom, 1985).

In another study of developmental toxicity in rabbits administered imazalil sulfate by gavage at a dose of 0, 5, 10 or 20 mg/kg bw per day on gestation days 6–18, the NOAEL for maternal toxicity was 5 mg/kg bw per day, on the basis of reduced feed consumption at 10 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 5 mg/kg bw per day, on the basis of an increased incidence of resorptions and a decrease in the number of live pups at 10 mg/kg bw per day (Dirkx et al., 1992a).

The Meeting concluded that imazalil is not teratogenic.

In an acute neurotoxicity study, rats were given a single oral dose of imazalil at 0, 60, 180 or 600 mg/kg bw and observed for 14 days. The NOAEL for systemic toxicity was 180 mg/kg bw, on the basis of decreased body weight gains in males, deaths in females, and FOB and motor activity alterations at the time of peak effect on day 0 in females at 600 mg/kg bw. The NOAEL for neurotoxicity was 600 mg/kg bw, the highest dose tested (Beck, 2006b). The Meeting noted that the highest dose used was above the LD_{50}, and therefore the FOB results are due to general toxicity.

In a published study of reproductive toxicity in which neurobehavioural end-points were measured, mice were fed imazalil in the diet at a concentration of 0, 120, 240 or 480 ppm (equal to 0, 19, 39 and 79 mg/kg bw per day for F_0 males and 0, 26, 45 and 102 mg/kg bw per day for F_0 females before conception). The NOAEL for developmental neurotoxicity was 120 ppm (equal to 19 mg/kg bw per day), based on effects on surface righting in males at 240 ppm (equal to 39 mg/kg bw per day) (Tanaka, 1995). The Meeting noted that parameters other than body weight were not measured in the other reproductive toxicity studies and that the lowest concentration used was higher than the NOAEL in other studies.

In a second published study of reproductive toxicity in which neurobehavioural end-points were measured, mice were fed imazalil in the diet at a concentration of 0, 6, 18 or 54 ppm (equal to 0, 0.85, 2.49 and 7.87 mg/kg bw per day, respectively, during gestation). Some neurobehavioural parameters were inconsistently affected at all doses (Tanaka et al., 2013).

The Meeting concluded that imazalil is not neurotoxic.

Toxicological data on metabolites and/or degradates

R061000 (rat and ruminant metabolite)

For metabolite R061000 (also known as rat metabolite 8), the acute oral LD_{50} in rats was greater than 2000 mg/kg bw (Haferkorn, 2010b). R061000 was negative for mutagenicity in a bacterial reverse mutation assay (Flügge, 2012), an in vitro chromosomal aberration test (Hargitai, 2017c) and an in vitro mammalian cell gene mutation test (Hargitai, 2017d).

The Meeting concluded that, based on the structure of R061000 and its low acute toxicity, this metabolite would be covered by the health-based guidance values for the parent compound.

R043449 (ruminant metabolite)

For metabolite R043449, the acute oral LD_{50} in rats was greater than 2000 mg/kg bw (Haferkorn, 2010a). R043449 was negative for mutagenicity in a bacterial reverse mutation assay (Flügge, 2010), an in vitro chromosomal aberration test (Hargitai, 2017a) and an in vitro mammalian cell gene mutation test (Hargitai, 2017b).

For R043449, the Meeting noted that the threshold of toxicological concern (TTC) approach (Cramer class III) could be applied for chronic toxicity.

R014821 (plant and rat metabolite)

A single-dose study was conducted for the metabolite R014821 (also known as rat metabolite 11). Rats were gavaged with R014821 at a single dose of 0, 125, 500 or 2000 mg/kg bw. The NOAEL was 125 mg/kg bw, based on reduced feed consumption and body weight gain at 500 mg/kg bw (Beerens-Heijnen, 2013). R014821 was negative for mutagenicity in an in vitro chromosomal aberration test (Hargitai, 2017f) and an in vitro mammalian cell gene mutation test (Hargitai, 2017e).

The Meeting concluded that, based on the structure of R014821 and its acute toxicity profile, this metabolite would be covered by the health-based guidance values for the parent compound.

Human data

A published case-study involving one woman indicated that imazalil used to treat a fungal infection was well tolerated after oral ingestion at doses of 50 mg/day progressing to 1200 mg/day over 6 months. The only adverse effect noted was nausea.

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

Toxicological evaluation

The Meeting reaffirmed the ADI of 0–0.03 mg/kg bw established by the 2001 Meeting. The present Meeting used the overall NOAEL of 2.5 mg/kg bw per day from 1-year and 2-year studies in dogs as the basis for this ADI and a safety factor of 100. The ADI is supported by a NOAEL of 2.4 mg/kg bw per day identified in a combined long-term toxicity and carcinogenicity study in rats. The Meeting noted that the LOAEL in the 2-year rat study was higher than the overall LOAEL in the two dog studies, and therefore the overall NOAEL from the dog studies was used as the basis of the ADI.

The Meeting reaffirmed the ARfD of 0.05 mg/kg bw established by the 2005 Meeting on the basis of a NOAEL of 5 mg/kg bw per day for both maternal (decreased feed consumption) and embryo/fetal toxicity (resorptions and decrease in number of live pups) in a developmental toxicity study in rabbits and a safety factor of 100. The Meeting was not able to determine whether the effects could occur following a single dose.

The ADI and ARfD for imazalil would also apply to the metabolites R061000 and R014821.

Levels relevant to risk assessment of imazalil

Species	Study	Effect	NOAEL	LOAEL
Mouse	Twenty-three-month study of toxicity and carcinogenicity[a]	Toxicity	50 ppm, equal to 8.1 mg/kg bw per day	200 ppm, equal to 33.4 mg/kg bw per day
		Carcinogenicity	50 ppm, equal to 8.1 mg/kg bw per day	200 ppm, equal to 33.4 mg/kg bw per day
	Developmental toxicity study[b]	Maternal toxicity	40 mg/kg bw per day	80 mg/kg bw per day
		Embryo and fetal toxicity	–	40 mg/kg bw per day[c]
	Developmental toxicity study[b]	Maternal toxicity	10 mg/kg bw per day	40 mg/kg bw per day

Species	Study	Effect	NOAEL	LOAEL
		Embryo and fetal toxicity	80 mg/kg bw per day	120 mg/kg bw per day
Rat	Two-year study of toxicity and carcinogenicity[a]	Toxicity	50 ppm, equal to 2.4 mg/kg bw per day	200 ppm, equal to 9.7 mg/kg bw per day
		Carcinogenicity	200 ppm, equal to 9.7 mg/kg bw per day	1 200 ppm, equal to 58 mg/kg bw per day
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	20 mg/kg bw per day	80 mg/kg bw per day
		Parental toxicity	20 mg/kg bw per day	80 mg/kg bw per day
		Offspring toxicity	20 mg/kg bw per day	80 mg/kg bw per day
	Developmental toxicity study[b]	Maternal toxicity	–	40 mg/kg bw per day[c]
		Embryo and fetal toxicity	40 mg/kg bw per day	80 mg/kg bw per day
Rabbit	Developmental toxicity study[b]	Maternal toxicity	5 mg/kg bw per day	10 mg/kg bw per day
		Embryo and fetal toxicity	5 mg/kg bw per day	10 mg/kg bw per day
Dog	One- and 2-year studies of toxicity[d,e]	Toxicity	2.5 mg/kg bw per day	5 mg/kg bw per day

[a] Dietary administration.
[b] Gavage administration.
[c] Lowest dose tested.
[d] Two or more studies combined.
[e] Capsule administration.

Acceptable daily intake (ADI) (applies to imazalil and metabolites R061000 and R014821, expressed as imazalil)

0–0.03 mg/kg bw

Acute reference dose (ARfD) (applies to imazalil and metabolites R061000 and R014821, expressed as imazalil)

0.05 mg/kg bw

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

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

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

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid and nearly complete

Dermal absorption	No data
Distribution	Extensive; highest concentrations in liver followed by kidney
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	Rapid: >90% (about 50% in urine and about 40% in faeces) within 24 hours
Metabolism in animals	Extensive metabolism by epoxidation, epoxide hydration, oxidative *O*-dealkylation, imidazole oxidation and scission, oxidative *N*-dealkylation
Toxicologically significant compounds in animals and plants	Imazalil
Acute toxicity	
Rat, LD_{50}, oral	227 mg/kg bw
Rat, LD_{50}, dermal	>2 000 mg/kg bw
Rat, LC_{50}, inhalation	1.84 mg/L
Rabbit, dermal irritation	Mildly irritating
Rabbit, ocular irritation	Severely irritating
Guinea-pig, dermal sensitization	Not sensitizing (Magnusson and Kligman; Buehler)
Short-term studies of toxicity	
Target/critical effect	Decreased body weight gain and liver toxicity
Lowest relevant oral NOAEL	2.5 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	40 mg/kg bw per day (rabbit)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Haematological changes, blood glucose, hepatic changes, follicular cell neoplasia
Lowest relevant NOAEL	2.4 mg/kg bw per day (rat)
Carcinogenicity	Carcinogenic in rats and mice; liver and thyroid tumours not relevant for humans based on mechanistic data[a]
Genotoxicity	
	No evidence of genotoxicity[a]
Reproductive toxicity	
Target/critical effect	Reduced pup viability
Lowest relevant parental NOAEL	20 mg/kg bw per day
Lowest relevant offspring NOAEL	20 mg/kg bw per day
Lowest relevant reproductive NOAEL	20 mg/kg bw per day
Developmental toxicity	
Target/critical effect	Increased number of resorptions, reduced number of live pups
Lowest relevant maternal NOAEL	5 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	5 mg/kg bw per day (rabbit)

Neurotoxicity	
Acute neurotoxicity NOAEL	600 mg/kg bw, highest dose tested (rat)
Subchronic neurotoxicity NOAEL	No data
Developmental neurotoxicity NOAEL	19 mg/kg bw per day (mouse)
Other toxicological studies	
Immunotoxicity	No data
Studies on toxicologically relevant metabolites	
R061000	
Acute oral LD_{50}	>2 000 mg/kg bw
Genotoxicity	No evidence of genotoxicity in vitro
R014821	
Single-dose study NOAEL	125 mg/kg bw
Genotoxicity	No evidence of genotoxicity in vitro
R043449	
Acute oral LD_{50}	> 2 000 mg/kg bw
Genotoxicity	No evidence of genotoxicity in vitro
Human data	
	High doses (up to 1 200 mg/day over 6 months) were well tolerated

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet.

Summary

	Value	Study	Safety factor
ADI[a]	0–0.03 mg/kg bw	One- and 2-year studies of toxicity in dogs	100
ARfD[a]	0.05 mg/kg bw	Developmental toxicity study in rabbits	100

[a] Applies to imazalil and metabolites R061000 and R014281, expressed as imazalil.

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Appendix 1. IPCS framework for the preliminary analysis of the relevance of a cancer mode of action for humans: liver and thyroid tumours

Introduction

Hepatic tumours are seen in mouse and rat studies with imazalil. In mice, liver adenomas were reported to be increased over controls at 200 and 600 ppm (equal to 33 and 100 mg/kg bw per day for male mice, respectively), with a NOAEL for carcinogenic effects of 50 ppm (equal to 8.1 mg/kg bw per day) (Verstraeten et al., 1993a). There was also a trend for the increase in the incidence of combined hepatocellular adenomas and carcinomas in female mice at 600 ppm (equal to 131 mg/kg bw per day), with a NOAEL of 200 ppm (equal to 41.6 mg/kg bw per day) (Verstraeten et al., 1993a). Increased incidences of liver adenomas in male rats over controls were reported at 2400 ppm (equal to 120 mg/kg bw per day), with a NOAEL for liver adenomas of 1200 ppm (equal to 58 mg/kg bw per day) (Van Deun, 1999). Thyroid follicular cell adenomas were reported in male rats at 1200 and 2400 ppm (equal to 58 and 120 mg/kg bw per day, respectively), with a NOAEL of 200 ppm (equal to 9.7 mg/kg bw per day) (Van Deun, 1999).

The postulated mode of action (MOA) for imazalil-induced rodent hepatocellular tumours is non-genotoxic activation of CAR, followed by the key events of altered gene expression specific to CAR activation, increased cell proliferation, formation of altered hepatic foci and, ultimately, the development of liver tumours. Elcombe et al. (2014) provided a review of the evidence that mouse or rat liver tumours that occur via a CAR MOA are not relevant to humans, because, although activation of CAR, the induction of CYP2B and other cytochrome P450 forms and liver hypertrophy can be observed in humans, the cell proliferation and subsequent altered hepatic foci and liver tumours do not occur in humans. Extensive data exist with known CAR-activating compounds that show a fundamental qualitative difference between the species and indicate that the key events leading to tumours in mice or rats are not operative in humans (Peffer et al., 2018). Imazalil did not demonstrate any increase in cell proliferation in human hepatocytes.

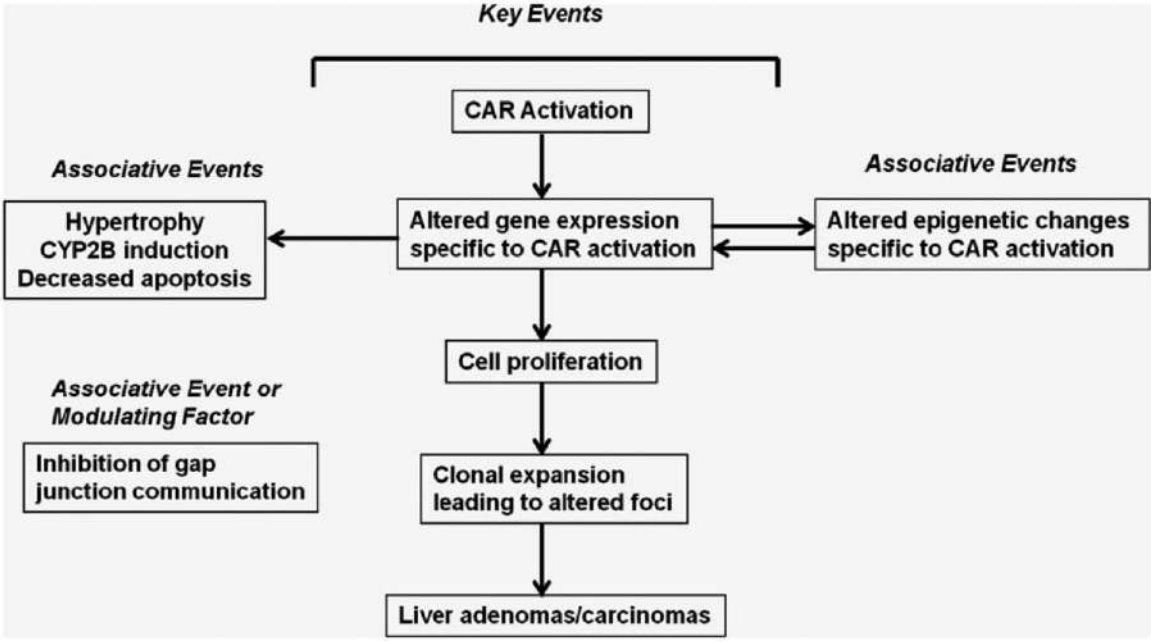
In addition, chronic exposure of male rats to imazalil resulted in neoplastic changes in thyroid follicular cells. The underlying cause is the increase in circulating TSH secondary to CAR activation with consequent hepatic enzyme induction, especially phase II enzymes responsible for clearance of thyroid hormones. The postulated MOA for the imazalil-induced thyroid follicular cell tumours involves the perturbation of homeostasis of the hypothalamic–pituitary–thyroid axis by an extra-thyroidal mechanism to which rats are particularly sensitive. Specifically, the MOA includes induction of conjugation of thyroid hormones with glucuronic acid through CAR activation and subsequent excretion in the bile, leading to a decrease in thyroid hormone plasma levels and activation of the hypothalamic–pituitary–thyroid axis. Rodents are much more sensitive to changes in thyroid hormone levels because of the lack of T_4 binding globulin (TBG), which acts as a buffer. This mechanism is not considered to be quantitatively relevant to humans, as the thyroid of humans is much less sensitive to this pathogenic phenomenon than that of rodents.

Postulated mode of action (theory of the case): hepatocellular tumours

The postulated MOA for imazalil-induced rodent hepatocellular tumours is non-genotoxic activation of CAR, followed by the key events of altered gene expression specific to CAR activation, increased cell proliferation, formation of altered hepatic foci and, ultimately, the development of liver tumours. Associative events in the MOA include altered epigenetic changes, induction of hepatic CYP2B enzymes, liver hypertrophy and decreased apoptosis, with inhibition of gap junctional intercellular communication being an associative event or modulating factor. The key events (i.e. CAR activation, altered gene expression, cell proliferation, altered foci and increased adenomas/carcinomas) can be demonstrated by measuring a combination of the key events themselves or associative events that are known markers for the key events (Peffer et al., 2018).

The MOA for imazalil-induced liver tumours is depicted in Fig. A1-1.

Fig. A1-1. MOA for imazalil-induced rodent liver tumour formation



Source: Elcombe et al. (2014)

The sequence of key events for the proposed MOA for rat and mouse liver tumours (designated LKE1 to LKE5) is:

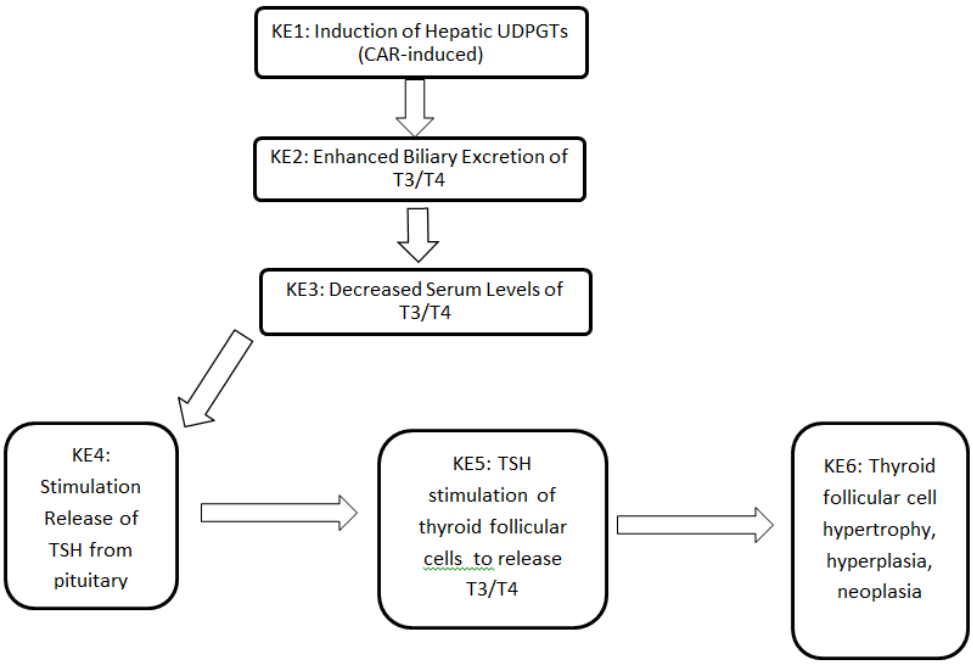
LKE1	Activation of CAR (molecular initiating event)
LKE2	Upregulation and downregulation of a set of genes secondary to CAR activation (*Cyp2b* up and *Cyp3a* down)
LKE3	Increased cell proliferation
LKE4	Increase in foci of altered hepatocytes
LKE5	Increase in hepatocellular adenomas/carcinomas (adverse outcome).

Postulated mode of action (theory of the case): thyroid follicular cell tumours

The postulated MOA for the imazalil-induced thyroid follicular cell tumours involves the perturbation of homeostasis of the hypothalamic–pituitary–thyroid axis by an extra-thyroidal mechanism to which rats are particularly sensitive. The typical MOA for extra-hepatic induction of thyroid tumours in rats includes induction of hepatic uridine diphosphate–glucuronosyltransferase (UGT, or UDPGT, as in Fig. A1-2 below) activity (secondary to CAR activation), leading to enhanced metabolism of the thyroid hormones T_4 and T_3 by conjugation and increased biliary excretion of the conjugated hormones. The result of this enhanced liver metabolism and excretion via UGT is a decrease in serum T_4 and T_3 levels. The reduced concentrations of circulating T_3 and T_4 are detected in the hypothalamus, which maintains homeostasis of thyroid hormones via the secretion of thyrotropin-releasing hormone (TRH). Increased TRH levels stimulate the pituitary to secrete more TSH. Prolonged elevation of circulating TSH levels stimulates the thyroid gland to deplete its stores of thyroid hormone (T_4) and continues to induce hormone production. Thus, the thyroid follicular cells enlarge (hypertrophy) and are induced to proliferate at an increased rate, increasing in number (hyperplasia). With chronic exposure, thyroid hyperplasia eventually progresses to thyroid neoplasia (USEPA, 1998; Dellarco et al., 2006). This mechanism is not considered to be quantitatively relevant to humans, as the thyroid of humans is much less sensitive to this pathogenic phenomenon than that of rodents due to the presence of TBG and longer T_4 and T_3 half-lives in humans.

The MOA for imazalil-induced thyroid tumours is depicted in Fig. A1-2.

Fig. A1-2. MOA for imazalil-induced thyroid tumours



KE: key event
Source: Elcombe et al. (2014)

The sequence of key events for the proposed MOA for rat thyroid tumours (designated TKE1 to TKE6) is:

TKE1	Induction of hepatic enzymes, including UGT activity
TKE2	Increased hepatic metabolism and biliary excretion of T_4/T_3
TKE3	Decrease in serum T_4/T_3 half-life and/or concentration
TKE4	Feedback to pituitary gland and increase in circulating TSH concentrations
TKE5	Thyroid follicular cell hypertrophy and/or hyperplasia
TKE6	Increased thyroid follicular cell adenomas/carcinomas.

Interplay between the two proposed MOAs

The toxicological database for imazalil is reviewed in this appendix in the context of the hepatic enzyme induction rodent-specific MOA for liver tumours and for the thyroid secondary specific responses detailed above. Because of the interplay between the two proposed MOAs for liver and thyroid tumours, the mechanistic studies that support similar key events are addressed together. For instance, the studies applicable to key events LKE1, LKE2 and TKE1 all evaluate liver gene expression and enzyme activity. Additionally, key events LKE3 and TKE2 are addressed together because the same set of data addresses increased hepatic cell proliferation and increased hepatic metabolism and supports the increased biliary excretion of T_4/T_3 (TKE2).

Liver key events 1 and 2 and thyroid key events 1 and 2: CAR activation (LKE1), induction of liver enzymes (LKE2) and hepatic UGT activity (TKE1) and increased hepatic metabolism and biliary excretion of T_4/T_3 (TKE2)

For imazalil, the adverse outcome for the liver (hepatocellular adenomas and carcinomas) was reported at 200 ppm (equal to 33 mg/kg bw per day) in male mice, with a NOAEL of 50 ppm (equal to 8.1 mg/kg bw per day), and at 600 ppm (equal to 130 mg/kg bw per day) in female mice, with a NOAEL of 200 ppm (equal to 42 mg/kg bw per day). In male rats, hepatocellular adenomas were reported at 2400 ppm (equal to 120 mg/kg bw per day), with a NOAEL of 1200 ppm (equal to 58 mg/kg bw per day). Evidence of LKE1 and LKE2 events include altered gene expression secondary to CAR activation (Elcombe, 2012c) and associative events supporting LKE1 and LKE2, including increased CYP2B and CYP3A enzyme activities, increased liver weight and hepatocellular hypertrophy.

The adverse outcome in the thyroid (thyroid follicular cell adenomas) was reported in male rats at 1200 ppm (equal to 58 mg/kg bw per day) and 2400 ppm (equal to 120 mg/kg bw per day), with a NOAEL of 200 ppm (equal to 9.7 mg/kg bw per day). Evidence of TKE1, induced hepatic UGT activity, was demonstrated in female mice and male and female rats (at 600 ppm, equal to 170 mg/kg bw per day, with a NOAEL of 200 ppm, equal to 55 mg/kg bw per day, in female mice; and at 400 ppm, equal to 42.0 mg/kg bw per day, with a NOAEL of 200 ppm, equal to 21.0 mg/kg bw per day, in male and female rats). Associative events supporting TKE2 include increased liver weight relative to body weight and increased incidence of hepatocellular hypertrophy, reported in male and female mice and male and female rats.

Mouse liver

Increased expression of Cyp2b10 and Cyp3a11 mRNA and protein and Gadd45β mRNA was demonstrated in male mice

In the study by Elcombe (2012c), male mice were administered imazalil in the diet for 7 days at 0, 50, 200 or 600 ppm. This study evaluated levels of hepatic murine *Cyp2b10*, *Cyp3a11* and *Gadd45β* mRNA.[1] Imazalil induced *Cyp2b10*, *Cyp3a11* and *Gadd45β* mRNA expression in a dose-dependent manner at all doses (50, 200 and 600 ppm), with maximal levels of induction at 600 ppm, at which target genes were induced 38.9-fold, 6.2-fold and 4-fold, respectively, in male mice when compared with controls. Elcombe (2102c) also evaluated the induction of murine CYP2B10 and CYP3A11 protein in liver microsomes. Marked dose-dependent increases in the microsomal expression of CYP2B10 protein were observed with imazalil at all doses (50, 200 and 600 ppm). The expression of CYP3A protein was induced by 600 ppm imazalil. Increased expression of *Cyp2b10* mRNA and CYP2B10 protein was reported at 50 ppm (the lowest dose tested; no NOAEL identified). Increased expression of *Cyp3a11* and *Gadd45β* mRNA was also reported at 50 ppm (the lowest dose tested), and increases in microsomal expression of CYP3A11 protein were reported at 600 ppm (NOAEL of 200 ppm). The response to CAR activation is apparent at lower doses in male mice than in female mice. In female mice, a trend for an increased incidence of combined hepatocellular adenomas and carcinomas was noted at 600 ppm (compared with 200 and 600 ppm in male mice), with a NOAEL for hepatocellular neoplasms in females of 200 ppm (compared with 50 ppm in male mice). Therefore, data available in male mice are also applicable to females.

[1] Mechanistically, GADD45β is an anti-apoptotic factor, a negative regulator of the p53 tumour suppressor. GADD45β has been implicated in pathways activated by CAR and contributory to the enhanced tumorigenic response in CAR wild-type animals (NIEHS, 2010).

An increase in CYP2B activation greater than the increase in CYP3A activation was demonstrated in both male and female mice

Microsomal PROD was used as a marker for CYP2B activity in all studies. CYP2B and CYP3A enzyme induction was evaluated in male mice after 7 days of dietary exposure in Elcombe (2012c) and after 1 month and 3 months of dietary exposure in Vermeir, Lavrijsen & Van Leemput (1994). CYP2B enzyme induction was highest at 7 days, but was also present at 1 month. In the study by Elcombe (2012c), after 7 days of dietary exposure, CYP2B activity was increased in male mice at 200 and 600 ppm by 3.2- and 6.7-fold over control values, respectively, with a NOAEL of 50 ppm. In the Vermeir, Lavrijsen & Van Leemput (1994) study, after 1 month, a statistically significant increase in PROD activity was reported at 600 ppm in male mice (92% increase over controls) and female mice (63% increase over controls[2]). After 3 months, PROD was not induced in male or female mice up to 600 ppm. Elcombe (2012a) evaluated CYP2B and CYP3A activity in freshly isolated female CD-1 mouse hepatocytes cultivated as monolayer cultures for 96 hours and treated with imazalil at 3, 10, 30 or 100 µmol/L or with vehicle alone (0.5% v/v DMSO). Treatment with imazalil at 3 µmol/L resulted in a statistically significant increase in CYP2B10 (PROD) activity (17% increase over controls). Treatment at 10 µmol/L did not result in an increase in CYP2B10 (PROD) activity in vitro, and excess toxicity prohibited evaluation of the results at 30 and 100 µmol/L.

Microsomal BQ was used as a marker for CYP3A11 activity by Elcombe (2012a,c). In Elcombe (2012c), administration of imazalil to male mice for 7 days at 600 ppm increased the BQ activity by 1.9- and 2.7-fold, respectively (NOAEL of 200 ppm). In Elcombe (2012a), BQ activity was increased dose-wise at 3, 10 and 30 µmol/L (34%, 100% and 105.3% increases over controls, respectively). Excess toxicity prohibited evaluation at 100 µmol/L. In Vermeir, Lavrijsen & Van Leemput (1994), EMD was used as a marker for CYP3A1 and CYP3A2 activities. Induction (expressed in nanomoles of product formed per milligram of protein per minute) by treatment with imazalil was observed for both male (41% increase over controls at 600 ppm) and female (71% increase over controls at 600 ppm) mice. However, the inductive effect in male mice was not revealed when the activity was expressed as nanomoles of product formed per nanomole of cytochrome P450 per minute. Dosing for 3 months with imazalil up to 600 ppm had hardly any effect on the EMD activity in male or female mice.

Significant increases in liver weight (relative to body weight) in male and female mice were reported in the toxicological database for imazalil

Elmore (2004a) reported a statistically significant increase in relative liver weight of male mice at 1200 ppm after 4 days of treatment (133% over controls). O'Neill (2002) reported statistically significant increases in relative liver weight in male mice after 2 weeks of dietary administration of imazalil at 600 and 1200 ppm (NOAEL of 400 ppm). Van Deun et al. (1994) reported statistically significant increases in relative liver weight in male and female mice at 600 ppm after 1 and 3 months (no effects reported at 50 or 200 ppm). Verstraeten et al. (1993b) reported a statistically significant increase in relative liver weight in male mice at 400 ppm and in male and female mice at 800 ppm after 3 months (NOAEL of 200 ppm for males and 400 ppm for females). O'Neill (2002) reported statistically significant increases in relative liver weight in male mice after 3 months of dietary administration of imazalil at 1200 ppm (NOAEL of 600 ppm). Verstraeten et al. (1993a) reported statistically significant increases in relative liver weight of male mice at 800 ppm (NOAEL of 400 ppm) and no statistically significant increases in relative liver weight in females (NOAEL of 800 ppm) in the mouse carcinogenicity study (23 months).

[2] PROD activity in female mice at 1 month was not increased, as reported in nanomoles per nanomole cytochrome P450 per minute.

Evidence of hepatocellular hypertrophy was reported in both male and female mice

Elmore (2004a) reported a statistically significant increase in centrilobular hepatocellular hypertrophy (minimal) in male mice at 1200 ppm after 4 days of treatment (6/6, compared with 0/6 in controls). O'Neill (2002) reported an increased incidence of hepatocellular hypertrophy in male mice after 2 weeks of dietary administration of imazalil at 200, 400, 600 or 1200 ppm (NOAEL of 100 ppm). A non-statistically significant increase in diffuse hepatocellular swelling was reported after 3 months in male mice at 600 ppm (Van Deun et al., 1994). Verstraeten et al. (1993b) reported a statistically significant increase in the incidence of centrilobular swelling in male mice at 400 ppm and in male and female mice at 800 ppm after 3 months (NOAEL of 200 ppm for males and 400 ppm for females). O'Neill (2002) reported an increased incidence of hepatocellular hypertrophy in male mice after 3 months of dietary administration of imazalil at 200, 400, 600 or 1200 ppm (NOAEL of 100 ppm).

Rat liver

Increased expression of Cyp2b10 and Cyp3a11 and Gadd45β mRNA was demonstrated in male rats

Cyp2b1, CAR (Nr113), Cyp3a1, Cyp3a2 and *Gadd45β* mRNA levels were evaluated after 14 and 28 days of dietary administration of 0, 200, 1200 or 2400 ppm imazalil to male rats by Mertens (2011). In rats exposed to imazalil for either 14 or 28 days, analysis of mRNA levels of *Cyp2b1* showed statistically significant and dose-related increases when compared with the mRNA levels of *Cyp2b1* in rats fed the basal diet; the LOAEL was 200 ppm (no NOAEL identified). Imazalil administered for 14 or 28 days induced the mRNA levels of *Cyp2b1, Cyp3a1, Cyp3a2* and *Gadd45β* in the liver of rats in a dose-dependent manner, with statistically significant changes reported at 1200 and 2400 ppm (NOAEL of 200 ppm).

An increase in CYP2B activation greater than the increase in CYP3A activation was demonstrated in both male and female rats

Microsomal PROD was used as a marker for CYP2B activity in all studies. Mertens (2011) evaluated CYP2B1/2 induction in male rats at 0, 200, 1200 and 2400 ppm via dietary exposure after 1, 2, 7, 14 and 28 days. In Mertens (2011), administration of imazalil to male rats at 0, 200, 1200 or 2400 ppm and phenobarbital (1200 ppm) across all treatment periods (1, 2, 7, 14 and 28 days) resulted in increases in CYP2B1/2 activity (as measured by PROD). Statistically significant increases were observed for all intervals at 1200 and 2400 ppm imazalil (with the exception of 14-day 1200 ppm imazalil) and the 1200 ppm phenobarbital treatment groups. The NOAEL for CYP2B induction in Mertens (2011) was 200 ppm. CYP2B enzyme induction was reported in male rats 1, 2 and 4 weeks after dietary administration of imazalil at 400, 1200 or 3200 ppm (Vermeir, Lavrijsen & Meuldermans, 2001). CYP2B enzyme induction was reported in male and female rats after 1 and 3 months of dietary administration of imazalil at 200, 400 or 800 ppm (Vermeir, Lavrijsen & Meuldermans, 1995). CYP2B enzyme induction was reported in male and female rats after 3 months of dietary administration of imazalil at 800, 1600, 2400 or 3200 ppm (Vermeir & Lavrijsen, 1996). The LOAEL was 200 ppm, the lowest dose tested.

EMD was used as a marker for CYP3A1 and CYP3A2 enzyme induction activity in Vermeir, Lavrijsen & Meuldermans (1995, 2001) and Vermeir & Lavrijsen (1996). In Vermeir, Lavrijsen & Meuldermans (2001), CYP3A enzyme induction (statistically significant) was reported in male rats after 2 weeks of dietary administration of imazalil at 400, 1200 or 3200 ppm. At 4 weeks, no statistically significant or biologically relevant increases were reported after 1 week of treatment. In Vermeir, Lavrijsen & Meuldermans (1995),[3] CYP3A enzyme induction was reported in females at 200, 400 and 800 ppm at 1 month and 3 months. Non-statistically significant increases were reported

[3] Livers taken from Van Deun et al. (1996b).

in males at 1 month at all doses and at 3 months at 400 and 800 ppm. CYP3A enzyme induction was reported in male and female rats after a 3-month dietary administration of imazalil at 800, 1600, 2400 or 3200 ppm (Vermeir & Lavrijsen 1996). The LOAEL was 200 ppm, the lowest dose tested; no NOAEL was identified.

Significant increases in liver weight (relative to body weight) in male and female rats were reported in the toxicological database for imazalil

Mertens (2011) reported a statistically significant increase in liver weight relative to body weight at 28 days in male rats (24.1% increase over controls) at 2400 ppm, with no increases recorded at 1, 2, 7 or 14 days at doses up to 2400 ppm. Statistically significant, dose-related increases in relative liver weight were reported in male rats at 200, 400 and 800 ppm and in females at 400 and 800 ppm at 1 month. Gur, Nyska & Crown (1990) reported statistically significant increases in relative liver weight in female rats at 1000, 2000 and 3000 ppm (NOAEL of 100 ppm). At 3 months, relative liver weight was increased only at 800 ppm in male rats (Van Deun et al., 1996b[3]). Van Deun et al. (1996a) reported increased relative liver weight in male rats at 800, 1600, 2400 and 3200 ppm after 3 months; no increase in relative liver weight was reported in female rats at any dose. Gur, Nyska & Crown (1990) reported statistically significant increases in relative liver weight in male and female rats at 1000 ppm (NOAEL of 200 ppm). Van Deun (1999) reported statistically significant increases in relative liver weight in male rats at 1200 and 2400 ppm (NOAEL of 200 ppm) and in female rats at 200, 1200 and 2400 ppm (NOAEL of 50 ppm).

Evidence of hepatocellular hypertrophy was reported in both male and female rats in the toxicological database for imazalil

Van Deun et al. (1996b) reported an increase in centrilobular swollen hepatocytes at 400 and 800 ppm in male rats (5/10 in controls, 5/10 at 200 ppm, 8/10 at 400 ppm and 8/10 at 800 ppm) after 1 month of treatment. No increases in female rats were reported at 1 or 3 months, and no changes were reported in males at 3 months. Van Deun et al. (1996a) reported an increase in the incidence of hepatocellular hypertrophy at 800, 1600, 2400 and 3200 ppm after 3 months in both male and female rats. Gur, Nyska & Waner (1991) reported an increase in the incidence of hepatocellular hypertrophy at 1000 ppm in male rats (15/19 compared with 0/20 in controls) and female rats (18/18 compared with 0/20 in controls) (NOAEL of 200 ppm). Van Deun (1999) reported an increased incidence (statistically significant) of hepatocellular hypertrophy (centriacinar and periacinar) after 24 months in male and female rats at 1200 and 2400 ppm (NOAEL of 200 ppm).

Rat thyroid

Evidence of UGT4 induction in male rats has been experimentally demonstrated

Vermeir, Lavrijsen & Meuldermans (2001) reported a statistically significant increase in T_4-UGT activity (T_4 substrate) in male rats after 1 week of treatment at 400, 1200 or 3200 ppm (LOAEL of 400 ppm; no NOAEL identified). Mertens (2011) reported increased UGT activity (UGT1A activity as measured by 4-methylumbelliferone glucuronidation) in male rats after 14 days at 2400 ppm and after 28 days at 1200 ppm and 2400 ppm (NOAEL of 200 ppm). After 2 and 4 weeks, UGT activity was statistically significantly increased in male rats at 3200 ppm (NOAEL of 1200 ppm). Vermeir, Lavrijsen & Meuldermans (1995) reported increased UGT activity (β-naphthoflavone) in male and female rats after 1 and 3 months at 800 ppm (NOAEL of 400 ppm). Van Deun et al. (1996a) reported non-statistically significant increases in UGT activity (β-naphthoflavone-induced) in male rats at 800, 1600, 2400 and 3200 ppm (LOAEL of 800 ppm; no NOAEL identified) and in female rats at 2400 and 3200 ppm.

Liver key events 3, 4 and 5: Cell proliferation (LKE3), foci (LKE4) and neoplasia (LKE5)

For liver key events 3, 4 and 5, the available toxicological database includes studies evaluating cell proliferation responses in vitro and in vivo and increased incidences of liver foci and tumours in chronic mouse and rat studies. An increase of murine hepatocyte cell proliferation in response to imazalil was demonstrated in vitro by Elcombe (2012a) and in vivo by Elmore (2004a) in male mice. An increase in eosinophilic foci is most commonly observed after long-term administration of CAR activators (Peffer et al., 2018), but mixed foci (i.e. including basophilic or clear cells) can at times be observed (Peffer et al., 2018).

Mouse and human liver: cell proliferation studies

Elcombe (2012a) evaluated the ability of imazalil to induce hepatocyte cell proliferation (measured as the change in replicative DNA synthesis [S-phase of the cell cycle]) in isolated male mouse hepatocyte cultures. The number of hepatocytes undergoing replicative DNA synthesis was measured by incorporation of BrdU into DNA and subsequent immunohistochemical detection. Treatment with imazalil at 10 or 30 µmol/L resulted in statistically significant increases in replicative DNA synthesis at 1.8- and 1.5-fold relative to controls, respectively. Elmore (2004a) evaluated the ability of imazalil to induce cell proliferation in male mice administered 1200 ppm imazalil in the diet for 4 days. Each mouse was implanted with mini-osmotic pumps (day −1) that delivered BrdU for the assessment of hepatic cell proliferation. There was a statistically significant increase in the BrdU labelling index of the imazalil-treated mice compared with controls.

Elcombe (2012b) evaluated the ability of imazalil to induce cell proliferation (measured as the change in replicative DNA synthesis [S-phase of the cell cycle]) in isolated female human hepatocyte cultures. No increase in cell proliferation was observed in human hepatocytes exposed to imazalil at concentrations up to and beyond the toxicity threshold.

Elcombe (2012c) demonstrated hepatocyte cell proliferation in wild-type mice at doses of 50 ppm and higher. Although cell proliferation was also observed in humanized mice, the response was lower than that observed in wild-type mice. This lack of cell proliferation differences between wild-type and hCAR/hPXR mice (Elcombe, 2012c) is further discussed in the "Uncertainties, inconsistencies and data gaps" section below.

Rat liver: cell proliferation studies

Mertens (2011) evaluated the ability of imazalil to induce hepatocyte proliferation in male rats. Osmotic pumps loaded with BrdU were implanted subcutaneously in all animals 4 days prior to the scheduled necropsy (study day −3, −2, 3, 10 or 24). Results of BrdU immunohistochemical staining of the liver revealed an increased level of BrdU incorporation in the liver of phenobarbital-treated rats on study day 7, indicating hepatocellular proliferative activity, with no similar effect noted in imazalil-treated rats. Only a single hepatocellular nucleus in a 200 ppm animal was found to be positive for BrdU at the study week 2 interim necropsy. Elmore (2004b)[4] reported an increase in hepatocyte cell proliferation in male rats on day 7 at 3200 ppm with intraperitoneal administration of BrdU 6 hours prior to necropsy.

Mouse liver: liver foci, hepatocellular adenoma and carcinoma

In addition to hepatocellular adenomas and carcinomas, liver foci, described as focal cellular changes by Verstraeten et al. (1993b) or as basophilic or eosinophilic focus of alteration by Sparrow (1993), were reported in male mice (NOAEL of 50 ppm) after 18 months of dietary administration of imazalil (Table A1-1).

[4] Samples obtained from Verbeek et al. (2000).

Table A1-1. Incidence of liver foci, hepatocellular adenoma and carcinoma in mice (18 months)

Parameter	Males				Females			
	0 ppm	**50 ppm**	**200 ppm**	**600 ppm**	**0 ppm**	**50 ppm**	**200 ppm**	**600 ppm**
Dose (mg/kg bw per day)	0	8.1	33	100	0	9.9	42	130
Focal cellular changes (Verstraeten et al., 1993b)	2/50	4/49	10/50*	8/50	0/50	0/50	0/50	0/50
Basophilic focus of alteration (Sparrow, 1993)	1/50	3/50	2/50	5/50	0/50	0/50	0/50	0/50
Eosinophilic focus of alteration (Sparrow, 1993)	0/50	0/50	1/50	0/50	0/50	0/50	0/50	0/50
Hepatocellular adenoma (Verstraeten et al., 1993b)	8/50	5/49	23/50**	17/50**	4/50	6/50	0/50	10/50
Hepatocellular carcinoma (Verstraeten et al., 1993b)	5/50	7/49	6/50	11/50	0/50	1/50	2/50	2/50

bw: body weight; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Verstraeten et al. (1993b)

Rat liver: liver foci, hepatocellular adenoma and carcinoma

Van Deun (1999) reported a statistically significant increase in eosinophilic foci at 2400 ppm in male rats (Table A1-2).

Table A1-2. Incidence of liver foci and hepatocellular adenoma and carcinoma in rats (24 months)

	Males					Females				
	0 ppm	**50 ppm**	**200 ppm**	**1 200 ppm**	**2 400 ppm**	**0 ppm**	**50 ppm**	**200 ppm**	**1 200 ppm**	**2 400 ppm**
Dose (mg/kg bw per day)	0	2.5	10	60	120	0	3.5	14	80	160
Clear cell focus/ foci	24/50	14/50	21/50	20/50	16/50	10/50	5/50	14/50	4/50	1/50
Basophilic focus/ foci of hepatocellular alteration	8/50	10/50	15/50	7/50	12/50	25/50	20/50	18/50	20/50	6/50
Eosinophilic focus/foci of hepatocellular alteration	20/50	19/50	22/50	28/50	37/50**	6/50	8/50	5/50	7/50	6/50
Hepatocellular adenomas	4/50 (8%)	2/50 (4%)	3/50 (6%)	4/50 (8%)	13/50* (26%)	2/50 (4%)	1/50 (2%)	2/50 (4%)	1/50 (2%)	2/50 (4%)
Hepatocellular carcinomas	1/50 (2%)	0/50 (0%)	0/50 (0%)	0/50 (0%)	1/50 (2%)	0/50 (0%)	0/50 (0%)	0/50 (0%)	0/50 (0%)	0/50 (0%)

bw: body weight; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Van Deun (1999)

Thyroid key events 3 and 4: Effects on serum T_3/T_4 (TKE3) and TSH (TKE4)

Verbeek et al. (2000) evaluated T_3, T_4 and TSH levels in male rats after 1, 2 and 4 weeks of dietary treatment followed by a recovery period of 4 or 9 weeks. The study demonstrated biologically relevant and statistically significant reductions in T_4 levels after 1 week at 1200 and 3200 ppm (12% and 17%, respectively) compared with the controls and non-statistically significant increases in TSH levels at 1200 and 3200 ppm (15% and 19%, respectively) compared with the controls. Similar non-statistically significant increases in TSH levels at 1200 and 3200 ppm were reported after 2 weeks of treatment (22% and 18%, respectively) compared with the controls. At 4 weeks of treatment, following the 1- and 2-week increases in TSH, serum levels of T_4 were increased compared with the controls, and TSH levels were decreased compared with the controls, indicating the homeostatic negative feedback relationship between T_4 and TSH.

Van Deun (1999) evaluated changes in thyroid hormone concentrations in the 30-month carcinogenicity study in rats. Changes in thyroid hormone concentrations in serum were inconsistent. In males, the concentrations of TSH tended to be higher and those of T_4 definitely lower at the two highest dietary concentrations (1200 and 2400 ppm); in females, the level of T_3 was decreased at the two highest dietary concentrations (1200 and 2400 ppm), and that of T_4 was decreased only at 1200 ppm. Piccirillo (2000) concluded that dose-related decreases in T_4 were reported at 1200 and 2400 ppm at the terminal interval, with a no-effect level of 200 ppm.

Thyroid key events 5 and 6: Thyroid hypertrophy, hyperplasia and tumours (TKE5 and TKE6)

Mertens (2011) evaluated BrdU incorporation in the thyroid of male rats exposed to 2400 ppm imazalil for 1–28 days. BrdU incorporation in the thyroid of imazalil-treated animals on study day 14 was higher, but not statistically significantly higher, than that in the control group. Phenobarbital-treated animals had statistically significantly higher thyroid BrdU incorporation on study days 7, 14 and 28.

In the study by Verbeek et al. (2000), male rats (10 per dose per time point) were administered imazalil in the diet at a concentration of 0, 400, 1200 or 3200 ppm or phenobarbital at 1200 ppm for 4 weeks with 1- and 2-week interim evaluations and 4- and 9-week recovery groups. Relative thyroid weight was increased at 3200 ppm after 2 weeks (119% compared with controls; statistically significant) and after 4 weeks (119% compared with controls; not statistically significant). A non-statistically significant increase in the incidence of follicular cell hypertrophy was reported after 2 weeks at 3200 ppm.

A statistically significant increase in relative thyroid weight was reported in male rats by Van Deun et al. (1996b) at 800 ppm after 1 month of dietary exposure to imazalil (118% compared with control value). At 3 months, relative thyroid weight was increased (110%), but not statistically significantly, compared with controls. A statistically significant increase in relative thyroid weight was reported by Van Deun et al. (1996a) at 3200 ppm after 3 months of dietary exposure to imazalil (126% compared with control value); increases over control were reported at 800, 1200 and 2400 ppm, but these values were not statistically significant and/or did not demonstrate a dose–response relationship.

In the 24-month rat carcinogenicity study (Van Deun, 1999), statistically significant increases in relative thyroid weight were reported only for male rats at all doses (50, 200, 1200 and 2400 ppm), and statistically significant increases in the incidence of follicular cell hyperplasia of the thyroid were observed at 1200 and 2400 ppm (Table A1-3).

Summary of dose concordance for the key events

Table A1-4 summarizes the dose concordance of the key events for imazalil-induced mouse liver tumours. Table A1-5 summarizes the dose concordance of the key events in the MOA for imazalil-induced rat thyroid tumours. For imazalil, the key events necessary to demonstrate the CAR-

induced liver tumour MOA and UGT-induced thyroid tumour MOA are available, and the key events demonstrate dose concordance with the adverse outcome.

Table A1-3. Thyroid effects in male rats (24 months)

Parameter	0 ppm	50 ppm	200 ppm	1 200 ppm	2 400 ppm
Dose (mg/kg bw per day)	0	2.5	10	60	120
Number of individuals	50	50	50	50	50
Absolute thyroid weight (mg)	44	51	101	76*	67*
Relative thyroid weight (mg/kg bw)	84	103*	202*	154*	145***
Follicular cell hyperplasia	4	8	6	11	12
Follicular cell adenoma	4	8	5	9	10
Follicular cell carcinoma	0	0	2	2	2

bw: body weight; ppm: parts per million; *: $P < 0.05$; ***: $P < 0.001$
Source: Van Deun (1999)

Table A1-4. Summary of the key event dose concordance for imazalil-induced mouse liver tumours

End-point	NOAEL (ppm)	LOAEL (ppm)	Reference
Key event 1: Activation of CAR (molecular initiating event)			
Not directly measured			
Key event 2: Upregulation and downregulation of a set of genes secondary to CAR activation			
Increased CYP2B and CYP3A mRNA	–	50 m[a]	Elcombe (2012c)
Associative event – Increased CYP2B enzyme activity	– 200 f	50 m[a] 600 f	Vermeir, Lavrijsen & Van Leemput (1994); Elcombe (2012c)
Associative event – Increased CYP3A enzyme activity	200 mf	600 mf	Vermeir, Lavrijsen & Van Leemput (1994); Elcombe (2012c)
Associative event – Increased liver weight	200 m 200 f	400 m 600 f	Verstraeten et al. (1993b) Verstraeten et al. (1993a)
Associative event – Hepatocellular hypertrophy	100 m 400 f	200 m 800 f	Verstraeten et al. (1993b); Van Deun et al. (1994); Vermeir, Lavrijsen & Van Leemput (1994); O'Neill (2002)
Key event 3: Increased cell proliferation			
BrdU in vitro	10 µmol/L	30 µmol/L	Elcombe (2012a)
BrdU in vivo (mouse)	<50 m	50 m	Elmore (2004a); Elcombe (2012c)
Key event 4: Increase in foci of altered hepatocytes			
Focal cellular changes (mouse)	50 m 600 f[b]	200 m –	Verstraeten et al. (1993b)
Eosinophilic focus/foci of hepatocellular alteration (rat)	1 200 m 2 400 f[b]	2 400 m –	Van Deun (1999)
Key event 5: Increase in hepatocellular adenomas/carcinomas (adverse outcome)			
Increase in hepatocellular adenomas	50 m	200 m	Verstraeten et al. (1993a)

End-point	NOAEL (ppm)	LOAEL (ppm)	Reference
	200 f	600 f (trend)	
Increase in hepatocellular carcinomas	200 m	600 m	Verstraeten et al. (1993a)
	600 f[b]	–	

BrdU: 5-bromo-2′-deoxyuridine; f: female; LOAEL: lowest-observed-adverse-effect level; m: male; NOAEL: no-oberved-adverse-effect level; ppm: parts per million

[a] Lowest dose tested.

[b] Highest dose tested.

Table A1-5. Summary of the key event dose concordance for imazalil-induced male rat thyroid tumours

End-point	NOAEL (ppm)	LOAEL (ppm)	Reference
Key event 1: CAR-induced increased in hepatic UGT activity (molecular initiating event)			
CAR-induced increase in T_4 UGT activity	200	400	Vermeir, Lavrijsen & Meuldermans (1995); Vermeir & Lavrijsen (1996); Vermeir, Lavrijsen & Meuldermans (2001); Mertens (2011)
Key event 2: Increased hepatic metabolism and biliary excretion of T_4/T_3			
Associative event – Increase in relative liver weight	50	100	Gur (1990); Van Deun et al. (1996a,b); Van Deun (1999); Mertens (2011)
Associative event – Hepatocellular hypertrophy	200	400	Gur, Nyska & Crown (1990); Van Deun et al. (1996a,b); Van Deun (1999); Mertens (2011)
Key event 3: Decrease in serum T_4/T_3 half-life and concentration			
T_3 decrease	3 200[a]	–	Verbeek et al. (2000)
T_4 decrease	400	1 200	Verbeek et al. (2000)
Key event 4: Increase in circulating TSH concentrations			
TSH increase	400	1 200	Verbeek et al. (2000)
Key event 5/6: Thyroid follicular cell hypertrophy, hyperplasia, adenomas			
Increase in relative thyroid weight	400	800	Van Deun et al. (1996a,b); Van Deun (1999)
Follicular cell hypertrophy	400	1 200	Verbeek et al. (2000)
Follicular cell hyperplasia	200	1 200	Van Deun (1999)
Follicular cell adenomas	200	1 200	Van Deun (1999)

CAR: constitutive androstane receptor; LOAEL: lowest-observed-adverse-effect level; NOAEL: no-oberved-adverse-effect level; ppm: parts per million; T_3: triiodothyronine; T_4: thyroxine; TSH: thyroid stimulating hormone; UGT: uridine diphosphate–glucuronosyltransferase

[a] Highest dose tested.

Temporal association

If an event (or events) is an essential element of tumorigenesis, it must precede tumour appearance.

For imazalil, the key events necessary to demonstrate the CAR-induced liver tumour MOA are available, and a temporal association of progression is demonstrated. Multiple exposure time data are available at 1, 2 and 4 days, 1 and 2 weeks, and 1, 3, 18 and 24 months in studies in which mice and rats were offered diets containing imazalil. Increased CAR-activated *Cyp2b* and *Cyp3a* mRNA levels and enzyme activity were demonstrated in mice in vitro at 96 hours (Elcombe, 2012a) and in vivo at 7 days (Elcombe, 2012c) and 1 month (Vermeir, Lavrijsen & Van Leemput, 1994); and in rats at 1 and 2 days, 1 and 2 weeks, and 1 and 3 months (Vermeir, Lavrijsen & Meuldermans, 1995, 2001; Vermeir & Lavrijsen, 1996; Mertens, 2011). Increased relative liver weights were recorded in mice at 4 days (Elmore, 2004a) and 2 weeks (O'Neill, 2002), 1 and 3 months (Verstraeten et al., 1993b; Van Deun et al., 1994; Vermeir, Lavrijsen & Van Leemput, 1994; O'Neill, 2002) and 18 months (Verstraeten et al., 1993a); and in rats at 1 and 3 months (Gur, Nyska & Crown, 1990; Gur, Nyska & Waner, 1991; Vermeir, Lavrijsen & Meuldermans, 1995, 2001; Vermeir & Lavrijsen, 1996; Mertens, 2011) and 24 months (Van Deun, 1999). Increased hepatocellular hypertrophy was reported in mice at 4 days (Elmore, 2004a), 2 weeks (O'Neill, 2002) and 3 months (Verstraeten et al., 1993b; Van Deun et al., 1994; Vermeir, Lavrijsen & Van Leemput, 1994; O'Neill, 2002); and in rats at 1 month (Van Deun et al., 1996a,b; Mertens, 2011), 3 months (Gur, Nyska & Waner, 1991) and 24 months (Van Deun, 1999). Increased cell proliferation was demonstrated in mouse hepatocytes at 96 hours (Elcombe, 2012a), in male mice at 4 days (Elmore, 2004a) and in male rats at 7 days (Elmore, 2004b). Increased incidences of liver foci were reported in male mice at 18 months (Verstraeten et al., 1993b) and in male rats at 24 months (Van Deun, 1999).

For imazalil, the key events necessary to demonstrate the MOA for UGT-induced thyroid tumours are available, and a temporal association of progression is demonstrated. Multiple exposure time data are available at 1, 2 and 4 days, 1 and 2 weeks and 1, 3 and 24 months in studies in which male rats were offered diets containing imazalil. Increased hepatic UGT activity in male rats was reported at 1 and 2 weeks and 1 and 3 months (Vermeir, Lavrijsen & Meuldermans, 1995, 2001; Vermeir & Lavrijsen, 1996; Mertens, 2011). Relative liver weights were increased at 1 and 3 months (Gur, Nyska & Crown, 1990; Gur, Nyska & Waner, 1991; Vermeir, Lavrijsen & Meuldermans, 1995, 2001; Vermeir & Lavrijsen, 1996; Mertens, 2011) and 24 months (Van Deun, 1999). Increased hepatocellular hypertrophy was noted at 1 month (Van Deun et al., 1996a,b; Mertens, 2011), 3 months (Gur, Nyska & Waner, 1991) and 24 months (Van Deun, 1999). Biliary excretion of T_4/T_3 was not measured in any study; however, serum T_4 levels were reduced at 1 week (Verbeek et al., 2000). Increased TSH levels in the serum were demonstrated at 1 and 2 weeks (Verbeek et al., 2000). Increased thyroid weight was demonstrated at 1 and 3 months (Van Deun et al., 1996b) and at 24 months (Van Deun, 1999). Microscopic examination of the thyroid demonstrated an increase in thyroid hypertrophy at 2 weeks (Verbeek et al., 2000), and thyroid hyperplasia was reported after 2 years of treatment (Van Deun, 1999).

Strength, consistency and specificity of association of tumour response with key events

The strength, consistency and specificity of the association can be established from the studies detailed above. The quantifiable precursor events, fundamental to the proposed MOAs, are consistent with the emergence of hepatocellular and thyroid follicular cell tumours in rodents.

Induction of CAR-induced CYP2B and CYP3A enzymes, associated with increased liver weight, hepatocellular hypertrophy and hepatocyte cell proliferation, is consistent with a CAR-mediated MOA. The rat appears to be more resistant than the mouse to imazalil-induced tumour formation, which is consistent with the findings for phenobarbital (Elcombe et al., 2014).

Observation of induction of hepatic UGT activity, associated with increased liver weight and hepatocellular hypertrophy, decreased T_4 and increased TSH levels in rats receiving imazalil in the

diet, is consistent with the perturbation of homeostasis of the hypothalamic–pituitary–thyroid axis by an extra-thyroidal mechanism.

Biological plausibility and coherence

Liver tumours

The MOA demonstrated for imazalil is consistent with the well-known MOA for CAR activation in rodents and is consistent with current understanding of cancer biology and nuclear receptor–mediated carcinogenesis. Phenobarbital is a standard example of a CAR-mediated cytochrome inducer (LeBaron et al., 2013; Elcombe et al., 2014). Treatment with phenobarbital induces a range of phase I (e.g. CYP2B, CYP2C and CYP3A subfamily enzymes) and phase II enzymes (e.g. microsomal epoxide hydrolase, some microsomal UGT enzymes), as well as phase III enzymes (Elcombe et al., 2014). The MOA includes activation of a nuclear receptor, selective cytochrome P450 induction, an increase in cell proliferation and suppression of apoptosis. This creates an environment in the liver where spontaneously initiated cells have a greater chance to survive and divide, eventually leading to tumour formation (Elcombe et al., 2014).

An important step in demonstrating the importance of CAR in liver tumour induction was the finding that phenobarbital does not increase liver weight or DNA synthesis or result in enzyme induction, and consequently there is no increased incidence of liver tumours in human hepatocytes or genetically modified mice lacking CAR. Similarly, studies in rats have demonstrated that CAR is needed for induction of enzymes and replicative DNA synthesis. Based on results in the knockout rodent systems and in human hepatocytes and a chimeric mouse/human model (in which the mouse liver is replaced with human hepatocytes), the lack of replicative DNA synthesis is identified as the key species difference in response to CAR activators, leading to the difference in tumour response (Peffer et al., 2018). Thus, there is a qualitative interspecies difference in tumour response between rats and humans.

The proposed MOA for imazalil-induced rodent liver tumour formation is biologically plausible and consistent with our current understanding of liver tumour formation by non-genotoxic mitogenic agents that can activate nuclear receptors. Imazalil did not induce cell proliferation in human hepatocytes.

Thyroid tumours

There exist considerable data from laboratory studies in rats that demonstrate the relationship between sustained perturbation of the hypothalamic–pituitary–thyroid axis, prolonged stimulation of the thyroid gland by TSH and the progression of thyroid follicular cells from hypertrophy to hyperplasia and eventually to neoplasia (McClain, 1995; Hard, 1998; Hurley, Hill & Whiting, 1998; Capen et al., 1999; IARC, 2001; Dellarco et al., 2006). Increased secretion of TSH may result via several mechanisms, including increased hepatic clearance of T_4, as is the case with imazalil.

Circulating levels of thyroid hormones (T_3/T_4) in the blood are checked and regulated by the thyrotropic cells of the hypothalamus (releasing TRH) and the pituitary gland, which is responsible for the synthesis of TSH. In the pituitary gland, T_4 is metabolized by 5′-deiodinase type II to T_3, which then binds to specific receptors in the cell nucleus. A decrease in T_3 receptor occupancy results in stimulation of TRH from the hypothalamus and TSH synthesis and secretion in the pituitary. Studies in vivo have shown that injection of rats with TSH leads to reductions in thyroid follicular cell nuclear statin, a non-proliferation-specific nuclear protein, indicating that these cells were leaving the non-dividing state to resume the cell cycle (Bayer et al., 1992). This study demonstrated that low, repeated doses of TSH (0.25 IU per rat twice daily) produced a cumulative response in nuclear statin levels over 10 days, which returned to normal resting levels within 5 days of cessation of TSH injections. Reduction in nuclear statin is also an early event that parallels the earliest known pinocytotic response to TSH. These data are consistent with increased TSH concentrations alone causing thyroid follicular cells of rats to enter a state of pre-proliferation. Therefore, the proposal that

imazalil causes thyroid follicular cell neoplasms in rats by initially inducing hepatic UGT is consistent with the known physiology of the hypothalamus–pituitary–thyroid dynamic control system, at least to the stage of hypertrophy and hyperplasia (Dellarco et al., 2006).

The tumour response elicited from chronic treatment with imazalil is typical of a rat thyroid carcinogen. For imazalil, the thyroid follicular cell tumours were found in male rats, but not in female rats or mice. Rats tend to be more sensitive to thyroid carcinogenesis than mice, and male rats are frequently found to be more sensitive than female rats with respect to the finding of thyroid follicular cell tumours in carcinogenicity studies (Hurley, Hill & Whiting, 1998; USEPA, 1998). The TSH levels are typically higher in male rats than in female rats (Hill et al., 1989). In addition, male rats are more prone to hepatic enzyme induction than females of the same strain, but this is dependent on the specific inducible enzyme, the dose of the inducing compound and the age of the animals (Sundseth & Waxman, 1992; Agrawal & Shapiro, 1996; Oropeza-Hernandez, Lopez-Romero & Albores, 2003).

Other modes of action

Liver tumours

Other potential modes of action for liver tumours include genotoxicity, cytotoxicity, hormonal perturbation, oxidative stress, peroxisome proliferator–activated receptor alpha (PPARα) activation, aryl hydrocarbon receptor (AhR)–mediated enzyme induction, infection and iron/copper overload (Elcombe et al., 2014).

Genotoxicity

The genetic toxicology studies with imazalil demonstrate that there is no concern for genotoxicity.

Hepatic cytotoxicity

Cytotoxicity is unlikely to be a relevant MOA for imazalil, as relevant toxicity data from numerous repeated-dose toxicity studies indicated a lack of treatment-related necrosis and necrosis-related end-points in rats and mice (e.g. increases in ALT, AST, ALP; data not shown). Taken together, the weight of evidence does not support a consistent association of cytotoxicity/necrosis in imazalil-treated animals with eventual hepatocellular tumours.

Elcombe (2012c) evaluated the effect of dietary administration of imazalil on the number of hepatocytes undergoing replicative DNA synthesis (S-phase of the cell cycle). Increased S-phase in liver was reported at all concentrations by up to 6-fold relative to control values.

Elcombe (2012a) evaluated cytotoxicity in response to imazalil in vitro in mouse hepatocytes as changes in intracellular ATP concentration and the number of hepatocytes undergoing replicative DNA synthesis (S-phase of the cell cycle). No effect on hepatocyte cytotoxicity (ATP) was reported at 10 or 30 μmol/L, whereas statistically significant increases in S-phase were demonstrated at 10 and 30 μmol/L.

As hepatic cytotoxicity was not evident, several specific regenerative proliferative MOAs can be excluded (e.g. iron/copper overload, infection).

Apoptosis

Imazalil experimentally did not demonstrate any increase in hepatocellular apoptosis (as measured by caspase immunohistochemistry) in male rats at doses up to 2400 ppm for up to 28 days (Mertens, 2011). Elcombe (2012c) evaluated the imazalil-induced increase in the concentration of cleavage products and reported that imazalil induction was less than that of the phenobarbital positive control.

Imazalil-induced *Gadd45β* mRNA[5] was reported in male rats at doses up to 2400 ppm for up to 28 days (Mertens, 2011) and in both wild-type and hPXR/hCAR mice (Elcombe, 2012c).

Oxidative stress

Imazalil experimentally did not demonstrate any increase in hepatocellular oxidative stress (as measured by 4-hydroxy-2-nonenal immunohistochemistry) in male rats at doses up to 2400 ppm for up to 28 daya (Mertens, 2011).

Elcombe (2012c) evaluated lipid peroxidation as thiobarbituric acid reactive substances (largely malondialdehyde) in liver homogenates. Dietary administration of phenobarbital or imazalil for 7 days had no effect on lipid peroxidation in the livers of wild-type or humanized mice.

PPARα

Although there can be considerable crosstalk between CAR and PXR receptors, the key and associative events for imazalil-induced liver tumour formation appear to be predominantly CAR dependent, as such effects are absent in mice lacking CAR. The imazalil-mediated induction of *Cyp2b10* and, to a lesser degree, *Cyp3a11* in the presence of increased hepatocellular S-phase strongly suggests that imazalil is an activator of the xenosensing nuclear receptors CAR and (possibly) PXR. Importantly, imazalil appeared to be a more potent activator of mouse CAR than human CAR, as noted by generally smaller responses in humanized CAR mice when compared with wild-type mice at comparable doses (Elcombe, 2012c).

Elcombe (2012c) evaluated the effect of dietary administration of imazalil on the number of hepatocytes undergoing replicative DNA synthesis (S-phase of the cell cycle) in wild-type and humanized (hPXR/hCAR) mice. Increased S-phase in liver was reported at all concentrations in wild-type mice by up to 6-fold relative to control values; administration of phenobarbital at 1000 ppm increased S-phase by 31.2-fold. Administration of imazalil at all concentrations to hPXR/hCAR mice increased S-phase by up to 3.4-fold relative to control values; administration of phenobarbital at 1000 ppm increased S-phase by 24.5-fold. S-phase was increased in all groups administered imazalil, but no dose–response relationship was observed, and, furthermore, the maximum response was less than 20% and 14% in wild-type and humanized mice, respectively, following the administration of phenobarbital. This lack of cell proliferation differences between wild-type and hCAR/hPXR mice (Elcombe, 2012c) has no effect on the validity of the proposed CAR MOA and is further discussed in the "Uncertainties, inconsistencies and data gaps" section below. It is important to note that imazalil-induced increases in hepatocellular cell proliferation were demonstrated in vitro in mouse hepatocytes (Elcombe, 2012a), but not in female human hepatocytes (Elcombe, 2012b). No concentration of imazalil induced any increases in replicative DNA synthesis (S-phase of the cell cycle) in human hepatocytes, indicating that, opposite to what occurs in mice, imazalil does not induce cell proliferation in human hepatocytes.

Thyroid tumours

Relevant alternative MOAs for rodent thyroid follicular cell tumours include genotoxicity, disruption of thyroid–pituitary functioning, chemicals inhibiting the uptake of iodide into the thyroid, chemicals inhibiting thyroid peroxidase, chemicals inhibiting release of thyroid hormone from the

[5] GADD45β is an anti-apoptotic factor, a negative regulator of the p53 tumour suppressor. GADD45β has been implicated in pathways activated by CAR and contributory to the enhanced tumorigenic response in CAR wild-type animals (NIEHS, 2010).

thyroid gland, chemicals damaging thyroid follicular cells and chemicals inhibiting the conversion of T_4 to T_3 (USEPA, 1998).

The genetic toxicology studies with imazalil demonstrate that there is no concern for genotoxicity.

Vermeir, Lavrijsen & Meuldermans (2001) evaluated the possible induction and/or inhibition of hepatic 5′-monodeiodinase (the enzyme that converts T_4 to T_3) and thyroid peroxidase activities by imazalil in male SPF Wistar rats after oral administration through the diet for 1, 2 and 4 weeks at concentrations of 0, 400, 1200 and 3200 ppm; phenobarbital (1200 ppm) was used as the positive control. Thyroid sections were obtained at 1, 2 and 4 weeks as well as after recovery periods of 4 and 9 weeks. 5′-Monodeiodinase activity was decreased with 3200 ppm imazalil and 1200 ppm phenobarbital at week 1, but not in the dose groups at weeks 2 and 4 or in the recovery groups. Thyroid peroxidase activities tended to be increased in the thyroids of the imazalil dose groups at weeks 1 and 4 and in the phenobarbital group at week 4.

Imazalil experimentally did not demonstrate any increase in thyroid follicular cell apoptosis (as measured by caspase immunohistochemistry) (Mertens, 2011).

Imazalil was included in the Hornung et al. (2018) screen for inhibition of deiodinase type 1 activity and did not demonstrate inhibition over 20%.

Imazalil experimentally did not demonstrate any increase in oxidative stress in thyroid follicular epithelial cells (as measured by 4-hydroxy-2-nonenal immunohistochemistry) in male rats at doses up to 2400 ppm for up to 28 days (Mertens, 2011).

Thyroid receptor effects of imazalil have been evaluated in the United States Environmental Protection Agency's Endocrine Disruption Screening Program for the 21st Century[6] and the Toxicity Forecaster[7] programmes. Imazalil was inactive in all assays above the limit of cytotoxicity.

Additional effects on the hypothalamic–pituitary–thyroid axis and disruption of other pathways of thyroid hormone metabolism are other possibilities for altering thyroid homeostasis. These variations would not differ in any fundamental way from the MOA that has been proposed for imazalil, in that all would lead to prolonged TSH stimulation with continuous exposure, and quantitatively humans are less sensitive than rodents to developing cancer from perturbations in the thyroid–pituitary axis (USEPA, 1998).

Uncertainties, inconsistencies and data gaps

Limited uncertainties, inconsistencies and data gaps exist for the proposed liver and thyroid tumour MOAs for imazalil. The identified inconsistencies and data gaps have no effect on the validity of the proposed liver or thyroid MOAs.

Liver tumours

The biological relevance of the liver tumours in female mice at 2400 ppm is uncertain. The incidence of hepatocellular adenomas was not statistically significant, but was increased over the available control and historical controls. There was no increase in the incidence of hepatocellular carcinomas in female mice. A trend for the increase in the incidence of hepatocellular adenomas and carcinomas combined was noted by Vandenberghe (2014). However, increases in CYP2B and CYP3A enzyme activity, liver weight and hepatocellular hypertrophy were reported in female mice. This does not affect the validity of the proposed imazalil-induced CAR MOA. The liver tumours in female mice may not be biologically relevant. If the liver tumours in female mice were considered to

[6] https://actor.epa.gov/edsp21/

[7] https://actor.epa.gov/dashboard/

be biologically relevant, the data generated in male mice in support of the CAR MOA are applicable to the female mice. The response to CAR activation in male mice and male rats is apparent at doses lower than those in female mice, as evidenced by the derived NOAEL values for hepatocellular neoplasms; therefore, data available in males are applicable to females. In addition, Peffer et al. (2018) stated that "in cases where both sexes were affected, in the interest of animal ethics, information gathered using just one sex that is representative of the responses in both males and females should be sufficient".

Increased *Cyp2b* and *Cyp3a* mRNA expression and LKE3 (increased cell proliferation) and LKE4 (increased in foci of altered hepatocytes) were reported only in male mice. As noted above, Peffer et al. (2018) stated that "in cases where both sexes were affected, in the interest of animal ethics, information gathered using just one sex that is representative of the responses in both males and females should be sufficient". This data gap has no effect on the validity of the proposed CAR MOA.

No direct measurement of CAR activation was made in mice or rats. Mertens (2011) did evaluate the levels of CAR (Nr113) and did not demonstrate induction at the mRNA level when compared with animals on the basal diet for any dose (0, 200, 1200 and 2400 ppm) of imazalil or the phenobarbital (1200 ppm) positive control at 24 or 28 days. Because phenobarbital, the positive control, did not elicit a response, the interpretation of this result is that the study was unable to detect changes in CAR mRNA levels. This data gap has no effect on the validity of the proposed CAR MOA, as the other key events and associative events were experimentally demonstrated in the toxicological database for imazalil.

Hepatocellular proliferation was reported in the in vitro mouse hepatocyte study (Elcombe, 2012a) and in male mice (Elmore, 2004a; Elcombe, 2012c) and male rats (Elmore, 2004b). Some studies, such as O'Neill (2002) and Mertens (2011), failed to demonstrate cell proliferation. This inconsistency has no effect on the validity of the proposed CAR MOA, as cell proliferation in both male mice and male rats was experimentally demonstrated within the toxicological database for imazalil. In addition, no cell proliferation was reported in the in vitro human hepatocyte study (Elcombe, 2012b).

Elcombe (2012c) did not report any differences in cell proliferation between wild-type and humanized (hCAR/hPXR) mice when dosed with imazalil or phenobarbital. This is also an identified inconsistency in the literature on phenobarbital (Elcombe et al., 2014). Elcombe et al. (2014) summarized two studies conducted with phenobarbital in wild-type and hCAR or hCAR/hPXR mice. One study demonstrated no cell proliferation in hCAR/hPXR mice, and the other demonstrated cell proliferation in hCAR mice. Elcombe et al. (2014) concluded that "the present data for effects on cell proliferation and apoptosis obtained with transgenic mice expressing either hCAR/hPXR or just hCAR is equivocal". Both phenobarbital and imazalil have been shown not to increase cell proliferation in cultured human hepatocytes (Elcombe, 2012b), whereas cell proliferation was demonstrated in cultured mouse hepatocytes (Elcombe, 2012a). Peffer et al. (2018) listed primary hepatocellular cultures in humans and rodents as specific tests to establish the non-human relevance of the CAR MOA. Therefore, this lack of cell proliferation differences between wild-type and hCAR/hPXR mice in vivo (Elcombe, 2012c) has no effect on the validity of the proposed CAR MOA. Furthermore, the hCAR/hPXR transgenic mouse model is an invalid model, because although the mice are humanized for CAR/PXR, all downstream events are still murine. As stated in describing the MOA for CAR-induced rodent tumours, the species differences are specific to the stimulation of cell proliferation, not to the activation of CAR. Elcombe et al. (2014) provided a review of the evidence that mouse or rat liver tumours that occur via a CAR MOA are not relevant to humans, because although activation of CAR, the induction of CYP2B and other cytochrome P450 forms, and liver hypertrophy can be observed in humans, the cell proliferation and subsequent altered hepatic foci and liver tumours do not occur in humans.

Thyroid tumours

No direct measurement of TKE2 (i.e. increased biliary excretion of T_3/T_4) was conducted in the data package. This is consistent with Dellarco et al. (2006), in that biliary excretion of T_3/T_4 was not explicitly measured for pyraziflumid. The associative events of increased hepatic metabolism and biliary excretion of T_4 are supported by the associative events that demonstrated increased phase I and phase II (T_4-UGT) enzymes as well as increases in liver weight and corresponding liver histopathology demonstrated in the toxicological database for pyraziflumid. T_4 is conjugated with glucuronic acid catalysed by T_4-UGT and excreted in the bile, which is generally accepted by the scientific community without the need for further animal testing (Japundžić, Bastomsky & Japundžić, 1976; McClain, 1989; Capen, 1997; Lecureux et al., 2009). This data gap has therefore no effect on the validity of the proposed thyroid MOA for imazalil.

Assessment of postulated mode of action: human applicability of the proposed MOA

Is the weight of evidence sufficient to establish an MOA in animals?

Liver tumours

As described in detail in this document, the rodent MOA consists of key events that are dose concordant, occur in a logical temporal sequence and are reproducible and consistent across multiple studies. Other potential MOAs have been considered and have been excluded. Thus, the weight of evidence is sufficient to establish an MOA for liver tumours in animals.

Thyroid tumours

As described in detail in this document, there is clear evidence that imazalil alters thyroid homeostasis by UGT induction, by reducing serum T_4 levels and consequently elevating serum TSH. Thus, the weight of evidence is sufficient to establish an MOA for thyroid tumours in animals.

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

Liver tumours

A number of effects of imazalil that are produced in the rodent liver can also be observed in humans. Such effects include activation of CAR, the induction of CYP2B and other cytochrome P450 forms, and liver hypertrophy. However, some clear species differences have been reported.

For example, although imazalil stimulates replicative DNA synthesis in cultured rodent (mouse) hepatocytes, as well as after in vivo administration, imazalil does not increase replicative DNA synthesis in cultured human hepatocytes (Elcombe, 2012a,b). Furthermore, imazalil inhibited apoptosis in mice and rats, which is not expected to occur in human hepatocytes. As described previously, the present literature data on phenobarbital for effects on cell proliferation and apoptosis obtained with transgenic mice expressing either hCAR/hPXR or just hCAR are equivocal. As discussed in the "Uncertainties, inconsistencies, and data gaps" section, the hCAR/hPXR transgenic mouse model is an invalid model, because although mice are humanized for CAR/PXR, all downstream events are still murine. The differences between humans and rodents are specific to the stimulation of cell proliferation, not to the activation of CAR. Elcombe et al. (2014) provided a review of the evidence that mouse or rat liver tumours that occur via a CAR MOA are not relevant to humans, because although activation of CAR, the induction of CYP2B and other cytochrome P450 forms, and liver hypertrophy can be observed in humans, the cell proliferation and subsequent altered hepatic foci and liver tumours do not occur in humans. The results of Elcombe (2012a) demonstrate the cell proliferation in mouse hepatocytes, and the results of Elcombe (2012b) demonstrate the lack of cell proliferation in human hepatocytes. Peffer et al. (2018) listed primary hepatocyte cultures (rodent and human) as a key study to evaluate the qualitative differences in hepatocyte response

between humans and rodents. Based on the known species differences data, it can be concluded that the animal CAR MOA for imazalil-induced rodent liver tumour formation is not qualitatively plausible for humans (Elcombe et al., 2014).

Thyroid tumours

The current understanding of the regulation of thyroid hormone homeostasis in humans and the resulting role of increased TSH levels as a risk factor for thyroid cancer was considered in order to assess the relevance of the key events in the MOA for thyroid follicular cell tumours in rats resulting from chronic exposure to imazalil. The general mechanisms involved in the function and regulation of the hypothalamic–pituitary–thyroid axis in humans and rats are qualitatively similar (Bianco et al., 2002). Therefore, an agent that decreases the serum levels of T_4 in rats could similarly reduce T_4 levels in humans and thereby could potentially lead to an increase in TSH levels.

However, there are quantitative differences between the rat and the human in the function and regulation of the hypothalamic–pituitary–thyroid axis. In contrast to rats, increases in TSH levels have not been demonstrated to result from agents that induce hepatic microsomal enzymes and reduce circulating T_4 levels (Lehman-McKeeman & Hill in Meek et al., 2003). Pharmaceuticals (rifampin, phenytoin and carbamazepine) induce hepatic microsomal enzymes in humans, including UGT, and reduce circulating T_4 levels, but do not result in increased TSH levels (Curran & DeGroot, 1991). Agents that produce thyroid tumours in rats by increasing glucuronidation and biliary excretion of T_4 at high experimental doses produce no changes to thyroid hormone levels at clinically relevant doses (Masubuchi, Hakusui & Okazaki, 1997). Differences in the basic physiological processes between humans and rats account for the dose–response differences and differences in the response of the hypothalamic–pituitary–thyroid axis in triggering increases in TSH levels.

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

Liver tumours

As the data suggest that there are sufficient qualitative differences between the species that develop liver tumours after imazalil treatment (rats and mice) and humans, it is not necessary to answer the third question regarding quantitative differences (Elcombe et al., 2014). The key events of CAR activation, cell proliferation, production of altered foci and production of tumours are well characterized in animals that develop liver tumours. However, although CAR activation does occur in humans, certain other key events do not appear to occur in humans. Both imazalil and phenobarbital have been shown not to increase cell proliferation in cultured human hepatocytes, and the development of altered hepatic foci has not been reported in the literature for phenobarbital (Elcombe et al., 2014). It is therefore not likely that liver tumours would occur through this MOA as a consequence of imazalil or phenobarbital exposure in humans.

As a further weight of evidence, although phenobarbital can act as a non-genotoxic carcinogen and tumour promoter in the rat and mouse, it does not appear to produce liver tumours in humans. Because of the therapeutic uses of phenobarbital, a number of epidemiological and other human studies are available. Moreover, in these studies showing no evidence of increased liver tumour risk, the subjects received phenobarbital for many years at doses producing plasma concentrations similar to those that are carcinogenic in rodents. For example, plasma levels of phenobarbital in three strains of mice given 500 ppm phenobarbital in the drinking-water ranged from 5 to 29 μg/mL, whereas plasma concentrations of phenobarbital in human subjects given therapeutic doses of 3–6 mg/kg bw ranged from 10 to 25 μg/mL (Munro, 1993). Overall, the human epidemiological studies support the conclusion that the MOA for phenobarbital-induced rodent liver tumours is not relevant to humans (Elcombe et al., 2014).

Thyroid tumours

The thyroid is not a direct or the initial target organ of imazalil. The primary target organ of imazalil is the liver and its hepatic metabolizing enzymes, and the increased metabolic activity indirectly increases the systemic clearance of T_4, leading to the hypothyroid state and compensatory increases in TSH in rats. Although there are no chemical-specific data on the possibility for imazalil to disrupt thyroid homeostasis in humans, a number of other microsomal enzyme inducers have been extensively studied, such as phenobarbital, primidone, phenytoin and carbamazepine (Benedetti et al., 2005). As discussed above, agents that produce hypothyroidism by altering the hepatic clearance of T_4 do not appear to result in elevated TSH levels in humans.

Conclusion: statement of confidence, analysis and implications

Liver tumours

There is sufficient experimental evidence to establish an imazalil-induced CAR MOA for rodent liver tumours. The data on species differences are sufficient to determine that this MOA would be qualitatively not relevant for humans (Table A1-6).

Table A1-6. A comparison of key events for CAR-induced liver tumours in rodents and humans

Key event	Evidence in rodents	Evidence in humans
CAR activation	Yes	Yes
Altered gene expression	Yes	Yes
CYP2B induction	Yes	Yes
Hypertrophy	Yes	Yes
Increased cell proliferation	Yes	No
Clonal expansion (foci)	Yes	No
Tumours	Yes	No

CAR: constitutive androstane receptor; CYP: cytochrome P450
Source: Adapted from Elcombe et al. (2014)

Thyroid tumours

There is sufficient experimental evidence to establish an extra-thyroidal disruption MOA for imazalil-induced thyroid follicular cell tumours in male rats. Although imazalil may potentially result in hypothyroidism in humans, there is sufficient quantitative evidence on the basic physiological processes in the general literature to conclude that thyroid tumours induced by a process involving increased hepatic clearance of thyroid hormone and altered homeostasis of the pituitary–thyroid axis in rodents is not likely to lead to an increase in susceptibility to tumour development in humans. Although there are no human data on imazalil, clinical data on other hepatic microsomal enzyme inducers were critical to this human relevance analysis. The general literature provided sufficient evidence to show that, unlike in the rat, decreased T_4 levels typically show no evidence of compensatory increases in TSH levels in humans. There is also cellular and biochemical evidence that the rat pituitary–thyroid axis is much more sensitive than that of humans to such perturbations. This sensitivity is likely the result of the rapid turnover of T_4 in rats coupled with the higher demand for TSH to maintain thyroid activity (Table A1-7). Table A1-8 summarizes the thyroid-specific species differences between rats and humans.

Table A1-7. A comparison of key events for thyroid tumours in rats and humans

Key event	Evidence in rats	Evidence in humans
Increased hepatic clearance of T_4	Yes	Plausible.
Decreased serum T_4	Yes	Plausible, but less likely due to buffering capacity of TBG.
Increased TSH levels	Yes	No. Microsomal enzyme inducers have not been shown to increase TSH levels even when T_4 is decreased.
Increased TSH increases thyroid cell proliferation and tumour formation	Yes	No. Induction of thyroid follicular cell tumours secondary to hypothyroidism is remote in humans, given the quantitative differences in thyroid function/homeostasis. Occurrence of thyroid cancer is rare even in severely hypothyroid individuals.

T_4: thyroxine; TBG: thyroxine binding globulin; TSH: thyroid stimulating hormone
Source: Adapted from Dellarco et al. (2006)

Table A1-8. Thyroid-specific species differences between rats and humans

Parameter	Rat	Human	Consequence
Protein binding of T_3 and T_4	Absent	Extensive binding to TBG and weak binding to albumin	Humans are able to replenish plasma T_3 and T_4 levels without the need for increased production by the thyroid gland.
Plasma TSH levels	60 times higher than in humans	Reference range 0.3–3.0 mIU/L	The rat thyroid is considerably more active than the human thyroid.
T_4 half-life	0.5–1 day	5–9 days	Much higher baseline rate of clearance from plasma in rats than in humans.
T_3 half-life	0.25 day	1 day	Much higher baseline rate of clearance from plasma in rats than in humans.
T_4 production rate/kg bw	10-fold higher than in humans	10-fold lower than in rats	Basal rate of thyroid hormone synthesis is much higher in rats than in humans.
Follicular cell morphology	Cuboidal	Low cuboidal	The rat thyroid produces more thyroid hormones than the human thyroid.

IU: international units; T_3: triiodothyronine; T_4: thyroxine; TBG: thyroxine binding globulin; TSH: thyroid stimulating hormone
Source: Adapted from Dellarco et al. (2006)

Implications of the IPCS human relevance framework

Imazalil is an illustration of an induced tumour response consistent with MOAs that have previously been defined and established as not likely to lead to an increase in susceptibility to tumour development in humans. The first question in the framework analysis, "Is the weight of evidence sufficient to establish an MOA in animals?", has been convincingly met by the data set on this chemical, which conforms to the same key events defined for the pathway of interest. The case for imazalil further demonstrates how data on the basic understanding of the biological processes involved in the MOA provide an important means to compare the rodent and human key events. Thus, the relevant human information on other chemicals was essential to evaluating the qualitative and quantitative differences between experimental animals and humans in addressing the plausibility of the cancer MOAs for humans (i.e. "Can human relevance of the MOA be reasonably excluded on the basis of fundamental, qualitative differences in key events between animals and humans?" and "Can human relevance of the MOA be reasonably excluded on the basis of quantitative differences in either kinetic or dynamic factors between animals and humans?" in the human relevance framework).

In conclusion, for liver tumours, the CAR MOA is qualitatively not relevant to humans. For thyroid tumours, whereas the thyroid MOA is qualitatively relevant to humans, quantitatively, humans are less sensitive than rodents in regard to developing cancer from perturbations in the thyroid–pituitary axis (USEPA, 1998).

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