FLUMIOXAZIN

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Explanation

Flumioxazin (code S-53482) is the International Organization for Standardization (ISO)–approved common name for *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboxamide (International Union of Pure and Applied Chemistry), with the Chemical Abstracts Service number 103361-09-7. It acts as a herbicide by inhibition of protoporphyrinogen oxidase (PPO), resulting in an accumulation of porphyrins. It is used against weeds and mosses.

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

This evaluation is based mainly on the study reports submitted by the sponsor. All critical studies contained statements of compliance with good laboratory practice (GLP), unless otherwise specified. However, GLP status was not specifically checked. In general, studies were conducted with technical material; however, neither impurity profiles of the tested materials nor current specifications were provided. Hence, it could not be assessed whether the tested material was representative of the currently available commercial technical material.

Most toxicity studies were conducted according to internationally recognized guidelines (generally United States Environmental Protection Agency [USEPA]/Federal Insecticide, Fungicide, and Rodenticide Act [FIFRA]), except where indicated.

The authors conducted a literature search on 7 May 2015 in the databases PubMed, PubMedCentral and Scopus (keyword: flumioxazin, no restriction for publication date). Thirty-six, 25 and 224 references were retrieved, respectively. Articles appearing to be obviously non-relevant for a toxicological or human health evaluation were excluded from the results list based on their titles and/or abstracts. For the remaining seven references, the full articles were retrieved. These articles were included in the evaluation and are described in the appropriate sections.

Evaluation for acceptable intake

1. Biochemical aspects

Two ^{14}C-labelled flumioxazin molecules were used for all absorption, distribution, metabolism and excretion (ADME) studies: [phenyl-^{14}C]flumioxazin and [tetrahydro-phthaloyl-1,2-^{14}C]flumioxazin (also indicated as [phthalimide-^{14}C]flumioxazin) (for the chemical structures and the positions of the label, see Fig. 1 and Fig. 2 below). According to the study reports cited below, some parts of the studies were not conducted according to GLP principles (e.g. no audit by a quality assurance unit).

1.1 Absorption, distribution and excretion

In the first study, groups of five male and five female rats were given, by gavage, 1) a single oral dose of [phenyl-^{14}C]flumioxazin at 1 mg/kg body weight (bw) (low dose), 2) a single oral dose of [phenyl-^{14}C]flumioxazin at 100 mg/kg bw (high dose) or 3) a dose of non-radiolabelled flumioxazin at 1 mg/kg bw per day for 14 days followed by a single oral dose of [phenyl-^{14}C]flumioxazin at 1 mg/kg bw (repeated-dose experiment). Excreta were collected for 7 days after the administration of the radiolabelled compound. Afterwards, rats were sacrificed, and specimens such as blood and selected organs were collected for further analysis.

Most (97.9–102.3%) of the radioactivity, expressed as a percentage of the administered dose, was eliminated within 7 days post-dosing, the majority within 2 days post-administration. Within 24 hours post-dosing, excretion via urine and faeces was 86% for both males and females in the low-dose group, 82% and 72% for males and females, respectively, in the high-dose group and 87% and 83% for males and females, respectively, in the repeated-dose group. Total recovery of radioactivity was nearly quantitative. In the low- and repeated-dose groups, slightly more radioactivity was excreted in faeces (56.4–71.5%) than in urine (28.6–42.8%). In the high-dose group, more of the radioactivity was excreted in faeces (78.1–85.2%) than in urine (13.0–23.4%). Urinary radiocarbon excretion was greater in females than in males in all dose groups.

Tissue ^{14}C residue levels, expressed as nanogram flumioxazin equivalents (eq) per gram tissue or parts per billion (ppb), were generally low on the seventh day post-dosing. Radiocarbon residue was higher in blood cells than in tissues. Tissue ^{14}C residue levels, including those for fat,

were lower than blood levels, which suggested little potential for bioaccumulation. Tissue ^{14}C residue levels were highest in the metabolic organs: liver (12.2–24.3 ppb in the low- and repeated-dose groups; 523–713 ppb in the high-dose group) and kidney (11.2–16.0 ppb in the low- and repeated-dose groups; 397 ppb in the high-dose group). Relative to the dose, tissue levels in the high-dose animals were lower than those in animals of the low- or repeated-dose groups (Matsunaga, 1993).

In the second study, groups of five male and five female rats were given, by gavage, 1) a single oral dose of [phthalimide-^{14}C]flumioxazin at 1 mg/kg bw (low dose) or 2) a single oral dose of [phthalimide-^{14}C]flumioxazin at 100 mg/kg bw (high dose). Further groups of eight males and eight females were given, via gavage, a dose of [phthalimide-^{14}C]flumioxazin at 1 mg/kg bw after pretreatment with unlabelled flumioxazin at 1 mg/kg bw per day for 14 consecutive days (repeated-dose experiment; however, samples of only five animals of each sex were analysed). Excreta were collected for 7 days after the administration of the radiolabelled compound. Afterwards, rats were sacrificed, and specimens such as blood and selected organs were collected for further analysis.

The radioactivity was almost completely eliminated from the body in all groups within 7 days after administration, the majority within 2 days post-administration. Within 24 hours post-dosing, excretion via urine and faeces was 76% and 71% for males and females, respectively, in the low-dose group, 84% and 69% for males and females, respectively, in the high-dose group and 86% and 87% for males and females, respectively, in the repeated-dose group. Nearly 100% of the dose was excreted via faeces and urine. Recoveries of ^{14}C (expressed as a percentage relative to the dosed ^{14}C) from faeces and urine were 59.6–65.8% and 30.7–36.8%, respectively, in the low-dose group, 83.4–87.5% and 11.8–14.1%, respectively, in the high-dose group and 62.5–65.7% and 31.7–35.3%, respectively, in the repeated-dose group. The proportion of urinary ^{14}C excretion in the high-dose group was significantly smaller than in the low- and repeated-dose groups in both sexes ($P < 0.001$). Urinary ^{14}C excretion in females of the low- and high-dose groups was significantly greater than that in males ($P < 0.005$ for the low-dose group, $P < 0.01$ for the high-dose group).

Tissue and blood ^{14}C residues on the seventh day after administration were generally low. In all groups, blood cells showed the highest ^{14}C residue levels, which were 41.2–46.6 ppb flumioxazin equivalents in the low- and repeated-dose groups and 2175–2268 ppb flumioxazin equivalents in the high-dose group. However, ^{14}C levels in the blood cells in the high-dose group did not increase in proportion to the dose. In addition, ^{14}C residue levels in blood, heart, kidney, liver and skin were relatively higher than those in any other examined tissues in both sexes for all groups. In all groups, tissue and blood contained less than 1.0% of the administered dose. Total residue in blood amounted to no more than 0.2% of the dose for all groups (Shiba, 1994).

The biliary excretion of flumioxazin was investigated in bile duct–cannulated male and female rats. Animals received a single oral dose of [phthalimide-^{14}C]flumioxazin at 1 mg/kg bw, and bile, urine and faeces were collected up to 72 hours post-dosing. The gastrointestinal tract (with contents) from each animal was also collected at terminal sacrifice.

Most of the radioactivity was excreted in the urine (37% in both males and females) or bile (41% and 37% in males and females, respectively) within 24 hours. At 72 hours after administration, mean radioactivity concentrations in the urine accounted for 43% and 41% of the dose in males and females, respectively, and those in bile, for 43% and 39% of the dose in males and females, respectively. Mean radioactivity concentrations in faeces accounted for 6% and 9%, and residues remaining in the gastrointestinal tract accounted for only 1% and 2%, in males and females, respectively. Total recoveries were above 90%. Flumioxazin was almost completely bioavailable in the rat after oral administration. Only a minor amount of the parent compound was detected in the faeces, amounting to approximately 1% of the dose (Gibson et al., 1997).

1.2 Biotransformation

In the samples of urine and faeces collected in the studies by Matsunaga (1993) and Shiba (1994), metabolites were separated by thin-layer chromatography using several different mobile phases, their retention times were compared with synthetic reference standards and spots were detected using autoradiography. The metabolites were quantified by liquid scintillation counting. Taking into account the results of all three reports, flumioxazin was extensively metabolized (Table 1). Thirty-five metabolites were detected and quantified after administration of [phenyl-^{14}C]flumioxazin, and seven were identified. Twenty-nine metabolites were detected and quantified after administration of [phthalimide-^{14}C]flumioxazin, and 10 were identified.

The main metabolic reactions were hydroxylation of the cyclohexene ring of the tetrahydrophthalimide moiety, cleavage of the imide linkage, cleavage of the amide linkage in the benzoxazine ring, reduction of the double bond in the tetrahydrophthalimide ring, acetylation of the amino group of the aniline derivative and incorporation of a sulfonic acid group in the tetrahydrophthalimide ring.

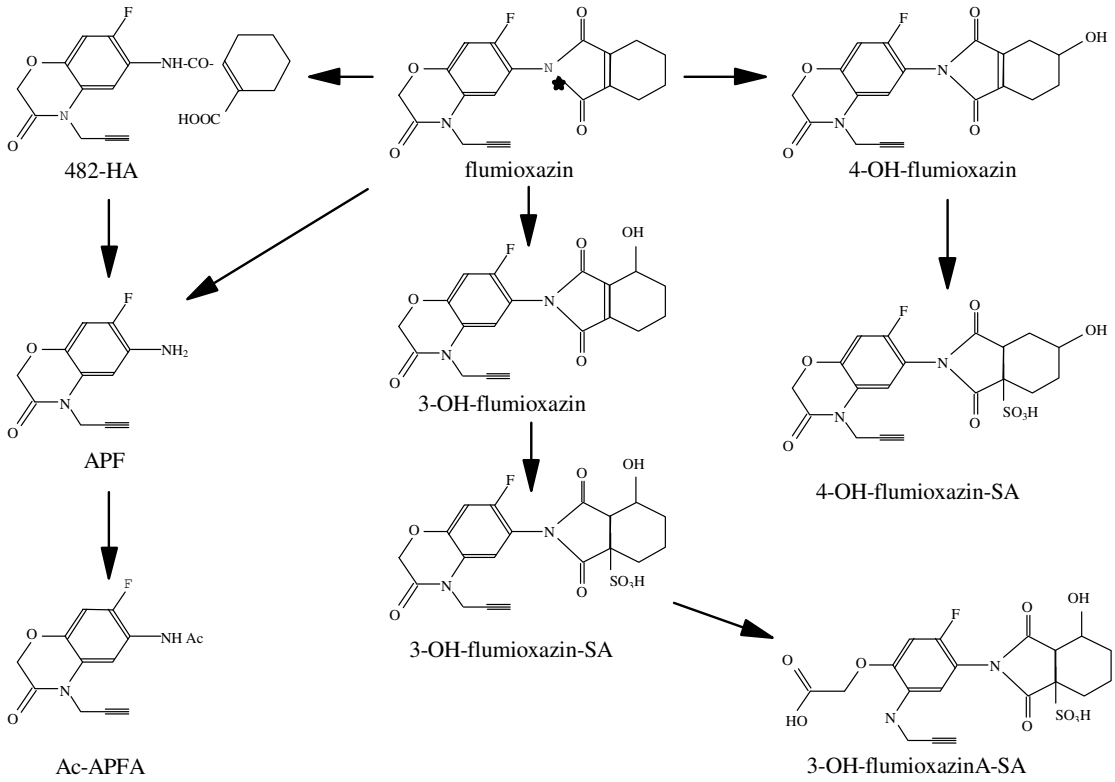
The proposed flumioxazin metabolic pathways in rats, based on the studies with [phenyl-^{14}C]flumioxazin and [phthalimide-^{14}C]flumioxazin, are summarized in Fig. 1 and Fig. 2, respectively.

Table 1. Amount of radioactive metabolites in urine and faecal extracts

	% of total administered radioactivity (male/female)					
	[phenyl-^{14}C]Flumioxazin			**[phthalimide-^{14}C]Flumioxazin**		
Metabolite[a]	**Low dose**	**High dose**	**Repeated dose**	**Low dose**	**High dose**	**Repeated dose**
Total excreted ^{14}C	102.3/99.2	98.2/101.5	97.9/98.9	96.5/96.4	99.4/97.5	97.5/97.8
Flumioxazin	0.3 / 0.4	51.0/46.6	0.3/5.2	2.8/1.9	66.1/63.0	1.2/1.3
3-OH-flumioxazin	3.2/3.9	2.3/3.1	3.1/4.5	6.2/5.3	2.5/2.5	6.4/6.3
4-OH-flumioxazin	2.0/3.0	1.8/3.2	2.3/3.7	5.8/6.1	2.1/2.7	6.5/7.0
APF	2.5/6.8	1.8/4.0	3.8/4.4	–	–	–
Ac-APFA	5.9/4.0	2.2/2.0	5.3/4.5	–	–	–
SAT-482	–	–	–	0.6/0.6	0.2/0.3	0.6/0.5
3-OH-SAT-482	–	–	–	2.2/2.0	0.7/0.7	1.9/1.6
4-OH-SAT-482	–	–	–	2.3/2.0	0.5/0.5	1.5/1.5
THPA	–	–	–	0.9/1.3	0.4/0.5	1.5/1.5
1-OH-HPA-1	–	–	–	1.3/1.4	0.7/0.8	1.8/2.0
3-OH-flumioxazin-SA	14.4/18.6	6.9/8.1	13.4/11.6	16.1/17.6	5.7/6.1	16.4/16.8
4-OH-flumioxazin-SA	5.2/7.0	2.9/3.1	6.0/4.7	7.4/7.8	2.3/2.4	8.3/8.3
3-OH-flumioxazin-A-SA	4.0/2.4	1.8/1.4	3.8/1.9	3.2/2.4	0.8/0.7	3.4/2.6
Identified metabolites	37.5/46.1	70.7/71.5	38.0/40.5	48.8/48.3	82.1/80.2	49.5/49.3
Sum of unknown metabolites	54.3/48.6	22.9/24.6	50.7/49.8	39.8/40.1	13.0/12.3	40.4/41.1
Unextractable ^{14}C	8.9/2.8	3.9/3.6	7.8/6.9	5.7/4.8	3.6/3.7	6.1/5.5

[a] See Figs 1 and 2 for chemical structures of metabolites.
Source: Matsunaga (1993); Shiba (1994)

Fig. 1. Proposed metabolic pathway of flumioxazin in the rat, based on [phenyl-^{14}C]flumioxazin



*: position of the label

Source: Matsunaga (1993)

Apparently, the results of the studies by Matsunaga (1993) and Shiba (1994) were also published in the open literature (Tomigahara et al., 1999a,b).

2. Toxicological studies

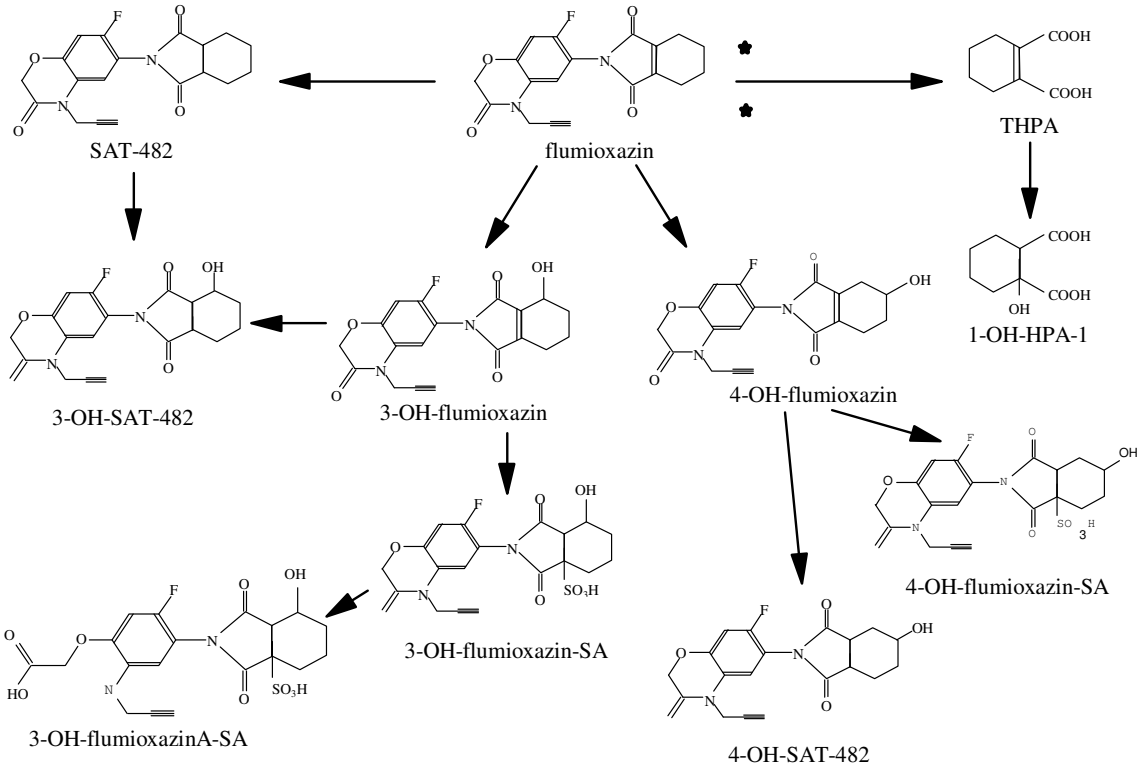
2.1 Acute toxicity

The results of acute toxicity studies with flumioxazin administered orally, dermally or by inhalation as well as those of the studies investigating skin and eye irritation/corrosion and skin sensitizing potential are summarized in Table 2.

(a) Lethal doses

Flumioxazin technical (purity 94.8%) was administered by gavage (suspension in 1% weight per volume [w/v] aqueous methyl cellulose, dosing volume of 20 mL/kg bw) to five male and five female Sprague-Dawley rats at a dose level of 0 or 5000 mg/kg bw. Mortality and clinical signs were recorded on five occasions on the day of dosing and daily thereafter for the remainder of the 14-day observation period. Individual body weights were measured on days 0, 7 and 14. At the end of the study, all animals were sacrificed, and any gross pathological changes were recorded. No mortality was observed, and there were no overt signs of reaction to treatment. No abnormalities were detected at necropsy. The acute oral median lethal dose (LD_{50}) of flumioxazin in rats was greater than 5000 mg/kg bw for both sexes under the conditions of the study (Hiromori, 1990a).

Fig. 2. Proposed metabolic pathway of flumioxazin in the rat, based on [phthalimide-^{14}C]flumioxazin



*: position of the labels

Source: Shiba (1994)

Table 2. Acute toxicity of flumioxazin

Species	Strain	Sex	Route	Purity (%)	Result	Reference
Rat	Sprague-Dawley	Male + female	Oral	94.8	LD_{50} > 5 000 mg/kg bw	Hiromori (1990a)
Rat	Sprague-Dawley	Male + female	Dermal	94.8	LD_{50} > 2 000 mg/kg bw	Hiromori (1990b)
Rat	Sprague-Dawley	Male + female	Inhalation	98.3	LC_{50} > 3.93 mg/L (maximum attainable concentration)	Kawaguchi (1990)
Rabbit	New Zealand White	Male + female	Skin irritation	94.8	Non-irritating	Nakanishi (1989)
Rabbit	New Zealand White	Male + female	Eye irritation	94.8	Minimally irritating	Nakanishi (1989)
Guinea-pig	Hartley	Male	Skin sensitization	94.8	Non-sensitizing (Magnusson & Kligman)	Nakanishi (1990)

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

Flumioxazin technical (purity 94.8%) suspended in 1% w/v aqueous methyl cellulose was applied to the clipped skin of five male and five female Sprague-Dawley rats at a dose level of 0 or 2000 mg/kg bw. The test material was covered for 24 hours with surgical tape, after which test material and the tape were removed. Mortality and clinical signs were recorded on five occasions on the day of dosing and daily thereafter for the remainder of the 14-day observation period. Individual body weights were measured on days 0, 7 and 14. At the end of the study, all animals were sacrificed, and any gross pathological changes were recorded. No mortality was observed, and there were no overt signs of reaction to treatment. No abnormalities were detected at necropsy. The acute dermal LD_{50} of flumioxazin in rats was greater than 2000 mg/kg bw for both sexes under the conditions of the study (Hiromori, 1990b).

Two groups of five male and five female Sprague-Dawley albino rats were subjected to a 4-hour nose-only exposure to a dust of flumioxazin (purity 98.3%) at an analytically determined concentration of 0 (control), 1550 or 3930 mg/m^3, the higher concentration being the maximum attainable. The dust had a mass median aerodynamic diameter of 4.99–6.18 µm. Mortality and clinical signs were recorded on five occasions on the day of exposure and at least daily during 14 days after dosing. Individual body weights were measured pre-dosing on day 0 and on days 3, 7 and 14. No mortalities occurred at any dose level. There were no clinical signs in the control group. In both treated groups, the following clinical signs were observed in both sexes: irregular respiration or bradypnoea from 0.5 hour after commencement of exposure, lasting up to 2 hours after cessation of exposure; and a decrease in spontaneous activity during exposure only. Body weight was unaffected by treatment. There were no gross necropsy findings that were attributable to treatment. Therefore, the acute inhalation median lethal concentration (LC_{50}) of flumioxazin in rats was greater than 3930 mg/m^3 air for both sexes under the conditions of the study (Kawaguchi, 1990).

(b) Dermal irritation

Flumioxazin technical (purity 94.8%) was applied at a dose of 0.5 g to the clipped dorsal region of three male and three female New Zealand White rabbits under a gauze patch moistened with corn oil and kept in contact with the skin for 4 hours under an occlusive dressing. Cutaneous observations for erythema and oedema were performed using the Draize scheme, at 4.5, 24, 48 and 72 hours after application. No cutaneous irritation was observed at any test site during the study (Nakanishi, 1989).

(c) Ocular irritation

Flumioxazin technical (purity 94.8%) was instilled at a dose of 100 mg into the conjunctival sac of one eye of each of three male and three female New Zealand White rabbits. Observations of ocular lesions were conducted at 1, 24, 48 and 72 hours after instillation. The test material induced slight redness and very slight chemosis of the conjunctiva in all rabbits and slight congestion in the iris in two rabbits 1 hour after application. Twenty-four hours after application, slight redness of the conjunctiva was observed in four rabbits. These reactions disappeared 48 hours after application. The test material caused slight, transient irritation to the eyes of rabbits (Nakanishi, 1989).

(d) Dermal sensitization

In a dermal sensitization study conducted according to the Magnusson & Kligman method, flumioxazin technical (purity 94.8%) was injected intradermally (for each animal: two injections of Freund's Complete Adjuvant [FCA] in water, two injections of flumioxazin as a 1% solution in corn oil, and two injections of flumioxazin in FCA and water) into 20 male Hartley guinea-pigs; 6 days later, a 10% sodium lauryl sulfate solution in petrolatum was applied, which was followed on the next day by an epidermal application of 25% test substance in petrolatum under occlusive dressing pads for 48 hours. Fourteen days after the epidermal application, the challenge treatment was performed. Flumioxazin at 25% in petrolatum was applied to the intact right flank of each animal for 24 hours. Observations were made 24 and 48 hours after removal of the patches. The control group (20 animals)

was treated in a similar fashion, but with no test item. 2,4-Dinitrochlorobenzene was used as the positive control (five animals). Intradermal injection concentrations were limited by precipitation. Topical application did not produce any skin reactions. No sensitization reactions were produced at the challenge site. The positive control produced satisfactory results. Under the conditions of the study, flumioxazin did not show skin sensitizing potential (Nakanishi, 1990).

2.2 Short-term studies of toxicity

(a) Oral administration

The short-term toxicity of flumioxazin was evaluated in mice, rats and dogs.

Mice

A 4-week dietary toxicity study in mice with flumioxazin technical (purity 94.8%) was conducted as a dose range–finding study to determine the dose levels for an oncogenicity study. Three groups of nine male and nine female Crj:CD-1 (ICR) mice received flumioxazin in the diet at a concentration of 1000, 3000 or 10 000 parts per million (ppm) for 4 weeks (equal to 152, 420 and 1367 mg/kg bw per day for males and 165, 482 and 1698 mg/kg bw per day for females, respectively). A similarly constituted control group received untreated diet.

No deaths or treatment-related clinical signs were noted during the study. No treatment-related changes in body weight, feed consumption, haematology, blood biochemistry or gross pathology were observed. Increases in both absolute and relative liver weights were observed in males at 10 000 ppm and in females at 3000 ppm, and relative liver weights of females were increased at 10 000 ppm (always less than a 15% increase). No treatment-related histopathological alterations were noted in the liver (other organs were apparently not evaluated). Flumioxazin (technical grade) affected liver weights in mice, but the toxicological significance of these changes was considered low, even at 10 000 ppm.

In conclusion, 7000 ppm was selected as the highest dose level for the oncogenicity study in mice, based on 7000 ppm (approximately 1000 mg/kg bw per day) being the highest USEPA guideline dose level for mouse oncogenicity studies. Considering the limited parameters evaluated, no no-observed-adverse-effect level (NOAEL) was identified for this study (Seki, 1990).

In a further study, four groups of 10 male and 10 female Crj:CD-1 (ICR) mice received flumioxazin (purity 98.4%) in the diet at a concentration of 100, 1000, 3000 or 10 000 ppm for 13 weeks (equal to 16.3, 164, 459 and 1062 mg/kg bw per day for males and 18.6, 202, 595 and 2163 mg/kg bw per day for females, respectively). A similarly constituted control group received untreated diet.

No deaths or treatment-related clinical signs were noted during the study. No treatment-related changes in body weight, feed and water consumption, urine analysis, haematology, blood biochemistry, ophthalmology or gross pathology were observed. Low reticulocyte counts and decreased calcium values in males at 10 000 ppm and increased creatinine values in females at 10 000 ppm were considered to be of little biological significance. Bone marrow smears were not examined. Increases in both absolute and relative liver weights were observed in males and females at 10 000 ppm (always less than a 15% increase). Ovary weights were reduced (by approximately 35%) in females at 10 000 ppm, No treatment-related histopathological alterations were noted.

The NOAEL was 3000 ppm (equal to 459 and 595 mg/kg bw per day for males and females, respectively), based on increased liver weights and decreased ovary weights at 10 000 ppm (equal to 1062 and 2163 mg/kg bw per day for males and females, respectively) (Hagiwara, 1990).

Rats

Flumioxazin (purity 98.4%) was offered to five groups of 12 male and 12 female Sprague-Dawley rats (aged 5 weeks) at a dietary concentration of 0, 30, 300, 1000 or 3000 ppm (equal to 0, 2.28, 20.7, 69.7 and 244 mg/kg bw per day for males and 0, 2.21, 21.7, 71.5 and 230 mg/kg bw per day for females, respectively) for 90 days. Achieved concentrations and homogeneity were verified by analysis. The animals were observed at least once daily for clinical signs, behaviour and mortalities. Ophthalmological examination was performed on all animals during the quarantine period and on all animals from the control and highest-dose groups after 90 days. Body weights, feed consumption and water consumption were determined at weekly intervals for all animals. Blood samples were taken after fasting at termination of the study for assessment of blood biochemical and haematological parameters. Urine analyses were conducted on all animals at termination. All animals were subjected to detailed postmortem gross examination, and all abnormalities were recorded. Organ weights (brain, heart, liver, spleen, thymus, kidneys, pituitary, adrenals, thyroid with parathyroid, ovaries or testes) were recorded. In the control and 3000 ppm groups, histological evaluation was performed on heart, spleen, lymph nodes (mandibular and mesenteric), thymus, tongue, salivary gland, oesophagus, stomach, small intestine (duodenum, jejunum, ileum), large intestine (caecum, colon, rectum), liver, pancreas, trachea, lungs, kidneys, urinary bladder, testes, ovaries, prostate, seminal vesicle, epididymides, uterus, mammary gland (female), vagina, pituitary, thyroid (with parathyroids), adrenals, brain (cerebrum and cerebellum), spinal cord (cervical, mid-thoracic and lumbar), sciatic nerve, eyes, Harderian glands, skin, skeletal muscle, bone and bone marrow (femur and sternum), aorta and gross lesions. In the remaining dose groups, histopathological examination was restricted to the lung, liver, kidneys, spleen, bone marrow and gross lesions.

No treatment-related effects on survival, clinical signs, feed consumption, water consumption or ophthalmological findings were observed throughout the study. Urine analysis parameters at week 13 were similar to control values. Body weights of rats receiving 3000 ppm were significantly reduced; feed consumption efficiency tended to be decreased. Haematological effects were observed at both 1000 and 3000 ppm. At 1000 ppm, these effects included significantly reduced mean corpuscular volume (males and females), significantly reduced mean corpuscular haemoglobin (females) and significantly increased platelet count (females). For animals at 3000 ppm, the haematological effects included significantly lower haemoglobin level, haematocrit value, mean corpuscular haemoglobin, mean corpuscular volume and myeloid/erythroid ratios for both sexes and significantly reduced red blood cell counts for females. Haematological parameters, which were significantly increased, included platelet count, reticulocyte count and erythroblast count for both sexes. Total bilirubin was increased in males at and above 1000 ppm. Liver and kidney weights relative to body weight were significantly higher in 1000 ppm males. In the 3000 ppm group, significant increases were observed in absolute spleen weights and relative liver and spleen weights in both sexes; absolute thyroid weights and relative brain, heart and thyroid weights in females; and relative kidney weights in males. Histopathological examination of tissues from the 3000 ppm group revealed increased incidences of extramedullary haematopoiesis in the spleen of both males and females. Haematopoiesis (erythroid hyperplasia) in the bone marrow and extramedullary haematopoiesis in the liver were seen in one female.

The NOAEL was 300 ppm (equal to 20.7 and 21.7 mg/kg bw per day for males and females, respectively), based on changes in haematological and clinical chemistry parameters at 1000 ppm (equal to 69.7 and 71.5 mg/kg bw per day for males and females, respectively) (Hagiwara, 1989).

Flumioxazin technical (purity 94.8%) was offered to five groups of 16 male and 16 female Sprague-Dawley rats (aged 5 weeks) at a dietary concentration of 0, 30, 300, 1000 or 3000 ppm (equal to 0, 1.9, 19.3, 65.0 and 197 mg/kg bw per day for males and 0, 2.2, 22.4, 72.9 and 218 mg/kg bw per day for females, respectively) for 90 days. After 5 weeks of dosing, six rats of each sex per dose group were used for an interim sacrifice. Achieved concentrations, stability and homogeneity were verified by analysis. The animals were observed at least twice daily for clinical signs, behaviour and mortalities, except on weekends, when the frequency was once per day. Ophthalmological

examination was performed on all animals of both sexes in the control group, on females at 1000 ppm and on both sexes at 3000 ppm in week 13 after application of a mydriatic agent. Body weights, feed consumption and water consumption were determined at weekly intervals for all animals. For assessment of blood biochemical and haematological parameters, blood samples were taken after overnight fasting at sacrifice of both the interim and main study groups. Urine analyses were conducted on samples collected in the week before sacrifice for all animals. All animals were subjected to detailed postmortem gross examination, and all abnormalities were recorded. Organ weights (liver, kidneys, spleen, heart, brain, testes, prostate, adrenals, ovaries, thymus, thyroid including parathyroid, and pituitary) were recorded. Histological evaluation was performed on abdominal skin, adrenals, aorta (thoracic), brain, epididymides, oesophagus, eyeballs (fixed in Davidson's fixative at scheduled sacrifice), femur (bone marrow), Harderian glands, heart, kidneys, large intestine (caecum, colon, rectum), liver, lungs, mammary glands (female only), mesenteric lymph nodes, ovaries, pancreas, parathyroids, pituitary, prostate, salivary gland (submandibular), sciatic nerve, seminal vesicles, skeletal muscle, small intestine (duodenum, jejunum, ileum), spinal cord (mid-thoracic area), spleen, sternum (bone marrow), stomach, submandibular lymph nodes, testes, thymus, thyroid, tongue, trachea, urinary bladder, uterus, vagina and macroscopically abnormal tissue.

One female from the 3000 ppm group was found dead in week 12. This death was considered related to treatment. Pallor of the auricles, eyes and/or all limbs was observed in this animal and in some additional females in this same group beginning at week 8. Blood vessels in fundus oculi were not clearly observed in two females in the 3000 ppm dose group. No treatment-related changes in body weights, water consumption or urine analysis were observed. The primary toxicological findings were related to changes in the haematopoietic system, including decreases in haemoglobin concentration, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and/or haematocrit values for males in the 1000 and 3000 ppm groups and for females at 300 ppm and above (Table 3). Signs of anaemia (decreased erythrocyte count) were also observed in females at 3000 ppm. Increases in reticulocyte and erythroblast ratios for females at 1000 ppm and above and for males at 3000 ppm and increases in leukocyte count or neutrophil count for females at 3000 ppm were also noted. Bone marrow differentiation revealed a decrease in myeloid/erythroid ratios for males at 3000 ppm and for females at 1000 ppm and above. Other indications of toxicity related to the haematopoietic system included dark and enlarged spleens and increased levels of plasma total bilirubin in animals exposed to 3000 ppm and extramedullary haematopoiesis in the spleen of males at 1000 ppm and above and in females at 300 ppm and above. Hypercellularity in the femoral bone marrow and extramedullary haematopoiesis in the liver were also noted in females at and above 1000 ppm. Increases in relative weights of liver, heart, kidney and thyroid were observed for males at and above 1000 ppm. For females, increases in liver weights were observed at 1000 and 3000 ppm, and increases in spleen, heart and kidney weights were observed at 3000 ppm. Liver sinusoidal cells containing brown pigments were reported for one male at the top dose and for females at 1000 ppm and above. In several females at 3000 ppm, centrilobular ballooning degeneration and necrosis of hepatocytes and brown pigment in hepatocytes and canaliculi in the liver, myelofibrosis and osteosis in the femoral bone marrow, brown pigment in tubular epithelial cells and tubular vacuolation in the kidney, cortical cytoplasmic vacuolation in the adrenal, atrophic changes with foam cells in the thymus, sinus histiocytosis in the lymph nodes, thrombosis in the lung and/or erosion in the glandular stomach were seen.

The lowest NOAEL was 30 ppm (equal to 2.2 mg/kg bw per day), based on haematological changes (including anaemia and extramedullary haematopoiesis) in females at 300 ppm (equal to 22.4 mg/kg bw per day), although the effects were marginal. For males, the NOAEL was 300 ppm (equal to 19.3 mg/kg bw per day), based on increases in liver, heart, kidney and thyroid weights at 1000 ppm (equal to 65.0 mg/kg bw per day) (Adachi, 1991).

Table 3. Selected findings in 90-day rat study

	Males					Females				
	0 ppm	**30 ppm**	**300 ppm**	**1 000 ppm**	**3 000 ppm**	**0 ppm**	**30 ppm**	**300 ppm**	**1 000 ppm**	**3 000 ppm**
Haemoglobin (g/dL)	14.6	14.8	14.8	13.9**	13.6**	13.7	14.1	13.8	12.4	8.8**
MCV (fL)	47.6	47.4	47.3	44.6**	42.2**	49.6	49.0	47.9*	42.9**	40.7**
MCH (pg)	17.5	17.6	17.3	16.2**	15.1**	17.9	17.7	17.3*	14.9**	13.4**
MCHC (g/dL)	36.8	37.2	36.5	36.3*	35.9**	36.2	36.1	36.0	34.6**	32.8**
Liver, relative weight (g%)	2.54	2.54	2.62	2.71*	2.88**	2.45	2.54	2.53	2.63*	2.95**
Heart, relative weight (g%)	0.32	0.31	0.34	0.34*	0.34*	0.35	0.34	0.33	0.36	0.49**
Kidney, relative weight (g%)	0.66	0.65	0.67	0.70*	0.71**	0.69	0.71	0.71	0.70	0.83**
Thyroid, relative weight (mg%)	5.0	5.8*	5.7	6.0*	7.0**	6.7	6.9	6.8	7.8	7.5
Spleen, increased extramedullary haematopoiesis	0	0	0	1	6	0	0	1	8	10

MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; MCV: mean cell volume; ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$ (significantly different from the vehicle control group; least significant difference test)
Source: Adachi (1991)

Dogs

Flumioxazin technical (purity 94.8%) was administered in gelatine capsules to three groups of four male and four female Beagle dogs (aged 6 months) at a dose of 10, 100 or 1000 mg/kg bw per day for 13 weeks. A concurrent control group of four males and four females received empty capsules. The animals were observed for clinical signs before administration (which took place at approximately 09:00), every 2–3 hours after administration until about 17:00 on working weekdays and at least 3 times per day on other days. Feed consumption was monitored daily, and body weight measurements were taken at weekly intervals from 2 weeks before commencement of dosing until study termination. Ophthalmological examination was performed on weeks 0, 5 and 11 of treatment. Electrocardiograms were recorded via standard limb lead II on weeks 0, 7 and 13 of administration. Haematology and clinical chemistry were undertaken at weeks 0, 4, 8 and 12. Bone marrow smear and myelocrit measurements were made using bone marrow samples obtained from sternal puncture prior to necropsy. Urine analysis was performed for all animals on weeks 0, 6 and 12. Faeces were collected during a 24-hour period on weeks 0, 6 and 12 and examined for faecal occult blood. To examine liver function, a bromosulfophthalein (BSP) retention test was performed at weeks 0, 5 and 11. To examine kidney function, a *para*-aminohippurate retention test was performed at weeks 0, 7 and 13. At necropsy, all animals were subjected to thorough gross examination and organ weight analysis: brain, pituitary, mandibular glands, thyroids (including parathyroids), thymus, lungs, heart, liver (including gallbladder), pancreas, spleen, kidneys, adrenals, testes, prostate, ovaries and uterus. Histopathology was also performed on these organs, as well as on eyeballs (including optic nerve), parotid glands, tongue, submandibular lymph nodes, larynx, trachea, aorta, urinary bladder (including ureters), oesophagus, stomach, small intestine (duodenum, jejunum, ileum), large intestine (caecum, colon, rectum), mesenteric lymph nodes, spinal cord (mid-thoracic), sciatic nerve, femoral skeletal

muscle, abdominal skin, mammary glands, sternal bone marrow, femoral bone marrow, epididymides and vagina and on gross lesions (the only one being the skin of the left forelimb of a high-dose animal). In addition to light microscopy, electron microscopy was also performed on suitably prepared samples of liver and kidney of all surviving animals at termination.

There were no deaths throughout the treatment period. As for clinical signs, a slightly higher frequency of loose faeces was noted in the 1000 mg/kg bw per day group. No abnormalities in feed consumption, body weight gain or feed efficiency were noted. No compound-related abnormalities in ophthalmology, electrocardiography, urine analysis, faecal examination, myelocrit measurement or myelogram examination of bone marrow were noted. No remarkable changes in haematology parameters were noted. No effects on kidney or liver function were noted. Treatment-related effects included a prolongation of activated partial thromboplastin time in females of the 1000 mg/kg bw per day group at weeks 4 and 12; increased total cholesterol and phospholipid levels and elevated alkaline phosphatase activity in the 100 and 1000 mg/kg bw per day groups (males and females); and increased absolute and relative liver weights, as well as histological changes in the liver, at 1000 mg/kg bw per day. Treatment-related changes, which were mild in severity (i.e. minimal or slight), were seen in the liver of both sexes from the 1000 mg/kg bw per day group. A minimal to slight proliferation of bile ductules was found in one male and two females from the 1000 mg/kg bw per day group. Two males from the 1000 mg/kg bw per day group exhibited minimal increased fibrous tissue around hepatic centrilobular veins. This change was also considered to be a response to hepatic injury due to this compound. Minimal capillary proliferation was seen in the gross lesion of the heart from one female in the 1000 mg/kg bw per day group. This change may be related to valvular telangiectasia, known as a spontaneous lesion. All other lesions were regarded as agonal and/or minor incidental changes of naturally occurring disease unrelated to the test compound. The incidence and relative severity of these changes were comparable among the control and treatment groups. On electron microscopic examination, proliferation and dilatation of the smooth endoplasmic reticulum were observed in the hepatocytes of all males and 3/4 females that received 1000 mg/kg bw per day. These morphological features, in the absence of damage to other organelles, were considered to represent an intracellular adaptive response to an increased metabolic demand presented by the test material, rather than evidence of overt hepatotoxicity per se.

The NOAEL was 10 mg/kg bw per day, based on increases in total cholesterol and phospholipid levels and elevated alkaline phosphatase activity at 100 mg/kg bw per day (Nakano, 1993).

Flumioxazin technical (purity 94.8%) was administered to three groups of four male and four female Beagle dogs (aged 6 or 7 months) by gelatine capsule at a dose level of 10, 100 or 1000 mg/kg bw per day for 52 consecutive weeks. In a concurrent control group, four males and four females received empty capsules. The animals were observed for clinical signs before administration (which took place at approximately 09:30), every 2–3 hours after administration until about 17:00 on working weekdays and at least 3 times per day on other days. Feed consumption was monitored daily, and body weight measurements were taken at weekly intervals from 2 weeks before commencement of dosing until study termination. Ophthalmological examination was performed at weeks 0, 25 and 51 of treatment. Haematology and clinical chemistry were undertaken at weeks 0, 13, 26, 39 and 52. Bone marrow smear and myelocrit measurements were made using bone marrow samples obtained from sternal puncture prior to necropsy. Urine analysis was performed for all animals at weeks 0, 13, 26, 39 and 52. Faeces were collected during a 24-hour period on weeks 0, 13, 26, 39 and 52 and examined for faecal occult blood. To examine liver function, a BSP retention test was performed on weeks 0, 12, 25, 38 and 51. At necropsy, all animals were subjected to thorough gross examination and organ weight analysis: brain, pituitary, mandibular glands, thyroids (including parathyroids), thymus, lungs, heart, liver (including gallbladder), pancreas, spleen, kidneys, adrenals, testes, prostate, ovaries and uterus. Histopathology was also performed on these organs as well as on eyeballs (including optic nerve), parotid glands, tongue, submandibular lymph nodes, larynx, trachea, aorta, urinary bladder (including ureters), oesophagus, stomach, small intestine (duodenum, jejunum,

ileum), large intestine (caecum, colon, rectum), mesenteric lymph nodes, spinal cord (cervical, mid-thoracic, lumbar), sciatic nerve, femoral skeletal muscle, abdominal skin, mammary glands, sternal bone marrow, femoral bone marrow, epididymides, vagina, lacrimal glands, membrana nictitans and abnormal sites. In addition to light microscopy performed on all the tissues listed above, electron microscopy was also performed on suitably prepared samples of liver of control animals and of the 1000 mg/kg bw per day group.

There were no deaths through the treatment period. As for clinical signs, a slightly higher frequency of loose faeces was noticed in the 1000 mg/kg bw per day group. No consistent abnormalities in feed consumption, body weight gain or feed efficiency were observed. No compound-related abnormalities in ophthalmology, urine analysis, faecal examination, myelocrit measurement or myelogram examination of bone marrow were noted, and no remarkable changes in haematology parameters were observed. No effects on liver function were noted in the BSP retention test. In blood biochemistry, increases in total cholesterol and phospholipid levels and an elevation in α_2-globulin ratio were noted in the 1000 mg/kg bw per day group, and an elevation in alkaline phosphatase activity was noted in the 100 and 1000 mg/kg bw per day groups at all intervals tested (i.e. weeks 13, 26, 39 and 52). The absolute and/or relative weights of the liver were elevated in one female animal in the 100 mg/kg bw per day group and one male and three females in the 1000 mg/kg bw per day group. At necropsy, no gross compound-related changes were observed. Light microscopic examination revealed minimal treatment-related changes in the liver in the 1000 mg/kg bw per day group, which consisted of hyperplasia of connective tissue at Glisson's capsule (adjacent to the gallbladder), accompanied by brown pigment and bile duct proliferation. Ultrastructural analysis showed proliferation and dilatation of smooth endoplasmic reticulum in the hepatocytes, which were presumably related to adaptive changes, in all animals from the 1000 mg/kg bw per day group.

The NOAEL in this study was 10 mg/kg bw per day, based on elevated alkaline phosphatase activity and increased liver weights at 100 mg/kg bw per day (Nakano, 1992).

(b) Dermal application

Rats

Groups of five rats of each sex were treated dermally with flumioxazin at a dose level of 100, 300 or 1000 mg/kg bw per day for a period of 21 days. An additional group of five rats of each sex was dermally exposed to the vehicle (corn oil) and served as a concurrent control group. All animals were observed twice daily for mortality and moribundity and once daily for obvious signs of a toxic response and for indications of dermal irritation. Detailed clinical observations were performed once weekly. Body weight measurements were recorded prior to initiation, and body weight and feed consumption measurements were recorded weekly for weeks 1 through 3 and at termination. At termination, blood was collected for evaluation of haematological effects and for serum chemistry analysis. Animals were subsequently killed and subjected to a complete gross necropsy, organ weight evaluations and a histomorphological examination of protocol-specified tissues.

No mortality, clinical signs of toxicity or signs of dermal irritation were observed. Evaluation of mean body weight and feed consumption revealed no differences between controls and treatment groups. Mean haemoglobin and haematocrit values in 1000 mg/kg bw per day females were reduced. Taking into account the low incidence and lack of dose–response relationship, no adverse findings were detected during gross necropsy examination of treated animals. No adverse changes in organ weight were observed; however, only spleen, kidney, liver and testis/epididymides were weighed. Histomorphological evaluation of gross lesions, spleen, kidneys, treated and untreated skin, and liver revealed no compound-related lesions.

The NOAEL of flumioxazin administered dermally to rats was 1000 mg/kg bw per day, the highest dose tested, for the male rats and 300 mg/kg bw per day for the female rats, based on haematological changes at 1000 mg/kg bw per day (Osherhoff, 1991).

(c) Exposure by inhalation

No short-term toxicity studies involving inhalation exposure were submitted.

2.3 Long-term studies of toxicity and carcinogenicity

Mice

Flumioxazin technical (purity 94.8%) was administered to groups of 51 male and 51 female Crj:CD-1 (ICR) mice (5 weeks old) at a dietary concentration of 300, 3000 or 7000 ppm (equal to 31, 315 and 754 mg/kg bw per day for males and 37, 346 and 859 mg/kg bw per day for females, respectively) for 78 weeks; a control group received basal diet only. In addition to these animals (designated "main group"), 15 mice of each sex per group were treated with these dietary dose levels for 52 weeks only, for interim sacrifice. Dose levels, homogeneity and stability in the diet were confirmed by analysis. In-life clinical investigations were performed on interim and terminal kill animals. All animals were observed at least once daily for clinical signs and mortality. Detailed physical examinations were performed once per week. Animals in the main groups were weighed on the first day of treatment, then approximately once per week during the first 13 weeks and approximately every 4 weeks thereafter. Animals in the satellite groups were weighed on the first day of treatment and approximately every 4 weeks thereafter. Each animal was weighed prior to necropsy. Feed consumption was determined for each cage (three animals of each sex per cage) approximately once per week in the first 13 weeks and then at approximately 4-week intervals thereafter in the main group and just every 4 weeks in the satellite group. Haematological examination was performed at necropsy, with blood collected from the abdominal vein. Animals were not fasted prior to collection. A detailed gross necropsy was performed at termination, as it was for those found dead or killed in extremis. The following organ weights were measured on the first 12 animals of each sex per dose in the main groups and 10 animals of each sex per dose in the interim sacrifice group: liver, kidneys, brain, testes, adrenals. When compared with current Organisation for Economic Co-operation and Development (OECD) Test Guideline 453, only a limited selection of organs from a limited number of animals were weighed. The following organs and carcasses from all animals (scheduled sacrifice, found dead or killed in extremis) were preserved, and those from all control and 7000 ppm main group animals and the first 10 animals of each sex per dose in the control and 7000 ppm interim sacrifice groups were subjected to histopathological examination: adrenals, aorta (thoracic), brain (medulla, pons, cerebellar and cerebral cortex), epididymides, oesophagus, eyeballs, femur (bone and marrow), gallbladder, Harderian glands, heart, kidneys, large intestine (caecum, colon and rectum), liver, lungs, lymph nodes (submandibular and mesenteric), mammary glands (female only), nerve (sciatic), ovaries, pancreas, parathyroids, pituitary, prostate, salivary glands (submandibular), seminal vesicles, skeletal muscle (femoral), skin (abdominal), small intestine (duodenum, jejunum and ileum), spinal cord (cervical, thoracic and lumbar), spleen, sternum (bone and marrow), stomach, testes, thymus, thyroid, tongue, trachea, urinary bladder, uterus and vagina, as well as any macroscopically abnormal tissue. The liver, gallbladder, kidney, lungs, brain and macroscopically abnormal tissues from all animals of the 300 and 3000 ppm main groups were also examined histopathologically. The liver, gallbladder, kidney, lungs, brain, ovaries and macroscopically abnormal tissues from the first 10 survivors of each sex per dose in the satellite group were also examined. When compared with current OECD Test Guideline 453, organs from a limited number of animals were examined. One male and one female died accidentally and were therefore excluded from histopathological evaluation. Tissues and organs showing autolysis were excluded from histopathological evaluation.

There were no changes in clinical signs, body weight, feed consumption, haematology, organ weight measurement or gross pathological examination related to administration of the test compound. Excluding accidental deaths, the mortality rates in the main group for the 0, 300, 3000, and 7000 ppm test groups were 27.5%, 28.0%, 29.4% and 25.5% for males and 35.3%, 31.4%, 29.4% and 30.0% for females, respectively. There were no significant differences in mortality rate between the control group and any of the compound-treated groups. No deaths were considered related to treatment with the test compound. Treatment-related increases in the incidence of hypertrophy of centrilobular hepatocytes were observed for males of the 3000 and 7000 ppm groups; in the absence

of further adverse liver-related effects in these animals, this was considered an adaptive finding. Increases in the incidence of diffuse hypertrophy and single-cell necrosis of hepatocytes were observed for females of the 3000 and 7000 ppm groups. No increase in the incidence of any tumours due to test compound administration was reported.

In this study, the NOAEL was 300 ppm (equal to 37 mg/kg bw per day), based on non-neoplastic changes in the liver in females at 3000 ppm (equal to 346 mg/kg bw per day). For males, the NOAEL was 7000 ppm (equal to 754 mg/kg bw per day), the highest dose tested (Seki, 1993a).

Rats

Flumioxazin technical (purity 94.8%) was administered to groups of 50 male and 50 female Sprague-Dawley rats (6 weeks old) at a dietary concentration of 50, 500 or 1000 ppm (equal to 1.8, 18 and 36.5 mg/kg bw per day for males and 2.2, 21.8 and 43.6 mg/kg bw per day for females, respectively) for 24 months; a control group received basal diet only. In addition to these animals (designated "main group"), 24 rats of each sex per group were treated for up to either 12 months (10 of the 24 animals) or 18 months (remainder of the 24 animals), for interim sacrifice. Dose levels, homogeneity and stability in the diet were confirmed by analysis. In-life clinical investigations were performed on interim and terminal kill animals. All animals were observed at least once daily for clinical signs and mortality. Detailed physical examinations were performed once per week. Animals in the main groups were weighed on the first day of treatment, then approximately once per week during the first 14 weeks and approximately every 4 weeks thereafter. Animals in the satellite groups were weighed on the first day of treatment and approximately every 4 weeks thereafter. Each animal was weighed prior to necropsy (including any decedents), except those necropsied at 18 months. Feed consumption was determined for each cage (animals housed three of each sex per cage) approximately once per week in the first 14 weeks and then at approximately 4-week intervals thereafter in the main groups and every 4 weeks in the satellite groups. Water consumption was measured for animals that underwent urine analysis at weeks 26–27, 50–51, 78–79 or 102–103. Urine analysis was performed from weeks 24 to 26, 51 to 52 and 76 to 77 in animals of the satellite groups and at week 103 in the main group. Ophthalmological examination was performed after application of a mydriatic agent in all animals of the control and 1000 ppm groups in the main group before the start of treatment and at week 53. At week 104, the examination was performed in all males of the control and 1000 ppm groups and all females of each dose group in the main group. Blood samples for haematology and clinical chemistry were taken from alternating sets of 10 animals of each sex per dose of the satellite group from the orbital sinus at weeks 14 and 27 after overnight fasting or from the abdominal aorta at weeks 53 and 79 under anaesthesia prior to necropsy. Blood samples for haematology and clinical chemistry were collected from the abdominal aorta under anaesthesia at necropsy (week 105) of the first 12 animals of each sex per dose of the main group. Serum samples were collected from the remaining animals after overnight fasting. Blood samples were also taken from decedents. Femur bone marrow smears were prepared at weeks 53 and 105. On necropsy, as well as a thorough gross examination, organ weight analyses were performed on the first 10 animals of each sex per dose at week 53 and the first 12 animals of each sex per dose at week 105 for the following organs: liver, kidneys, spleen, heart, brain, testes, adrenals and ovaries. When compared with current OECD Test Guideline 453, only a limited selection of organs in a limited number of animals were weighed in week 53. The organs listed below as well as carcasses collected from the first 10 animals of each sex per dose of the satellite group at week 53, from all survivors at week 105 and from all decedents or animals killed in extremis were prepared for histology: abdominal skin, adrenals, aorta (thoracic), brain (medulla/pons, cerebellar cortex and cerebral cortex), epididymides, oesophagus, eyeball, femur (bone marrow), Harderian glands, heart, kidneys, large intestine (caecum, colon and rectum), liver, lungs, mammary glands (female only), mesenteric lymph nodes, ovaries, pancreas, parathyroids, pituitary, prostate, salivary gland (submandibular), sciatic nerve, seminal vesicles, skeletal muscle, small intestine (duodenum, jejunum and ileum), spinal cord (cervical, thoracic and lumbar), spleen, sternum (bone marrow), stomach, submandibular lymph nodes, testes, thymus, thyroid, tongue, trachea, urinary bladder, uterus and vagina, as well as macroscopically abnormal tissues. Slides were examined for all scheduled sacrifices in the control group and 1000

ppm group at weeks 53 and 105 and for all decedents and those killed in extremis (except those decedents or humane kills taking place between weeks 53 and 79). Only the liver, spleen, kidneys, lungs and macroscopically abnormal tissues of animals in the 50 and 500 ppm groups were examined microscopically.

No treatment-related changes were observed in regard to body weight, feed consumption, water consumption, urine analysis, ophthalmology, blood biochemistry, gross pathological examination or organ weight. Mortality was not affected by treatment. In the animals that were killed owing to extreme deterioration of their physical condition, no relationship to treatment was found. Haematological examination revealed a decrease in haemoglobin concentrations in both sexes of the 500 and 1000 ppm groups, decreases in mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration in males of the 1000 ppm group and in females of the 500 and 1000 ppm groups, a decrease in haematocrit values in females of the 500 and 1000 ppm groups, an increase in erythrocyte counts in females of the 1000 ppm group, an increase in erythroblast counts in both sexes of the 1000 ppm group and an increase in reticulocyte incidence in females of the 500 and 1000 ppm groups. Bone marrow differentiation revealed a low myeloid to erythroid ratio in females of the 1000 ppm group at week 53. Histopathological examination revealed an increase in the incidence and severity of extramedullary haematopoiesis in the spleen of females in the 500 and 1000 ppm groups at week 53 and of males in the 500 and 1000 ppm groups at week 105 (Table 4). An increase in the incidence and severity of chronic nephropathy in the kidney was observed in males of the 500 and 1000 ppm groups at week 105 (Table 4). No treatment-related increase in tumour incidence during the 2-year treatment was reported.

Under the conditions of this study, the NOAEL was 50 ppm (equal to 1.8 and 2.2 mg/kg bw per day for males and females, respectively), based on anaemia observed at 500 ppm (equal to 18 and 21.8 mg/kg bw per day for males and females, respectively) (Seki, 1993b).

Table 4. Selected findings in 2-year rat study

	Incidence of findings							
	Males				**Females**			
	0 ppm	**50 ppm**	**500 ppm**	**1 000 ppm**	**0 ppm**	**50 ppm**	**500 ppm**	**1 000 ppm**
Number of animals evaluated for spleen findings (week 53, satellite group)	10	10	10	10	10	10	10	10
Spleen, increased extramedullary haematopoiesis (week 53) Slight-mild-moderate	2-1-0	4-0-0	5-1-0	1-1-0	5-0-1	6-0-0	3-4-0	0*-5-3
Number of animals evaluated for spleen findings (week 105, main group)	50	48	49	49	50	50	50	49
Spleen, increased extramedullary haematopoiesis (week 105) Slight-mild-moderate-severe	4-0-0-0	5-0-0-0	11*-0-2-0	12*-1-0-0	9-8-0-2	14-2-3-0	16-8-2-0	14-4-5-0
Number of animals evaluated for kidney findings (week 105, main group)	50	50	50	50	50	50	50	50
Kidney, chronic nephropathy (week 105) Slight-mild-moderate-severe	8-13-1-2	7-8-8-0	12-15-11-1	17-11-5-0	3-3-0-0	3-4-0-0	6-6-2-0	6-2-0-0

ppm: parts per million; *: $P \leq 0.05$ (Mann-Whitney's *U*-test)
Source: Seki (1993b)

Table 5. Results of genotoxicity studies performed with flumioxazin

Type of study	Organism/cells	Dose range tested	Batch purity (%)	Result	Reference
In vitro gene mutation	Ames: *Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538, and *Escherichia coli* WP2 uvrA	±S9: 0, 50, 100, 200, 500, 1 000, 2 000 µg/plate	94.8	±S9: Negative	Kogiso (1989)
	Chinese hamster V79 *hprt* forward mutation	±S9: 0, 14.1, 28.1, 56.3, 112.5, 225 µg/mL[a] −S9: 0, 14.1, 28.1, 56.3, 112.5, 225 µg/mL[b] +S9: 0, 28.1, 56.3, 112.5, 337.5, 450 µg/mL[b]	99.6	±S9: Negative	Wollny (2011)
In vitro chromosomal aberration assay	Chinese hamster ovary K1	±S9: 0, 10.6, 35.4, 70.9 µg/mL[c]	98.2	−S9: Negative +S9: Positive	Kogiso (1988)
In vivo micronucleus assay	Mouse bone marrow micronucleus (intraperitoneal injection)	0, 300, 1 000, 5 000 mg/kg bw	98.4	Negative	Hara & Kogiso (1988)
In vivo chromosomal aberration assay	Rat bone marrow chromosomal aberration (oral administration)	Male: 0, 1 250, 2 500, 5 000 mg/kg bw Female: 0, 1 250, 2 500, 4 400, 5 000 mg/kg bw	94.8	Negative	Hara (1990)
In vivo unscheduled DNA synthesis assay	Rat hepatocytes (oral administration)	1 250, 2 500, 5 000 mg/kg bw	94.8	Negative	Kogiso (1990)

bw: body weight; DNA: deoxyribonucleic acid; hprt: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] First mutation assay.

[b] Second mutation assay.

[c] Concentrations equivalent to 0, 0.03, 0.1 and 0.2 mmol/L, respectively.

2.4 Genotoxicity

Flumioxazin was negative in a bacterial mutation assay and a mammalian cell mutation assay in both the presence and absence of metabolic activation and did not induce unscheduled DNA synthesis in rat hepatocytes in an in vivo assay (Table 5).

Treatment of Chinese hamster ovary cells with flumioxazin resulted in an increase in chromosomal aberrations in the presence, but not in the absence, of metabolic activation. However, there was no evidence of clastogenic activity in either an in vivo chromosomal aberration test in rat bone marrow cells or an in vivo mouse micronucleus test in bone marrow cells, which are the appropriate follow-up studies.

(a) In vitro

Flumioxazin technical (purity 94.8%) was tested for its mutagenic potential on *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 and *Escherichia coli* strain WP2 uvrA in a preincubation assay conducted according to the Ames test procedure at concentrations

up to 2000 µg/plate (where precipitation of the test article was observed) in the absence or presence of S9 mix. Two experiments were performed, although concentrations were plated only in duplicate in each experiment. Hence, the study design was less powerful than a study conducted according to current test guidelines (triplicate plating is recommended). The top dose was limited by the presence of precipitate above 2000 µg/plate, which made it difficult to count the colonies. Flumioxazin was not cytotoxic up to 5000 µg/plate in a range-finding experiment. A slight increase of revertants (in the presence of S9 mix) was observed, but the incidence did not reach a factor of 2 when compared with concurrent controls. In summary, flumioxazin did not display mutagenic potential under the conditions of this test. Satisfactory positive control results were obtained (Kogiso, 1989).

Flumioxazin technical (purity 99.6%) was tested for its mutagenic potential at the *hprt* locus in V79 cells at concentrations up to 450 µg/mL in the presence of S9 mix and up to 225 µg/mL without metabolic activation, in an OECD Test Guideline 476–compliant study. The assay was performed in two independent experiments using two parallel cultures each. The first main experiment was performed with and without liver microsomal activation and a treatment period of 4 hours. The second experiment was performed with a treatment time of 4 hours with metabolic activation and 24 hours without metabolic activation. The maximum concentration of the pre-experiment (3600 µg/mL) was equal to a concentration of approximately 10 mmol/L (the maximum recommended dose, in accordance with the guideline). No cytotoxic effects were observed under the experimental conditions. The concentration ranges of the main experiments were limited by the solubility of the test article in aqueous medium. No substantial and reproducible dose-dependent increase in mutation frequency was observed in either main experiment. The positive controls (ethyl methanesulfonate in the absence of S9 and 7,12-dimethylbenz(*a*)anthracene in the presence of S9) induced marked increases in mutant frequency, thereby demonstrating the sensitivity of the test system. It is concluded that flumioxazin did not induce mutation at the *hprt* locus of V79 Chinese hamster cells when tested under the conditions employed in this study (Wollny, 2011).

Flumioxazin (purity 98.2%) was tested for its clastogenic potential in Chinese hamster ovary K1 cells at concentrations up to 70.9 µg/mL in the presence and absence of S9 mix. The study was conducted according to USEPA FIFRA Guideline 84-2, but was not compliant with GLP (no authorized protocol, study director not identified in report, quality assurance unit not involved in any part of the study). Cells (10^5/mL) were seeded 24 hours before treatment. In the absence of S9, cells were exposed to flumioxazin for 24 hours. The exposure time in the presence of S9 was 6 hours, after which the cells were washed and cultured in fresh media for a further 18 hours. Appropriate positive controls were used. One hundred metaphases from each dose were examined, and the frequencies of cells with structural aberrations (including and excluding gaps) were recorded. The study design was less powerful than required for a study conducted according to the current test guideline (single cultures only, 200 metaphases scored instead of 300, no short-term treatment). Cell survival was calculated by counting the number of cells 48 hours after exposure in the absence of S9 and 42 hours after cultivation in fresh medium in the presence of S9. Some evidence of clastogenic activity was observed in the presence of metabolic activation (Table 6). In the absence of metabolic activation, no evidence of clastogenicity was observed up to a maximum concentration of 70.9 µg/mL (0.2 mmol/L), a concentration that was limited by cytotoxicity (Kogiso, 1988).

(b) In vivo

Flumioxazin (purity 98.4%) was tested for its in vivo clastogenic potential in the mouse micronucleus assay. Groups of four male ICR mice were administered flumioxazin by intraperitoneal injection at a dose of 0, 300, 1000 or 5000 mg/kg bw. The vehicle was corn oil. The total dosing volume was 20 mL/kg bw. The positive control material was cyclophosphamide. The study was conducted according to USEPA FIFRA Guideline 84-2 but was not GLP compliant (study director not identified in report, quality assurance unit not involved in any part of the study). The bone marrow harvest time was 24 hours after intraperitoneal injection. The incidence of micronucleated polychromatic erythrocytes in 1000 polychromatic erythrocytes and the ratio of polychromatic erythrocytes to whole erythrocytes (polychromatic erythrocytes + normochromatic erythrocytes) were

Table 6. Results of a non-GLP mammalian cell in vitro clastogenicity assay performed with flumioxazin

Chemical	Dose (mol/L)	No. of cells examined	Cells with aberrations (%)[a]		Chromosomal aberrations[b]							Survival (%)
			TAG	TA	ctg	ctb	cte	csb	cse	frg	MT	
−S9												
DMSO[c]	–	100	1	1	0	1	0	0	0	0	0	100.0
Flumioxazin	3×10^{-5}	100	1	1	0	0	1	0	0	0	0	101.3
	1×10^{-4}	100	1	0	1	0	0	0	0	0	0	73.2
	2×10^{-4} [d]	100	1	1	0	0	0	1	0	0	0	44.5
	5×10^{-4} [d]	– [e]	–	–	–	–	–	–	–	–	–	22.7
MMC	1.5×10^{-7}	100	40**	37**	6	13	34	1	1	0	0	66.8
+S9												
DMSO[c]	–	100	9	6	3	4	4	0	0	0	0	100.0
Flumioxazin	3×10^{-5}	100	13	9	4	5	3	0	0	0	0	96.5
	1×10^{-4} [d]	100	34**	26**	13	13	23	0	1	0	0	90.8
	2×10^{-4} [d]	100	56**	53**	28	47	41	0	0	1	0	59.4
	5×10^{-4} [d]	– [e]	–	–	–	–	–	–	–	–	–	21.5
BP	2.5×10^{-5}	100	48**	45**	6	12	58	1	1	0	0	94.0

BP: benzo(*a*)pyrene; DMSO: dimethyl sulfoxide; MMC: mitomycin C; *: $P < 0.05$; **: $P < 0.01$ (significantly different from the vehicle control group; chi-squared test)

[a] TAG: aberrations including gaps; TA: aberrations excluding gaps.

[b] ctg: chromatid gaps; ctb: chromatid breaks; cte: chromatid exchanges; csb: chromosome breaks; cse: chromosome exchanges (including chromosome rings and dicentric chromosomes); frg: fragmentation; MT: cells with multiple aberrations (more than 10).

[c] Final concentration in the medium was 0.5%.

[d] A small amount of precipitation was observed in the medium.

[e] No metaphase.

determined for each animal. The study design was less powerful than required for a study conducted according to the current test guideline (only 1000 polychromatic erythrocytes evaluated instead of 2000, no second evaluation after 48 hours). In this study, there was no increase in the incidence of micronucleated polychromatic erythrocytes following sampling at 24 hours post-dosing of male mice when tested up to a dose of 5000 mg/kg bw. The ratio of polychromatic erythrocytes to whole erythrocytes was reduced (Hara & Kogiso, 1988).

Flumioxazin technical (purity 94.8%) was tested for its clastogenic potential in the in vivo chromosomal aberration assay in rat bone marrow. Groups of five male and five female Sprague-Dawley rats were administered flumioxazin by oral gavage at a dose of 1250, 2500 or 5000 mg/kg bw. The vehicle was corn oil. The total dosing volume was 20 mL/kg bw. The positive control material was cyclophosphamide. The bone marrow harvest time was 24 hours after oral administration. A time course experiment was also performed in which groups of five males were administered 5000 mg/kg bw and groups of five females received 4400 mg/kg bw (the actual dose for

female rats was due to an error and should have been 5000 mg/kg bw) and sacrificed at 6, 12, 24 or 48 hours after administration. Rats received an intraperitoneal dose of colchicine in saline 2 hours prior to sacrifice. After sacrifice, bone marrow cells were collected from the femur, and slides were prepared. Slides were examined blind to treatment. One thousand erythrocytes were counted to determine the ratio of polychromatic erythrocytes to whole erythrocytes. At least 500 cells per animal were analysed to determine the mitotic index. Fifty metaphases with $2n \pm 1$ chromosomes were analysed from each animal. The study design was less powerful than required for a study conducted according to the current test guideline (100 metaphases scored instead of 200). There were no signs of toxicity on administration of test substance, although body weight gain was suppressed in treated animals at 24 hours after treatment. Suppression of the mitotic index was not seen in males, but a decrease in mitotic index was observed at 6 and 24 hours in females, followed by an increase at 48 hours. A decrease in the ratio of polychromatic erythrocytes to normochromatic erythrocytes was seen at 12, 24 and 48 hours after treatment at 5000 mg/kg bw in males and at 24 and 48 hours after treatment at 4400 mg/kg bw in females, demonstrating exposure of the bone marrow in this assay. Under the conditions of this study, there was no increase in the incidence of chromosomal aberrations in the bone marrow cells following sampling of male and female rats at 6, 12, 24 and 48 hours post-dosing when tested up to a dose of 5000 mg/kg bw (Hara, 1990).

Flumioxazin technical (purity 94.8%) was tested for its DNA damaging potential in an in vivo unscheduled DNA synthesis assay. The study was conducted according to USEPA FIFRA Guideline 84-4. Groups of three male Sprague-Dawley rats were gavaged with 5000 mg/kg bw per day (the vehicle was corn oil, the dosing volume was 20 mL/kg bw), and the occurrence of unscheduled DNA synthesis in hepatocytes was examined 3, 12 and 24 hours after administration (time course study). Groups of three males were also dosed with 1250, 2500 or 5000 mg/kg bw and examined for the occurrence of unscheduled DNA synthesis in hepatocytes 12 hours after administration (dose–response study). At the allotted time, rats were sacrificed, and the hepatocytes were isolated. The hepatocytes were cultured in a medium containing ^{3}H-labelled thymidine for 4 hours. To determine the occurrence of unscheduled DNA synthesis, incorporation of the radiolabelled thymidine was detected by autoradiography and evaluated in 50 cells per animal. The study design was less powerful than required for a study conducted according to the current test guideline, which requires 100 cells per animal from at least two slides. No increase in the incidence of unscheduled DNA synthesis in hepatocytes was observed in this study following sampling of males at 3, 12 and 24 hours post-dosing when tested up to a dose of 5000 mg/kg bw (Kogiso, 1990).

2.5 Reproductive and developmental toxicity

(a) One- and two-generation studies

In a dose range–finding one-generation study for a two-generation reproductive toxicity study, Sprague-Dawley rats were fed diets containing 0, 100, 500, 1000 or 5000 ppm flumioxazin technical (purity 94.8%; during premating period, equal to 0, 5.6, 27.5, 54.9 and 263.9 mg/kg bw per day for males and 0, 6.2, 30.9, 61.4 and 306.0 mg/kg bw per day for females, respectively). The male rats were given diets containing the test material for a total of 63 days, beginning 35 days prior to cohabitation. The female rats were provided the diet containing the test material for 35 days prior to cohabitation, through cohabitation, gestation and delivery, and for 21 days postpartum.

No male rats died; however, three females from the 5000 ppm group died during gestation. Other signs of toxicity in both males and females, including clinical signs, gross lesions (e.g. enlarged spleen) and reduced body weights and/or feed consumption, were observed at doses of 100 ppm and above. Females were most significantly affected during gestation at doses of 500 ppm and above. Concentrations as high as 5000 ppm did not affect mating performance or fertility; however, no live litters were produced in the 500, 1000 or 5000 ppm groups (total resorptions), precluding evaluation of the lactation period for these groups. All pregnant dams in the control and 100 ppm groups delivered litters. The 100 ppm concentration did not affect the averages for the duration of gestation, litter size, pup sex ratio or pup body weight. Similarly, this concentration did not affect the viability or lactation indices or the external or necropsy observations for the pups (Hoberman, 1991a).

Based on the results of this first study, a second pilot study was initiated using 500 ppm as the highest test concentration. In the second study, rats were exposed for a similar period of time to a dose of 0, 100, 200, 300, 400 or 500 ppm flumioxazin (purity 94.8%) in the diet (during premating period, equal to 0, 5.6, 11.3, 17.1, 22.2 and 28.4 mg/kg bw per day for males and 0, 6.9, 13.6, 19.7, 27.1 and 32.3 mg/kg bw per day for females, respectively). The male rats were given diets containing the test material for a total of 72 days, beginning 28 days prior to cohabitation. The female rats were provided with diet containing the test material for 28 days prior to cohabitation, throughout cohabitation, gestation and delivery, and for 21 days postpartum.

Decreased body weight gains and feed consumption values occurred in the 300, 400 and 500 ppm group female rats, and decreases in the gestation indices, litter sizes, average number of pups per litter and pup survival tended to occur in the 300 and 400 ppm groups. No live litters were produced in the 500 ppm group, and the number of implantation sites was reduced in this dose group. Gestation length tended to be increased in the 400 ppm dose group. Based on these data, concentrations as high as 300 ppm were recommended for the multigeneration rat reproductive toxicity study (Hoberman, 1991b).

A two-generation (one litter per generation) reproductive toxicity study was conducted in which flumioxazin technical (purity 94.8%) was administered to Sprague-Dawley rats at dietary levels of 0, 50, 100, 200 and 300 ppm (for test substance intake, see Table 7). F_0 rats (30 of each sex) were exposed continuously for approximately 12 weeks prior to cohabitation and throughout breeding, gestation and lactation, until scheduled sacrifice. The second generation (F_1) (adjusted to 30 rats of each sex) was exposed for a minimum of 86 days before cohabitation and throughout breeding, gestation and lactation, until scheduled sacrifice. Body weights and feed consumption were determined weekly throughout the study until termination for F_0 and F_1 males. For F_0 and F_1 females, body weights and feed consumption were recorded weekly during the premating periods, on days 0, 6, 10, 15, 20 and 25 of gestation, and on days 1, 4, 7, 10, 14, 16, 18 and 21 of lactation. Feed consumption during lactation for the F_0 and F_1 generations was recorded up to postpartum day 14. Estrous cycling of F_0 and F_1 animals was evaluated for 1 week before cohabitation. Rats were cohabited for mating until the presence of sperm or a copulatory plug was observed (designated day 0 of gestation) or until completion of the 21-day cohabitation period. The day of parturition was defined as day 1 of lactation. F_1 and F_2 pups were evaluated daily for external abnormalities, and individual pup body weights were recorded on days 1, 4, 7, 14 and 21 of lactation. Where appropriate, litters were culled (random pup selection) to four male and four female pups after weighing on day 4 of lactation. Culled pups were necropsied. At least one F_1 generation pup of each sex (whenever possible) per litter was selected at weaning for continuation as the F_1 generation (30 pups of each sex). F_1 pups not selected for continued observation were sacrificed and necropsied on postpartum day 21. Organ weights and histopathological evaluation were limited to reproductive organs, mammary gland (females only), pituitary and other organs with gross findings.

In F_0 males, there were no adverse effects on clinical signs, body weight or body weight gain (Table 8), feed intake or findings during necropsy (including histopathological evaluation) in any dose group. No effects on F_0 male mating performance or fertility at concentrations up to and including the highest concentration tested (300 ppm) were reported. The average duration of cohabitation, the number of male rats mating and the number of male rats siring litters were similar among the groups.

In F_1 males, clinical observations, significantly reduced terminal body weight, reduced absolute weights of the left and right epididymides, left and right testes, and brain, reduced weight of the right epididymis relative to brain weight, and a tendency to reduced absolute weight of the prostate were observed at 300 ppm in male rats. There was also one male death at 300 ppm on day 3 post-weaning, which was attributed to treatment, because the rat shared treatment-related signs with two other rats in the same group (appearing pale). The rat that died also appeared dehydrated and had urine-stained abdominal fur and remarkably reduced body weight before death. Necropsy showed black ingesta in the stomach and intestines. Histopathology showed hypoplasia of the coagulating gland, epididymides, prostate, seminal vesicles and testes. The 300 ppm group also tended to have

Table 7. Mean test substance intake in reproductive toxicity study in rats

	Mean test substance intake (mg/kg bw per day)							
	50 ppm		100 ppm		200 ppm		300 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
F_0 generation								
Premating (days 1–83)	3.2	3.8	6.3	7.6	12.7	15.1	18.9	22.7
Gestation	–	3.3	–	6.7	–	13.0	–	20.8
Lactation	–	5.9	–	12.3	–	24.1	–	29.6
F_1 generation								
Premating (days 1–99)	3.7	4.3	7.5	8.5	15.0	17.2	22.4	25.6
Gestation	–	3.3	–	6.5	–	13.3	–	20.2
Lactation	–	5.7	–	12.0	–	23.0	–	30.5

bw: body weight; ppm: parts per million
Source: Hoberman (1992)

Table 8. Body weight gain in F_0 and F_1 generations during selected study periods

	Body weight gain (g)									
	0 ppm		50 ppm		100 ppm		200 ppm		300 ppm	
	M	F	M	F	M	F	M	F	M	F
F_0 generation										
Days 1–83 premating	393.4	169.3	408.8	175.2	384.9	166.5	385.9	172.1	403.0	169.4
Day 1–termination	489.3	–	517.4	–	487.0	–	486.0	–	498.6	–
Days 0–20 gestation	–	137.6	–	140.6	–	137.2	–	133.0	–	95.5**
Days 1–4 lactation	–	2.5	–	1.7	–	1.4	–	−1.8	–	−10.8
Days 1–21 lactation	–	−3.6	–	−0.8	–	3.7	–	−5.1	–	4.7
F_1 generation										
Days 1–99 premating	544.8	290.9	584.4	300.7	574.1	291.0	553.2	288.7	514.0*	274.7
Day 1–termination	631.3	–	669.4	–	663.9	–	629.6	–	591.4	–
Days 0–20 gestation	–	145.8	–	123.8*	–	139.8	–	125.7*	–	104.8**
Days 1–4 lactation	–	−2.3	–	−1.9	–	4.1	–	−24.5	–	−6.3
Days 1–21 lactation	–	6.3	–	1.7	–	16.0	–	−7.5	–	3.4

F: females; M: males; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (significantly different from the vehicle control group; Dunnett's test, Dunn's test or Fisher's test)
Source: Hoberman (1992)

reduced numbers of rats that mated and a reduced proportion of pregnant rats in cohabitation. Necropsy of four rats in the 300 ppm group that did not sire a litter revealed changes in one or both testes and epididymides (small, purple or flaccid testes and small, purple or thread-like epididymides; one of these rats also had a small prostate and a small right seminal vesicle).

In F_0 generation female rats, a concentration of 300 ppm produced statistically significant effects on body weight gains and body weights during gestation (Table 8) and on absolute or relative feed consumption values during gestation (increased values) and lactation (decreased values). The averages for the duration of cohabitation, the number of female rats that mated and the number of mated female rats that were pregnant were similar among the groups. At 300 ppm, significantly increased numbers of F_0 generation rats with red vaginal substance present during gestation were observed; this observation was assumed to be related to resorption of litters. Five F_0 generation female rats in the 300 ppm group were pregnant but did not deliver until sacrificed on day 25 of gestation. Organ weights were not affected in F_0 generation female rats.

In the F_1 generation, a statistically significantly increased number of deaths and clinical, necropsy and histopathological observations were seen in adult animals at 300 ppm. During necropsy of deceased females, livers were described as yellow and revealed bile stasis and/or necrosis upon histopathological examination. Dose-related reductions (sometimes statistically significant) in body weight gains (Table 8), body weights, and absolute and relative feed consumption were observed in F_1 females from the 300 ppm group. Lower body weight gain in the 200 ppm dose group was restricted to the late gestational period. The F_1 generation female rats in the 100 and 200 ppm groups tended to have transient reductions in feed consumption values on days 4–7 of lactation; however, these observations did not persist. The averages for the duration of cohabitation, the number of female rats that mated and the number of mated female rats that were pregnant were similar among the groups.

The gestation index in F_0 and F_1 generation female rats was reduced at 300 ppm (Table 9) and was statistically significantly reduced in the F_1 females, which was related to the fetal deaths in utero. This concentration also significantly increased the number of dams in the F_0 generation with all F_1 pups dying on days 1–21 postpartum and the number of dams in the F_0 generation that did not deliver a litter.

The 200 ppm concentration of the test substance significantly reduced F_1 generation pup body weights on day 1 postpartum. The 100 ppm concentration and above tended to increase the number of dying pups per litter on days 1 and 4 (preculling) postpartum and reduced viability index in the F_1 generation. In the 200 ppm dose group, F_1 pup weight per litter was reduced. The 300 ppm concentration of the test substance tended to reduce or significantly reduced the number of pups delivered, number of liveborn pups, viability index, litter size at weighing, average pup weight per litter and surviving pups per litter in both generations; additionally, clinical and necropsy observations related to pup morbidity (reduced live births and viability) were reported in both generations (Table 9). Offspring in the 300 ppm F_1 group appeared weak upon clinical observation.

The NOAEL for parental toxicity was 200 ppm (equal to 12.7 mg/kg bw per day), based on clinical signs of toxicity and reductions in body weight, body weight gain, feed consumption and organ weights.

The NOAEL for reproductive toxicity was 200 ppm (equal to 12.7 mg/kg bw per day), based on reduced gestation index in both the F_0 and F_1 generations and an increased number of F_0 dams that did not deliver a litter.

The NOAEL for offspring toxicity was 50 ppm (equal to 3.2 mg/kg bw per day), based on increased postnatal pup mortality in the F_1 generation (Hoberman, 1992).

(b) Developmental toxicity

Rats

A non-GLP pilot dose range–finding study was conducted in pregnant rats to determine appropriate doses for the oral teratology study. Flumioxazin (purity 98.2%) was suspended in a 0.5% aqueous solution of carboxymethyl cellulose and administered by gavage at a dose of 0, 30, 100, 200 or 500 mg/kg bw per day to six pregnant rats per dose on days 6 through 15 of gestation. Current test guidelines require daily administration from implantation to the day prior to scheduled caesarean

Table 9. Mating and gestation parameters in the rat dietary two-generation reproductive toxicity study with flumioxazin

	F_0 generation					F_1 generation				
	0 ppm	**50 ppm**	**100 ppm**	**200 ppm**	**300 ppm**	**0 ppm**	**50 ppm**	**100 ppm**	**200 ppm**	**300 ppm**
Pregnancy rate, *N*/*N* (%) (fertility index)	23/28 (82.1)	22/28 (78.6)	26/28 (92.8)	28/30 (93.3)	21/27 (77.8)	23/28 (82.1)	21/29 (72.4)	19/26 (73.1)	18/26 (69.2)	18/22 (81.8)
Mated within days 1–7, 1st male, *N* (%)	24 (85.7)	22 (81.5)	24 (88.9)	27 (90.0)	23 (92.0)	23 (85.2)	23 (82.1)	22 (88.0)	19 (76.0)	15 (75.0)
Mated days 8–14, 1st male, *N* (%)	2 (7.1)	2 (7.4)	3 (11.1)	0 (0.0)	1 (4.0)	3 (11.1)	0 (0.0)	2 (8.0)	2 (8.0)	1 (5.0)
Mated with 2nd male, days 15–21, *N* (%)	2 (7.1)	3 (11.1)	0 (0.0)	3 (10.0)	1 (4.0)	1 (3.7)	5 (17.8)	1 (4.0)	4 (16.0)	4 (20.0)
Pregnant *N*/*N* cohabited (%)	23/30 (76.7)	22/30 (73.3)	26/30 (86.7)	28/30 (93.3)	21/30 (70.0)	23/30 (76.7)	21/30 (70.0)	19/30 (63.3)	18/30 (60.0)	18/26 (69.2)
Gestation index: Live litter delivered / pregnant rats, *N*/*N* (%)	23/23 (100)	22/22 (100)	25/26 (96.2)[a]	28/28 (100.0)	16/21[##] (76.2)	23/23 (100.0)	20/21 (95.2)	19/19 (100.0)	18/18 (100.0)	16/18 (88.9)
Dams that did not deliver a live litter, *N* (%)	0	0	1 (3.8)[a]	0	5 (23.8)[##]	0	1 (4.8)	0	0	2 (11.1)
Duration of gestation (days)	23.0	22.8	23.1	22.8	23.2	22.9	23.0	22.8	22.9	23.0
Day of delivery[b,c]										
Day 21	3	3	3	4	1	2	1	3	3	0
Day 22	17	18	18	24	10	16	12	10	8	10
Day 23	3	0	3	0	2	4	5	5	6	4
Day 24	0	0	1	0	1	0	1	0	0	0
Implantation sites per delivered litter	16.1	15.9	15.5	16.5	15.0	17.6	15.4	17.3	15.4	16.2
Total implantation sites[d]	16.1	15.9	15.5	16.5	15.4	17.6	15.1	17.3	15.3	16.3
Dams with stillborn pups, *N* (%)	3 (13.0)	3 (13.6)	3 (11.5)	2 (7.1)	1 (4.8)	2 (8.7)	3 (15.0)	2 (10.5)	4 (22.2)	2 (12.5)
Dams with all stillborn pups, *N* (%)	0	0	1 (3.8)	0	0	0	0	0	0	0
Dams delivering one or more liveborn pups, *N* (%)	23 (100.0)	22 (100.0)	25 (96.2)[a]	28 (100.0)	16 (100.0)	23 (100.0)	20 (100.0)	19 (100.0)	18 (100.0)	16 (100.0)

	F_0 generation					F_1 generation				
	0 ppm	**50 ppm**	**100 ppm**	**200 ppm**	**300 ppm**	**0 ppm**	**50 ppm**	**100 ppm**	**200 ppm**	**300 ppm**
Dams with all liveborn pups dying days 1–21 postpartum, *N* (%)	0	0	0	0	2 (12.5)[##]	0	0	0	0	0[e]
Pups delivered *N*	322	324	360	409	115	363	271	298	242	153
Pups delivered/ dam	14.4	15.2	13.8	14.6	7.2[##]	15.8	13.6	15.7	13.4	9.6[##]
Pups dying[j]										
Day 1	2	2	3	5	5[##]	0	0[f]	2	1	5[g##]
Days 2–4	0	3	10[##]	15[##]	9[##]	5	6[f]	6	7	26[g##]
Days 5–7	1	0	0	0	1	2	1	0	0	1
Days 8–14	0	0	0	0	0	2	0	0	0	0
Viability index[h] *N/N* (%)	324/326 (99.4)	321/326 (98.5)	341/354[#] (96.3)	387/407[##] (95.1)	98/112[##] (87.5)	353/358 (98.6)	245/251[f] (97.6)	288/296 (97.3)	222/230 (96.5)	118/149[g] (79.2[##])
Lactation index[i] *N/N* (%)	176/177 (99.4)	172/172 (100.0)	192/192 (100.0)	215/215[e] (100.0)	80/81 (98.8)	179/183 (97.8)	139/140 (99.3)	152/152 (100.0)	138/138 (100.0)	93/94 (98.9)
Live litter size										
Day 1	14.1	14.7	14.0	14.4	6.7[##]	15.6	13.2	15.5	12.7	9.1[##]
Day 4 precull	14.1	14.6	13.6	13.8	7.0[##]	15.3	12.9	15.2	12.3	7.9[##]
Day 4 postcull	7.7	7.8	7.7	7.7	5.8[##]	8.0	7.4	8.0	7.7	6.3[##]
Day 21	7.6	7.8	7.7	7.7	6.2[#]	7.8	7.3	8.0	7.7	6.2[#]
Pup weight per litter (g)										
Day 1	6.9	6.5	6.7	6.1**	5.7**	6.4	6.7	6.3	6.1	5.6**
Day 4 precull	10.5	9.7	10.1	9.5	8.5**	9.0	9.8	8.9	9.2	8.0*
Day 4 postcull	10.6	9.8	10.2	9.6	8.6**	9.2	9.9	9.1	9.3	8.0*
Day 7	17.9	16.8	17.3	16.3	14.1*	15.7	16.6	15.3	15.2	13.1**
Day 14	35.3	33.2	36.3	33.9	29.5	33.8	34.7	32.8	33.1	30.1**
Day 21	54.0	51.7	56.1	52.7	47.8	52.8	55.3	52.9	53.5	50.0

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (significantly different from the vehicle control group; Dunnett's test, Dunn's test or Fisher's test); [#]: $P < 0.05$; [##]: $P < 0.01$ (significantly different from the vehicle control group; Dunn's test)

[a] One dam delivered a litter consisting of only two stillborn pups.

[b] Restricted to rats with confirmed mating dates.

[c] Excludes values for one dam, which delivered a litter on day 18 of gestation (the mating date was incorrectly identified).

[d] Includes pregnant rats that did not deliver a litter and were sacrificed on day 25 of gestation.

[e] Excludes value for one pup missing on day 18 postpartum. It was found on the floor on day 20 postpartum and sacrificed.

[f] Excludes values for dam found dead on day 2 of lactation. There were three pups stillborn, one pup found dead, and 13 pups sacrificed on the same day.

[g] Excluded value for one dam found dead on day 2 of lactation. The one surviving pup was sacrificed on the same day.

[h] No. of live pups on day 4 (preculling) postpartum/no. of liveborn pups on day 1 postpartum.

[i] No. of live pups on day 21 postpartum/no. of live pups on day 4 (postculling) postpartum.

[j] Historical control data: mean mortality postnatal days 1–4: 2.6% (range: 0–8.7%).

Source: Hoberman (1992)

section; hence, the study design may have been less sensitive than required according to current standards.

No animals died during the course of the study, and maternal toxicity was limited to decreased weight gain in all treatment groups. No changes in feed intake were reported. High embryolethality was observed in all dose groups. Litters from dams exposed to 200 and 500 mg/kg bw per day were completely resorbed, and only four live fetuses were produced in the 100 mg/kg bw per day group. Fetuses from 30 mg/kg bw per day dams had significantly reduced weights and were found to have both skeletal and visceral abnormalities, primarily wavy ribs and ventricular septal defects (Table 10). One fetus in the low-dose group was reported to have curvature of scapula. Because of the high degree of embryolethality at doses of 100 mg/kg bw per day and above, the highest dose selected for the definitive study was 30 mg/kg bw per day (Kawamura, 1989).

Flumioxazin technical (purity 94.8%) was administered by gavage to four groups of 22 pregnant rats (Slc:SD) at a dose of 1, 3, 10 or 30 mg/kg bw per day during organogenesis (days 6 through 15) to evaluate the effects on dams and fetuses. Current test guidelines require daily administration from implantation to the day prior to scheduled caesarean section; hence, the study design may have been less sensitive than required according to current standards. The material was suspended in a 0.5% aqueous methyl cellulose vehicle. A control group received vehicle only. Animals were mated overnight, and the day on which sperm were observed in the vaginal smear was designated day 0 of gestation. All females were observed daily for mortality and clinical signs. Each female was weighed on days 0, 6, 9, 12, 15 and 20 of gestation. Terminal body weight was the weight of the animal at sacrifice without the gravid uterus. Feed consumption was measured for days 6, 9, 12, 15 and 20 of gestation. On the morning of day 20, all dams were sacrificed and examined grossly for abnormalities of the thoracic, abdominal and pelvic viscera. Corpora lutea were counted. The uteri were weighed, including contents, and examined for the number of implantations and the numbers and sites of live fetuses and dead embryos/fetuses. Each live fetus was sexed, weighed and observed externally, including the oral cavity. The fetuses with external abnormalities were fixed in formalin and were not examined for skeletal or visceral abnormalities. Approximately one half of all externally normal fetuses from each litter were randomly selected, fixed and stained with alizarin red S and examined for skeletal abnormalities, variations and ossification. The remaining live fetuses were fixed in Bouin's solution and examined for visceral abnormalities or variations. Statistical analyses were performed using appropriate methods.

No maternal deaths were observed in any dose group, and no treatment-related effects on clinical signs or feed consumption were noted. A decrease in maternal body weight gain was reported for the 30 mg/kg bw per day dose group (only on gestation day [GD] 20); this was related to a decrease in the number of live fetuses and fetal weights. Necropsy findings included dark reddish fluid or material in the uterus of dams receiving 30 mg/kg bw per day, which was also considered to be related to the death of the litter. The numbers of live fetuses and fetal weights were decreased in the 30 mg/kg bw per day group, and the incidence of embryo mortality tended to be higher in this dose group, but was not statistically significant. No effects on the number of implantations, sex ratios or external abnormalities were found. The incidence of fetuses with cardiovascular abnormalities (i.e. ventricular septal defects) was increased in the 30 mg/kg bw per day group (Table 10). The incidence of cardiovascular abnormalities in the 10 mg/kg bw per day group was not significantly different from the incidence in controls, but was above the historical control range. Double aortic branch was observed in three fetuses at 30 mg/kg bw per day. Other developmental effects observed at 30 mg/kg bw per day included an increase in the incidence of wavy ribs and curvature of the scapula and ulna and a decrease in the number of ossified sacrococcygeal vertebral bodies.

The NOAEL for embryo and fetal toxicity was 3 mg/kg bw per day, based on an increased incidence of malformations (cardiac ventricular septal defects) in the rat fetuses. The maternal toxicity NOAEL was 30 mg/kg bw per day, the highest dose tested (Kawamura, 1990a).

Table 10. Incidence of selected fetal findings with laboratory historical control data (affected fetuses/litters[a])

Kawamura (1989): range-finding study	**0 mg/kg bw per day**	**30 mg/kg bw per day**	**100 mg/kg bw per day**	**200 mg/kg bw per day**	**500 mg/kg bw per day**
Visceral examination					
No. of examined fetuses	38	25	2	0	0
No. of examined litters	6	5	2	0	0
Heart: ventricular septal defects	0/0	11**/5[##]	1/1	–	–
Skeletal examination					
No. of examined fetuses	42	28	2	–	–
No. of examined litters	6	6	1	–	–
Curvature of scapula	0/0	1/1	0/0	–	–
Wavy ribs	0/0	9/3	1/1	–	–
Kawamura (1990a): main study	**0 mg/kg bw per day**	**1 mg/kg bw per day**	**3 mg/kg bw per day**	**10 mg/kg bw per day**	**30 mg/kg bw per day**
Visceral examination					
No. of examined fetuses	147	138	144	144	102
No. of examined litters	22	21	21	22	18
Heart: ventricular septal defects	2/2	1/1	2/2	6/5	26**/12[##]
	HC[b]: 21 studies with no affected fetuses 4 studies with 1 fetus (in 1 litter) affected 1 study with 2 affected fetuses (in 2 litters) Maximum: 2.1% of fetuses / 10% of litters				
Artery: double aortic branch	0/0	0/0	0/0	0/0	3/2
Skeletal examination					
No. of examined fetuses	154	144	152	153	111
No. of examined litters	22	21	21	22	18
Curvature of scapula	0/0	0/0	0/0	0/0	10/4[#]
Curvature of ulna	0/0	0/0	0/0	0/0	3/2
Wavy ribs	1/1	1/1	7/3	0/0	27**/12[##]
Kawamura (1991): dermal study	**0 mg/kg bw per day**	**30 mg/kg bw per day**	**100 mg/kg bw per day**	**300 mg/kg bw per day**	
Visceral examination					
No. of examined fetuses	153	133	144	88	
No. of examined litters	23	22	22	17	
Heart: ventricular septal defects	1/1	1/1	2/2	13**/9[##]	
	HC[b]: see above (Kawamura, 1990a)				
Skeletal examination					
No. of examined fetuses	159	136	149	90	
No. of examined litters	23	22	22	17	
Curvature of scapula	0/0	0/0	0/0	2/1	

Table 10 (continued)

Kawamura (1991): dermal study	0 mg/kg bw per day	30 mg/kg bw per day	100 mg/kg bw per day	300 mg/kg bw per day
Wavy ribs	0/0	0/0	2/1	18**/10[##]

bw: body weight; HC: historical control; *: $P < 0.05$; **: $P < 0.01$ (Wilcoxon's rank sum test); [#]: $P < 0.05$; [##]: $P < 0.01$ (Fisher's exact test)

[a] The numbers of affected litters were not included in the study reports but were summarized by the sponsor's representative, Exponent (email dated 24 July 2015).

[b] These 26 studies were conducted from 1982 to 1989; four studies with nine or fewer litters were excluded from the total list of 30 studies submitted; the study reports did not include HC data on further fetal findings.

Apparently, parts of the results of the study by Kawamura (1990a) were also published in the open literature (Kawamura et al., 1995).

The developmental toxicity of flumioxazin in rats was evaluated in a dermal teratology study compliant with USEPA FIFRA Guideline 83-3. On days 6–15 of gestation, pregnant rats (SLc:SD) were exposed dermally to flumioxazin technical (purity 94.8%) in corn oil at a dose level of 30, 100 or 300 mg/kg bw per day, applied to dorsal skin clipped free of hair and secured under a non-occlusive dressing for 6 hours. After 6 hours, the dressing was removed, and the application site was wiped clean of material. A control group received a dermal dose of corn oil alone. Current test guidelines require daily administration from implantation to the day prior to scheduled caesarean section; hence, the study design may have been less sensitive than required according to current standards. The day of successful mating (sperm in vaginal smear) was designated as day 0 of gestation. All females were observed daily for mortality and clinical signs. Each female was weighed on days 0, 6, 9, 12, 15 and 20 of gestation. Feed consumption of each female was measured over a 1-day period on days 6, 9, 12, 15 and 20 of gestation. On the morning of day 20 of gestation, all dams were sacrificed and examined for gross abnormalities of the thoracic, abdominal and pelvic viscera. Corpora lutea were counted. Uteri were weighed, including contents, and examined for the number of implantations and the number and site of live fetuses and dead embryos/fetuses. Each live fetus was weighed and observed externally, including the oral cavity. The fetuses with external abnormalities were fixed in 10% formalin and not examined for skeletal or visceral abnormalities. Approximately one half of all externally normal fetuses from each litter were randomly selected, fixed in alcohol and stained with alizarin red S. All specimens were examined for skeletal abnormalities or variations and ossification. The remaining live fetuses were fixed in Bouin's solution and examined for visceral abnormalities or variations. Statistical analyses were performed using appropriate methods.

No adverse effects were observed in the dams throughout the study. Uterine weight was reduced in dams of the top-dose group. Increased fetal mortality was accompanied by decreases in the number of live fetuses and fetal weights at 300 mg/kg bw per day. Reddish material was reported in top-dose dams. No external abnormalities were observed at any dose level. An increase in cardiovascular abnormalities (i.e. ventricular septal defect), an increase in wavy ribs and curvature of scapula and a decrease in the number of ossified sacrococcygeal vertebral bodies were observed at 300 mg/kg bw per day (Table 10).

The NOAEL for embryo/fetal toxicity was 100 mg/kg bw per day, based primarily on increased incidences of cardiac ventricular septal defects, wavy ribs and curvature of scapula and a decrease in the number of ossified sacrococcygeal vertebral bodies in the fetuses of rats. The maternal toxicity NOAEL was 300 mg/kg bw per day, the highest dose tested (Kawamura, 1991).

Apparently, the results of the study by Kawamura (1991) were also published in the open literature (Kawamura et al., 2014).

Rabbits

A pilot dose range–finding study was conducted in pregnant rabbits to determine appropriate doses for the oral teratology study. The study was not GLP compliant, but was quality assured. Flumioxazin technical (purity 94.8%) was administered to New Zealand White rabbits on days 7 through 19 of gestation. Current test guidelines require daily administration from implantation to the day prior to scheduled caesarean section; hence, the study design may have been less sensitive than required according to current standards. The test material was suspended in aqueous 0.5% (w/w) methyl cellulose and given via oral intubation at a dose of 0, 300, 500, 1000 or 1500 mg/kg bw per day to five or six rabbits per dose. Clinical observations were recorded at least twice daily during the dosing period and at least once daily during the post-dosing period. Body weights were recorded on day 0 and days 7 through 29 of gestation. Feed consumption was recorded daily. On day 29 of gestation, animals in all dose groups were sacrificed, caesarean sectioned and examined for gross lesions, number of corpora lutea, number and placement of implantation sites, early and late resorptions, and live and dead fetuses.

No deaths, abortions or premature deliveries occurred during this study. Doses of flumioxazin technical as high as 1500 mg/kg bw per day did not result in clinical or necropsy observations, nor did they affect maternal body weight gains or feed consumption values. Similarly, there were no adverse effects of doses of flumioxazin technical up to 1500 mg/kg bw per day on embryo/fetal viability, sex ratios, body weights or external morphology (Hoberman, 1991c).

Based on the results of the pilot study, groups of 20 pregnant New Zealand White rabbits were administered flumioxazin technical (purity 94.8%) at a dose of 0, 300, 1000 or 3000 mg/kg bw per day by oral gavage. The highest dose was well in excess of the 1000 mg/kg bw per day limit dose for developmental toxicity studies. The vehicle used was 0.5% methyl cellulose. The rabbits were artificially inseminated. Rabbits were administered the test material on days 7–19 of gestation. Current test guidelines require daily administration from implantation to the day prior to scheduled caesarean section; hence, the study design may have been less sensitive than required according to current standards. The rabbits were observed twice daily during the dosing period and once daily during the post-dosing period for clinical signs of toxicity, abortions, premature deliveries and deaths. Body weights were recorded on the day of insemination and daily during the dosing and post-dosing periods. The rabbits' feed consumption was measured daily, starting with the day of insemination and continuing through the dosing and post-dosing periods. On day 29 of gestation, all rabbits were sacrificed. Caesarean sections were performed, corpora lutea were counted and the uterine contents were examined for the number and placement of implantation sites, live and dead fetuses, early and late resorptions, and any abnormalities. All live fetuses were weighed, sexed and examined for external alterations. Live fetuses were sacrificed and examined for soft tissue alterations and subsequently were eviscerated, stained with alizarin red S and examined for skeletal alterations. Gross lesions in the fetuses and does were preserved in formalin. Adult tissues and carcasses that appeared normal were discarded. Statistical analyses were performed using appropriate methods.

The 3000 mg/kg bw per day dose tended to reduce maternal body weight gains and absolute and relative feed consumption values, which reached statistical significance at certain time ranges. No gross lesions were produced at any dose level. The 3000 mg/kg bw per day dose group litters tended to have reduced fetal weights, but these differences were not statistically significantly different. No fetal external, soft tissue or skeletal findings were attributable to the treatment with the test substance.

Based on the absence of adverse effects, the NOAEL for embryo and fetal toxicity was 3000 mg/kg bw per day, the highest dose tested. The maternal toxicity NOAEL was 1000 mg/kg bw per day, based on reductions in maternal body weight gains and absolute and relative feed consumption values (Hoberman, 1991d).

Apparently, parts of the results of the study by Hoberman (1991d) were also published in the open literature (Kawamura et al., 1995).

2.6 Special studies

(a) Neurotoxicity

Flumioxazin does not belong to a class of chemicals known to be associated with neurotoxicity, and no signs of neurotoxicity were reported in acute or repeated-dose toxicity studies. According to the sponsor, acute and subchronic neurotoxicity studies of flumioxazin were performed in order to comply with the USEPA's data requirements for registration. A delayed neurotoxicity study was not considered necessary, as flumioxazin has no organophosphate or organocarbamate structural groups. It is noted that the purity of the flumioxazin tested was greater than that of the technical material. The respective dose range–finding studies were not submitted by the sponsor, but were cited in the study reports.[1]

In an acute neurotoxicity study, flumioxazin (purity 99.6%) in aqueous 0.5% methyl cellulose was administered once via gavage at a dose of 0, 200, 700 or 2000 mg/kg bw to groups of young male and female adult rats (12 animals of each sex per group); treatment was followed by a 14-day observation period. A neurobehavioural test battery consisting of motor activity and functional observational battery assessments was conducted on all rats prior to test substance administration to obtain baseline measurements and then repeated again on study day 0 (approximately 8 hours post-administration) and on study days 7 and 14. On study day 15, all surviving rats underwent whole-body in situ perfusion under anaesthesia. Brain weights and brain dimensions (excluding olfactory bulbs) were recorded. Central and peripheral nervous system tissues from six rats of each sex in the control and 2000 mg/kg bw groups were processed for histopathological examination.

There were no deaths or clinical signs of toxicity. Mean body weights and body weight gains were unaffected by treatment. No treatment-related effects were observed in functional observational battery measurements or motor activity assessments on the day of dosing or subsequently during the observation period. A few statistically significant differences in brain weights and measurements (Table 11) were considered to be unrelated to dosing with flumioxazin, because they were within or very close to historical control ranges and, with the exception of male brain width measurements, showed no dose–response relationship. Furthermore, there were no associated morphological changes in the brain, and no similar finding was observed in the 90-day repeated-dose neurotoxicity study.

There were no further treatment-related effects on brain parameters or histopathological changes in the nervous system. In this study, the NOAEL for acute neurotoxicity of flumioxazin was 2000 mg/kg bw, the highest dose tested (Herberth, 2011a).

In a subchronic dietary neurotoxicity study, flumioxazin (purity 99.6%) was administered orally via the diet at a dose of 0, 500, 1500 or 4500 ppm over 90 days to groups of young male and female adult rats (12 animals of each sex per group). The mean daily intakes of flumioxazin over study days 0–91 were equal to 0, 37, 110 and 323 mg/kg bw per day for males and 0, 41, 124 and 358 mg/kg bw per day for females, respectively. A neurobehavioural test battery consisting of motor activity and functional observational battery assessments was conducted on all rats prior to test material administration to obtain baseline measurements, and the testing was repeated in weeks 3, 7 and 12 of dietary administration. Ophthalmic examinations were conducted before and near the end of the administration period. After completion of the final neurobehavioural test in week 13, all surviving animals underwent whole-body in situ perfusion under anaesthesia. Brain weights and brain dimensions (excluding olfactory bulbs) were recorded. Central and peripheral nervous system tissues

[1] Herberth MT (2010). An oral (gavage) dose range–finding acute neurotoxicity study of flumioxazin in rats. Unpublished report from WIL Research Laboratories, LLC, Ashland, OH, USA (Report no. WIL-194032).

Crittenden PL (2011). Flumioxazin – A 28-day dietary dose range–finding study in Sprague Dawley rats for immunotoxicity and neurotoxicity studies. Unpublished report from WIL Research Laboratories, LLC, Ashland, OH, USA (Report no. WIL-194034).

Table 11. Summary of brain weights and measurements with laboratory historical control data

	Control	200 mg/kg bw	700 mg/kg bw	2 000 mg/kg bw	HC mean (range)[a]
Males					
Weight (g)	1.97	2.05	2.03	2.01	2.0 (1.80–2.12)
Length (mm)	20.0	19.9	20.6*	20.3	20.9 (19.7–25.2)
Width (mm)	15.1	15.4*	15.5**	15.6**	15.1 (14.7–15.5)
Females					
Weight (g)	1.94	1.88	1.90	1.86*	1.9 (1.67–1.98)
Length (mm)	20.0	19.5	19.8	19.7	20.4 (19.2–24.3)
Width (mm)	15.1	15.0	15.0	14.9	14.7 (14.2–15.2)

bw: body weight; HC: historical control; *: $P < 0.05$; **: $P < 0.01$ (Dunnett's test)
[a] From studies conducted in 2004–2008.
Source: Herberth (2011a)

from six rats of each sex in the control and 4500 ppm groups were processed for histopathological examination.

An isolated mortality in the female high-dose group was considered not to be related to treatment with flumioxazin. Mean body weights and body weight gains were not consistently affected by treatment. Haematological changes characteristic of regenerative microcytic, hypochromic anaemia were observed at 500 ppm (males only), 1500 ppm (both sexes) and 4500 ppm (both sexes). The sponsor considered the haematological changes in males at 500 ppm as not adverse, because they were of low magnitude and were within the historical control range. However, as no details on the historical control data (e.g. underlying studies, dates of studies, individual data, age of animals) were provided and because of the clear dose–response relationship and the similarity to the findings in other studies, these findings in males at 500 ppm are considered to be adverse. No treatment-related effects were reported in ophthalmic, functional observational battery or motor activity assessments during the study. There were no consistent treatment-related effects on brain parameters or histopathological changes in the nervous system. There was no indication of neurotoxicity up to 4500 ppm.

Therefore, under the conditions of the study, the NOAEL for subchronic neurotoxicity was 4500 ppm (equal to 323 mg/kg bw per day for males and 358 mg/kg bw per day for females), the highest dose tested. Haematological changes were reported for all dose groups from 500 ppm (equal to 37 mg/kg bw per day) (Herberth, 2011b).

(b) Immunotoxicity

A study was performed to evaluate the potential immunotoxic effects of flumioxazin when administered orally via the diet to female Sprague-Dawley rats. The assessment of immunotoxicity was based primarily on the results of a splenic antibody-forming cell (AFC) assay to assess the T cell–dependent antibody response (TDAR) to sheep red blood cells (sRBCs). Groups of 15 animals were treated with diet containing flumioxazin (purity 99.6%) at 0, 500, 1500 or 4500 ppm (equal to 0, 42, 126 and 371 mg/kg bw per day, respectively) for 28 consecutive days. In addition, cyclophosphamide, a known immunosuppressant, was administered to a further group for 4 consecutive days (study days 24–27) via intraperitoneal injection at 50 mg/kg bw per day. Ten animals of each group received an intravenous immunization injection of sRBCs on study day 24 (TDAR groups). The remaining five animals from each group were used for haematological evaluation at the scheduled necropsy. On study day 28, all rats were euthanized. During the in-life period, all animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily, and detailed physical examinations were performed weekly. Individual body weights were recorded twice weekly. Feed consumption and water consumption were recorded weekly. Serum samples for possible

immunoglobulin M (IgM) antibody analysis were collected from all rats in the TDAR groups at the scheduled necropsy. Complete necropsies were conducted on all animals, and the spleen, lymph nodes, bone marrow, adrenal glands, Peyer's patches and thymuses were collected. Spleen and thymus weights were obtained from all rats in the TDAR groups, and spleens were collected for the AFC analysis.

There were no test substance–related effects on survival, clinical observations, body weight, feed consumption, water consumption or macroscopic findings in any of the test substance–treated groups.

In the haematology groups, test substance–related alterations in haematology parameters (white blood cells, haemoglobin, haematocrit, mean cell volume [MCV], mean corpuscular haemoglobin [MCH], mean corpuscular haemoglobin concentration [MCHC], and reticulocyte, neutrophil and lymphocyte counts) were noted in the 1500 and/or 4500 ppm groups. Lower MCV and MCH values were noted in the 1500 and 4500 ppm groups when compared with the vehicle control group. Higher white blood cell count, absolute and per cent reticulocyte counts, and absolute neutrophil and lymphocyte counts and lower haemoglobin, haematocrit and MCHC values were also noted in the 4500 ppm group when compared with the vehicle control group.

In the TDAR groups, there were no test substance–related effects on thymus weights (absolute or relative) or spleen cell numbers in any of the treated groups. Higher absolute and relative spleen weights were noted for the 4500 ppm group.

There were no test substance–related effects on the AFC response, as measured by the AFC IgM specific activity (AFC/10^6 spleen cells) and total spleen activity (AFC/spleen), at any dose level tested. A higher AFC response was noted for the 500 ppm group; however, this result was not considered test substance related based on the presence of one high-responding individual in the 500 ppm group. In addition, statistically significant differences in specific and/or total spleen activity were not noted in the 1500 or 4500 ppm groups when compared with the vehicle control group.

Under the conditions of the study, haematological changes were reported at 1500 and 4500 ppm. No adverse effects on AFC response were reported up to the highest dietary concentration of 4500 ppm (equal to 371 mg/kg bw per day) (Crittenden, 2011).

(c) Mechanistic data regarding developmental toxicity in rats

Species differences were clearly evident in the developmental toxicity studies. Flumioxazin caused embryolethality, teratogenicity (e.g. ventricular septal defects, curvature of the scapula and ulna and wavy ribs) and growth retardation in rats at 30 mg/kg bw per day in the absence of maternal toxicity, but not in rabbits at the 100-fold higher and maternally toxic dose of 3000 mg/kg bw per day. A research programme was initiated by the sponsor to elucidate the reasons for the species differences and to support the assessment of the human relevance of these findings. The results of the submitted studies are presented below.

Critical period of embryonic sensitivity

To determine the critical period of embryonic sensitivity, pregnant rats were administered a single dose of flumioxazin (purity 94.8%) in 0.5% methyl cellulose by gavage at 400 mg/kg bw on 1 day of gestation beginning on day 11 through day 15. Groups of 4–5 animals were treated per gestation day. This 400 mg/kg bw dose was selected as one that would produce fetuses with ventricular septal defects following a single dose, without producing excessive fetal deaths. Rats were sacrificed on day 20, and uterine and fetal examinations were performed. Live fetuses were sexed, weighed, examined externally and fixed in Bouin's solution for examination for ventricular septal defects. The study was not GLP compliant (no authorized protocol, quality assurance unit not involved in any part of the study). GD 12 was determined to be the most sensitive day, based on the incidence of embryonic deaths, fetal weight reduction and incidence of ventricular septal defects (Kawamura, 1993).

Apparently, some of the results of the study by Kawamura (1993) were also published in the open literature (Kawamura et al., 1995).

Pathogenesis of developmental toxicity in rats

Flumioxazin (purity 94.8%) was given to groups of six female rats at a dose of 0, 3000 or 10 000 ppm in the diet for a maximum of 5 weeks. The effects of flumioxazin on the haematological system (haematological end-points, blood chemistry, urinary coproporphyrin, free erythrocyte protoporphyrin) were examined periodically to elucidate the mechanism by which it induces anaemia. The study was not conducted according to GLP principles and was not quality assured.

Throughout the treatment period, no deaths occurred, nor were there any treatment-related changes in body weight or gross pathology. Feed consumption decreased minimally at 3000 ppm and in the higher-dose groups, but this change was transient. The haematological changes at both dose levels included decreases in erythrocyte count, haemoglobin, haematocrit, MCV, MCH, MCHC and bone marrow myeloid/erythroid ratio and increases of siderocytes, erythroblasts and platelets. The neutrophils and reticulocytes decreased during the early treatment period, but increased thereafter. The urinary coproporphyrin and erythrocyte protoporphyrin levels were increased in the 3000 ppm group (but were not measured for the 10 000 ppm group). In the blood biochemistry tests, the abnormal findings in treated groups included increases in serum iron, total cholesterol, blood urea nitrogen, sodium and potassium levels, as well as decreases in aspartate aminotransferase activity and uric acid, calcium and triglyceride levels. Increased liver and spleen weights were observed in treated groups.

These findings suggest that flumioxazin-associated anaemia in rats can be classified as sideroblastic anaemia, resulting primarily from the defective haem pathway during the process of haemoglobin biosynthesis. Increased blood porphyrin suggested that flumioxazin induces porphyria in rats (Yoshida, 1996).

A study was designed to investigate the ontogeny and pathogenesis of a variety of developmental end-points in rat embryos and fetuses. The study was not conducted according to GLP principles (no authorized protocol, quality assurance unit not involved in any part of the study). Flumioxazin (purity 94.8%) was administered to pregnant rats by gavage on day 12 of gestation at 400 mg/kg bw, and embryos/fetuses were removed from the uterus on days 13 through 20 of gestation (number of treated animals was eight on GD 13, 16 on GDs 14, 15, 17 and 20, and 18 on GD 16). A control group received vehicle only (number of treated animals was seven on GD 13 and eight on GDs 14, 15, 16, 17 and 20). Embryos/fetuses were examined externally for enlargement of heart and oedema of the whole body. One half of each litter was examined internally for closure of interventricular foramen of the heart. The other half was used to measure the number of red blood cells, haemoglobin and serum protein and was then examined for wavy ribs.

The indicators of developmental toxicity were noted remarkably on day 14, when treated embryos were observed to have enlarged heart, oedema and anaemia (decreased red blood cell count and haemoglobin). These effects were also observed on days 15 and 16 of gestation, after which the values for treated litters were similar to control values. Beginning on day 15 and continuing to day 20, mortality in the treated litters was increased. The mortality rate was relatively constant on GDs 17 and 20, indicating that all deaths occurred during the earlier period. This is consistent with the early resorptions observed in other developmental toxicity studies with flumioxazin. Closure of interventricular foramen began on day 16 of gestation in control fetuses (72.7% closed). Closure of interventricular foramen did not occur in any treated fetuses until day 16, and even then, the percentage with closure on day 17 was well below control values (89.7% control versus 23.9% treated). On day 20, the foramen of 95.2% of the control fetuses had closed, with only 57.7% of the treated fetuses reaching this milestone. Serum protein concentration was reduced on days 15 and 16 of gestation in treated animals and recovered by day 17 to values similar to control values. In treated litters, evidence of incomplete chondrification of the ribs was observed on day 16, delayed

ossification was observed on day 17, and wavy ribs and other skeletal findings (bent scapula, radius and ulna) were observed on day 20.

These data suggest that the enlarged heart, oedema and anaemia preceding the occurrence of the fetal mortality may be instrumental in the cause of death. Similarly, the occurrence of enlarged heart preceding the failure of the interventricular closure would be related to the pathogenesis of this finding (Kawamura, 1997a).

Comparative histopathological changes in rat and rabbit embryos

An examination of the histopathological changes in rat and rabbit embryos was undertaken for fetuses exposed to flumioxazin at the most sensitive point of fetal development. Pregnant rats and rabbits were administered flumioxazin (purity 94.8%) in 0.5% methyl cellulose at 1000 mg/kg bw on day 12 of gestation. Control animals received 0.5% methyl cellulose on day 12 of gestation. One to four animals were used per treatment regimen. The study was not conducted according to GLP principles (no authorized protocol, quality assurance unit not involved in any part of the study). Rats were sacrificed at 6, 12, 24, 36 or 48 hours after treatment, and rabbits at 6, 24 or 48 hours. Embryos were examined externally and then subjected to examinations of umbilical blood smears and light or electron microscopic examination of tissues. Umbilical blood smears were stained with Berlin blue for detection of cellular iron. Sagittal or transverse sections of the thoraco-abdominal region of the fetuses were prepared and stained with haematoxylin and eosin for light microscopic examinations. Some sections with heart and liver were stained with Berlin blue and examined. The hearts and livers of some fetuses were prepared for examination by electron microscopy.

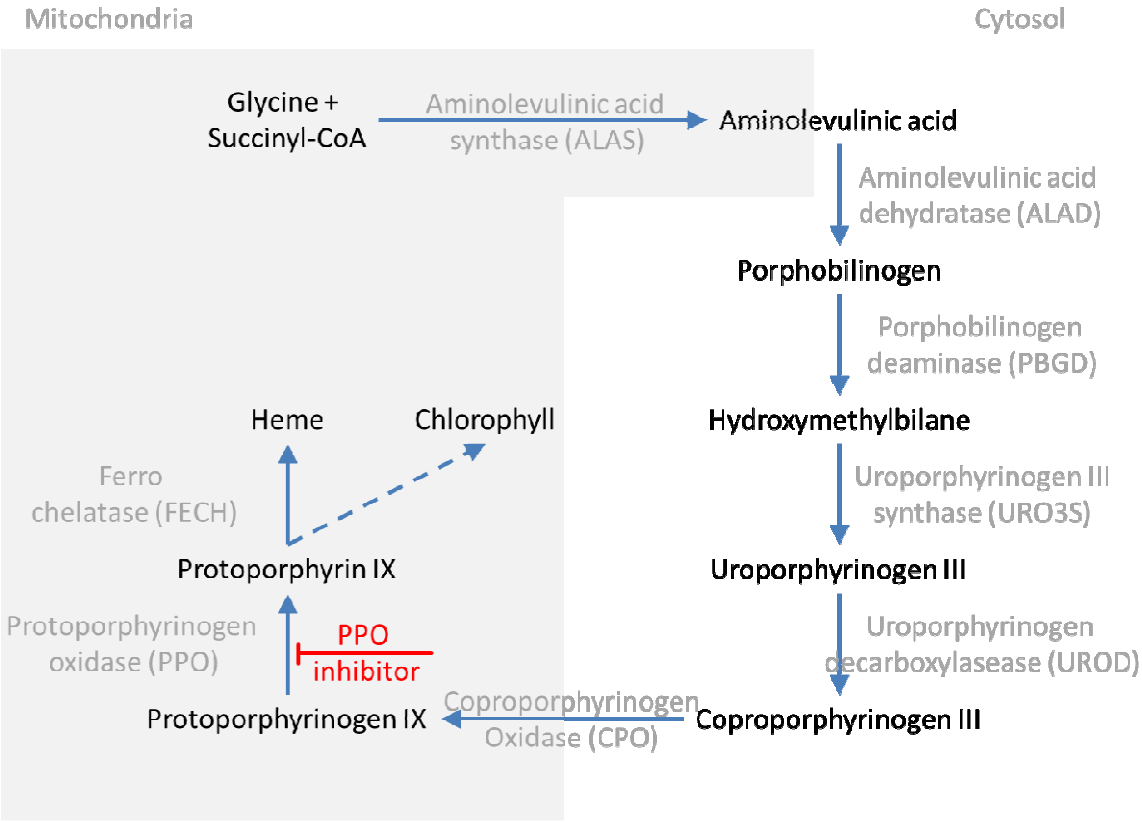
No embryonic deaths were observed in rats at 24 hours after treatment. The first intrauterine deaths were observed at 36 hours, and embryonic mortality increased to 93.2% at 48 hours after treatment. A primary effect was observed in circulating erythroblasts. Mitochondrial iron deposits in polychromatophilic erythroblasts, dilatation of the mitochondrial matrix in polychromatophilic erythroblasts at 6 hours post-dosing, and erythroblastic cell death evident after the appearance of the mitochondrial lesions were observed. These findings were suggested to reflect the observed anaemia in rat embryos. No histopathological signs in the embryonic rat heart were observed up to 24 hours after treatment, and no cell death in the embryonic rat heart was observed up to 48 hours after treatment, suggesting that no primary injury to the embryonic heart was produced by flumioxazin. Histological changes in the rat embryonic hearts at 36 or 48 hours after treatment included thin ventricular wall, poorly developed ventricular trabeculae, and hypoplasia of the muscular septum and endocardial cushions of the atrioventricular canal. In rat fetuses, dilatation of hepatic sinusoidal vessels was observed at 24 and 36 hours; at 48 hours, hepatic necrosis was reported. No treatment-related changes in the external appearance of embryos or intrauterine deaths were reported in rabbits. Likewise, no iron deposits in erythroblasts of rabbit embryos or histopathological changes similar to those produced in rat embryos were seen in the rabbit embryo (Kawamura & Yoshioka, 1997).

Apparently, parts of the results of the study by Kawamura & Yoshioka (1997) were also published in the open literature (Kawamura et al., 1996a).

Protoporphyrin IX accumulation

According to the sponsor, flumioxazin is a photo-bleaching agent whose herbicidal activity is considered to derive from inhibition of porphyrin biosynthesis, a key step in chlorophyll production in plants (Fig. 3).

Fig. 3. Haem and chlorophyll biosynthesis pathway



Source: Adapted from Ajioka, Phillips & Kushner (2006) and Nordmann & Puy (2002)

The sponsor stated that treatment of plants with flumioxazin resulted in the accumulation of protoporphyrin IX (PPIX) in plant cells, probably due to inhibition of PPO and auto-oxidation of protoporphyrinogen IX to PPIX. Porphyrin biosynthesis is common to plants and animals as part of chlorophyll and haem synthesis, respectively. Therefore, considering the biological activity of flumioxazin in plants and indications of haematotoxicity in rats in subchronic and chronic toxicity studies, mechanistic studies were conducted examining the accumulation of PPIX in animal embryos exposed to flumioxazin.

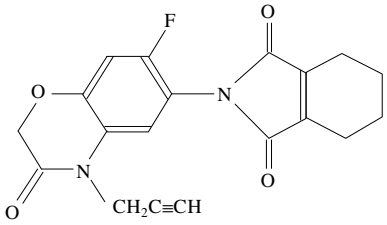
To determine whether a species difference exists in accumulation of PPIX in rat and rabbit embryos, pregnant rats and rabbits were administered a single oral dose of 1000 mg/kg bw flumioxazin in 0.5% methyl cellulose, and the concentrations of PPIX in embryos and maternal livers were measured with a high-performance liquid chromatographic (HPLC) method at 2, 6, 12, 18 and 24 hours after administration for rats and at 2, 6, 12, 24 and 48 hours after administration for rabbits. Two to four rats and 2–3 rabbits were used per treatment regimen. The study was not conducted according to GLP principles (no authorized protocol, quality assurance unit not involved in any part of the study). The concentrations of PPIX in rat embryos and maternal livers were much higher than those in the corresponding tissues of the rabbit, with peak concentrations observed at 12 hours for embryos (concentration change from pretreatment to maximum concentration; rats: 0.15 versus 19 µg/g tissue; rabbits: not detected versus 0.1 µg/g tissue) and 6–12 hours for maternal livers (rats: 0.1 versus approximately 1.3 µg/g tissue; rabbits: 0.05 versus 0.22 µg/g tissue) (Kawamura, 1996a).

Apparently, parts of the results of the study by Kawamura (1996a) were also published in the open literature (Kawamura et al., 1996b).

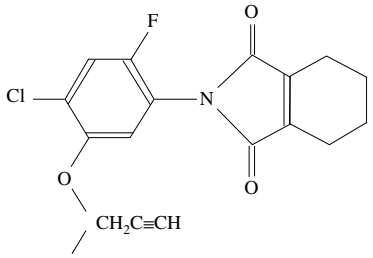
To allow further comparisons, a selection of structurally related chemicals was included in the investigations: S-23121 and S-23031 (chemical structures are depicted in Fig. 4).

Fig. 4. Chemical structures of the three related chemicals (flumioxazin, S-23121 and S-23031) of the N-phenylimides family

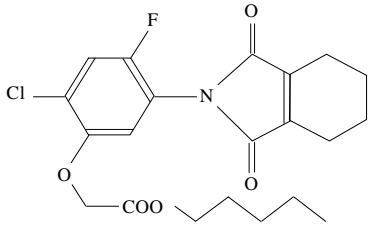
Flumioxazin



S-23121



S-23031



Source: Holmes (2014)

Both flumioxazin (Kawamura, 1990a) and S-23121 (Kawamura, 1990b) were found to produce the same pattern of developmental toxicity in rats at a dose level of 10 or 20 mg/kg bw per day, respectively, with no evidence of developmental effects when tested in rabbits (S-23121: highest dose tested was 15 mg/kg bw per day, Hoberman, 1990; flumioxazin: Hoberman, 1991d). The maternal NOAEL in rabbits treated with S-23121 was 2 mg/kg bw per day, based on abortions and decreased body weight, body weight gain and feed intake at 4 mg/kg bw per day and above. S-23031, however, did not produce maternal or developmental toxicity, even when tested up to 1500 mg/kg bw per day in rats (Lemen, 1991a), nor did it produce developmental toxicity when tested up to 800 mg/kg bw per day in rabbits, a dose that produced maternal toxicity (mortality) with a NOAEL of 400 mg/kg bw per day (Lemen, 1991b).

Apparently, parts of the results of the studies by Kawamura (1990b), Hoberman (1990) and Lemen (1991a,b) were also published in the open literature (Kawamura et al., 2013).

To investigate whether PPIX accumulation in rat embryos was different for the three similar compounds, pregnant rats were administered flumioxazin, S-23121 or S-23031 at 1000 mg/kg bw on day 12 of gestation, and PPIX accumulation was measured 14 hours later in maternal livers and in embryos with an HPLC method. The 14-hour time period approximates the peak PPIX accumulation time point reported previously. The study was not conducted according to GLP principles (no authorized protocol, quality assurance unit not involved in any part of the study). Both flumioxazin

and S-23121 induced PPIX accumulation to a similar extent in rat embryos, about 250 times that of control fetuses (approximately 25 µg/g versus 0.095 µg/g tissue). The PPIX concentration in maternal livers of animals treated with flumioxazin or S-23121 was approximately 3 times that observed in livers of control animals (0.7–0.8 µg/g versus 0.236 µg/g). The PPIX concentration in embryos of S-23031-treated rats was similar to the value for control embryos, whereas the concentration in maternal livers was slightly higher than the control value (0.379 µg/g versus 0.236 µg/g) (Kawamura, 1996b).

To investigate the possible link between developmental toxicity and PPO inhibition, the critical period for PPIX accumulation, presumably resulting from PPO inhibition, in rat and rabbit embryos and maternal livers was investigated. Pregnant rats and rabbits (3–5 animals per group) were administered a single oral gavage dose of flumioxazin (purity 94.8%) at 400 mg/kg bw (rats) or 1000 mg/kg bw (rabbits) at 19:30 on 1 day of gestation from day 10 through 15. A control group of pregnant rats received 0.5% methyl cellulose in the same dosing scheme. The study lacked control animals for rabbits. The study was not conducted according to GLP principles (no authorized protocol, quality assurance unit not involved in any part of the study). PPIX was extracted from embryos and maternal livers 14 hours after treatment and quantified with an HPLC method. The most sensitive period for inducing the highest accumulation of PPIX in rat embryos was treatment with flumioxazin at 19:30 on GD 11 to 19:30 on GD 12. PPIX accumulation was not observed at any developmental stage in the rabbit. Accumulation of PPIX was not observed in maternal rat or rabbit liver at the doses used in this study. However, reported results for rabbits need to be evaluated with caution, taking into account the missing control animals (Kawamura, 1997b).

Apparently, parts of the results of the study by Kawamura (1997b) were also published in the open literature (Kawamura, Kato & Fantel, 2014).

Species differences in the accumulation of PPIX due to the inhibition of PPO by flumioxazin were investigated in vitro using cryopreserved primary hepatocytes from female rats, rabbits, monkeys and humans. The study was not conducted according to GLP principles (the report contained neither GLP nor quality assurance statements). Cells were thawed and seeded at 0.5×10^6 cells/mL in a 24-well plate for approximately 24 hours. Flumioxazin was dissolved in dimethyl sulfoxide (DMSO) and added at final concentrations of 0, 0.01, 0.03, 0.1 and 0.3 µg/mL. No fewer than six replicates of each concentration were prepared, with each experiment conducted in triplicate for each lot of hepatocytes. Following exposure to flumioxazin for 24 hours, the medium was removed, and cells were harvested and processed for potential PPIX accumulation using analysis by liquid chromatography/mass spectrometry (LC/MS). The concentration of PPIX was expressed relative to protein amount. The basal PPIX concentration in rat hepatocytes was 293 pg/mg protein and was dose-dependently increased by the addition of flumioxazin, with approximately a 10-fold increase above the basal level when cells were exposed to 0.3 µg/mL of flumioxazin. The basal PPIX concentration in rabbit hepatocytes was similar to that in the rat; however, the PPIX concentration was not increased in rabbit hepatocytes when exposed to flumioxazin. A similar effect was seen in monkey hepatocytes: whereas the basal PPIX concentration was much lower (42 pg/mg protein), no accumulation of PPIX was observed when monkey hepatocytes were exposed to flumioxazin. In all three batches of human hepatocytes, basal PPIX levels were lower than those of the rat and rabbit, and the mean PPIX concentration of the three lots in the control samples was 180 pg/mg protein. An approximate 4.4-fold increase in mean PPIX concentration was observed following exposure to flumioxazin up to 0.3 µg/mL. In two of the three human hepatocyte samples, the increase was approximately 6-fold (with individual experiments reaching 10-fold increases).

In conclusion, the induction ratios of PPIX following treatment with flumioxazin at 0.3 µg/mL were 10.3-, 1.1-, 1.4- and 4.4- to 6-fold in primary hepatocytes of rat, rabbit, monkey and human, respectively. When judging the validity of the experiments, it should be taken into account that in the report, no data on the viability of the hepatocytes were given, and the interindividual variability was not evaluated for rat, rabbit or monkey samples. Human hepatocyte samples were taken from Caucasian women 64, 38 and 68 years of age (Abe, 2011a).

Table 12. Mean IC_{50} values for test chemicals in PPO assays

	Mean (range) IC_{50} values (µmol/L)						
	Rat liver			Rabbit liver			Human liver
Chemical	**Study 1 Green & Dabbs (1993)**	**Study 2 Green & Dabbs (1996)**	**Study 3 Noda (1995)**	**Study 1 Green & Dabbs (1993)**	**Study 2 Green & Dabbs (1996)**	**Study 3 Noda (1995)**	**Study 2 Green & Dabbs (1996)**
Flumioxazin	0.008 08 (0.005 62–0.010 9)	0.007 15 (0.005 69–0.009 50)	0.023	0.051 9 (0.019 8–0.069 9)	0.138 (0.076 4–0.220)	0.30	0.017 3 (0.012 2–0.020 5)
S-23121	0.010 8 (0.009 75–0.011 8)	–	0.036	1.56 (0.540–2.57)	–	0.690	–
S-23031	0.793 (0.212–1.27)	–	2.23	4.75 (0.413–9.06	–	12.50	–

IC_{50}: median inhibitory concentration; PPO: protoporphyrinogen oxidase

Inhibition of protoporphyrinogen oxidase

Inhibition of PPO activity by flumioxazin and two chemically related compounds in rat, rabbit and human liver mitochondria. As discussed previously, PPO is a key enzyme in haem synthesis, catalysing the transformation of protoporphyrinogen IX to PPIX, which proceeds to formation of protohaem (see Fig. 3 above). Three in vitro studies were initiated to determine whether flumioxazin would inhibit PPO activity in adult liver and/or whole embryos. They were not conducted according to GLP principles or quality assured. In the first study, flumioxazin and two chemically related compounds (S-23121 and S-23031; see Fig. 4 for structures) were tested for their effects on PPO activity in adult liver, day 12 embryos and day 15 embryos of rats and rabbits. Viable and metabolically active mitochondria were prepared from each of these tissues and incubated with substrate, protoporphyrinogen and selected concentrations of each test chemical (flumioxazin: 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} mol/L; S-23121: 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} mol/L; S-23031: 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} mol/L).

The mean relative potency for inhibition of PPO from mitochondria of all tissues was flumioxazin > S-23121 >> S-23031 (Table 12). A difference in sensitivity between the two species was observed, with rat tissue exhibiting a greater sensitivity (100-fold) to PPO inhibition by the test chemicals compared with rabbit tissues. Adult liver and embryo mitochondria showed similar sensitivity to PPO inhibition by the test chemicals (Green & Dabbs, 1993).

Because rat mitochondria from adult liver showed properties similar to those of embryonic tissue in the prior study, only adult liver samples were used in the two following studies. The effect of flumioxazin on PPO activity in adult female human liver samples, in addition to specimens from rats and rabbits, was investigated. Mitochondria were prepared from the livers of adult female rats, rabbits and humans (six Caucasian donors, aged 15–71 years). The inhibition of PPO activity was studied in freshly prepared mitochondria. The final test concentrations covered a broad range when tested in specimens from humans (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} mol/L) or from rats and rabbits (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} mol/L).

The calculated median inhibitory concentration (IC_{50}) values are shown in Table 12. The relative sensitivity of the species to PPO inhibition by flumioxazin was rat > human > rabbit. The mean IC_{50} for human liver PPO was 0.0173 ± 0.0044 µmol/L. The IC_{50} determined in the current study for rat liver mitochondria agreed very closely with that determined in the previous study

(0.007 15 ± 0.0021 µmol/L compared with 0.008 08 ± 0.0027 µmol/L). The IC_{50} for flumioxazin to rabbit liver PPO determined in the present study was slightly higher than the values obtained previously (0.138 ± 0.0739 µmol/L compared with 0.0519 ± 0.028 µmol/L). IC_{50} values in human samples were 2.4-fold higher than those determined in rat samples. Interindividual differences in the enzyme kinetic parameters K_m (Michaelis-Menten constant) and V_{max} (maximum velocity) were in the range of 4- to 6-fold, respectively, in liver samples from humans (Green & Dabbs, 1996).

The third study (Noda, 1995) examined the effects of flumioxazin and the other two structurally related chemicals on PPO activity in adult female rat and rabbit liver. IC_{50} values are summarized in Table 12. Whereas the individual experiments by Noda (1995) were done in triplicate, they were not reproduced in a second experiment. The experiments by Green & Dabbs (1993, 1996) were repeated, to gain a total of three experiments each with triplicate determinations.

Although there were some differences in absolute values between studies, the relative potency for inhibition of PPO from mitochondria of all tissues was flumioxazin > S-23121 > S-23031 (Green & Dabbs, 1993). The relative sensitivity of the species to PPO inhibition by flumioxazin was rat (0.007 15 µmol/L) > human (0.0173 µmol/L) > rabbit (0.138 µmol/L) or rat (1) > human (2.4) > rabbit (19.3) (Green & Dabbs, 1996). The relative relationship of PPO inhibition between the three species tested is rat > human > rabbit, with the two agents (flumioxazin and S-23121) that produced developmental effects in rats both producing the greatest inhibition of PPO (Noda, 1995).

Inhibition of PPO activity by flumioxazin and its major metabolites in rat liver mitochondria. The PPO IC_{50} was determined for flumioxazin and its major metabolites – 3-OH-flumioxazin, 4-OH-flumioxazin and APF – in rat liver mitochondrial fraction in a non-GLP study. The assay was conducted in a 96-well format. Serial dilutions of the test substances were made in DMSO (or DMSO alone as a vehicle control) and were aliquoted in duplicate along with diluted mitochondrial solution into wells. To correct for the auto-oxidation rate of PPIX, heat-inactivated mitochondria (denatured at 80 °C for 15 minutes) were also assayed in parallel with the samples. Following preincubation and the addition of protoporphyrinogen IX, the 96-well plate was loaded into a plate reader, and changes in fluorescence (excitation wavelength, 410 nm; emission wavelength, 630 nm) derived from the produced PPIX were measured automatically for 60 minutes at 1-minute intervals. The results suggest that flumioxazin had the highest PPO inhibitory activity among the four substances tested, followed by 3-OH-flumioxazin and 4-OH-flumioxazin (Table 13). APF had only slight inhibitory activity against PPO up to 100 µmol/L (below 20%) in this study; therefore, no IC_{50} value could be determined (Abe, 2011b).

Table 13. Results for PPO inhibition by flumioxazin and its major metabolites

	Flumioxazin	3-OH-flumioxazin	4-OH-flumioxazin	APF
IC_{50} (µmol/L)	0.004 55	0.062 5	0.667	ND
Fold change relative to flumioxazin	1	13.7	147	ND

ND: not determined
Source: Abe (2011b)

In vitro studies for effects of flumioxazin on haem synthetic pathway and cell proliferation in human and rat cells

Effects of flumioxazin on haem synthetic pathway and cell proliferation in K562 cells. The objective of this study was to investigate the effect of flumioxazin on the haem synthetic pathway in the human K562 cell line (derived from a patient with chronic myeloid leukaemia in the acute phase); this cell line can differentiate into erythroid cells by treatment with sodium butyrate. The study was not conducted according to GLP principles. K562 cells were plated at 1×10^5 cells/mL per well

(24-well plate) in RPMI medium containing sodium butyrate and flumioxazin (dissolved in DMSO). Flumioxazin was examined in the presence of sodium butyrate at concentrations of 0, 0.01, 0.1, 1 and 5 µmol/L. Cell densities were determined on days 2, 4, 6 and 8 (following subculturing at day 4); PPIX and haem content were determined at the same intervals using LC/MS or liquid chromatography with ultraviolet (UV) detection, respectively. The effect of flumioxazin treatment on K562 cells in the absence of sodium butyrate was not reported. No positive control substances were included in the investigations.

In conclusion, PPIX accumulation in K562 cells at 1 µmol/L and above was observed in a dose-dependent manner, with no effect on cell proliferation or haem synthesis up to the highest dose of 5 µmol/L (Kawamura, 2012a).

Effects of metabolites of flumioxazin on haem synthetic pathway and cell proliferation in K562 cells. The objective of this study was to investigate the effects of selected metabolites of flumioxazin (3-OH-flumioxazin, 4-OH-flumioxazin and APF) on the haem synthetic pathway in K562 cells. The study was not conducted according to GLP principles. K562 cells were plated onto 6 cm dishes at 5×10^5 cells/5 mL per dish in RPMI medium containing flumioxazin or its metabolites at a concentration of 5 µmol/L and sodium butyrate. Test substances and sodium butyrate were dissolved in DMSO. Cell densities were determined on days 2, 4, 6 and 8 (following subculturing at day 4), with PPIX and haem content determined at the same intervals using LC/MS or LC/UV detection, respectively. The effects of the treatment with flumioxazin or metabolites on K562 cells in the absence of sodium butyrate were not reported. No positive control substances were included in the investigations. A possible dose–response relationship was not determined.

In conclusion, flumioxazin and the tested metabolites had no effect on haem synthesis or proliferation of K562 cells. PPIX was increased in K562 cells treated with flumioxazin, but this was not observed with any of the tested metabolites (Kawamura, 2012b).

Effects of flumioxazin on haem synthetic pathway and cell proliferation in rat erythroleukaemia cells. The inhibition of haem synthesis by flumioxazin and accumulation of PPIX were investigated in a study with a rat erythroleukaemia (REL) cell line (derived from transplantable tumours from 7,12-dimethylbenz(*a*)anthracene-induced erythroleukaemia in the Long-Evans rat) that had been induced to differentiate into an erythroid form by treatment with hexamethylenebisacetamide (HMBA). The study was not conducted according to GLP principles. REL cells were suspended in RPMI medium containing flumioxazin (at 5, 1, 0.3, 0.1, 0.01 or 0 µmol/L) and HMBA (both were dissolved in DMSO), plated onto 60 mm dishes at 5×10^5 cells/5 mL per dish. The seeding day was defined as day 0. Control cell suspension at day 0 was analysed for haem and PPIX content. The cells were counted on days 2, 4, 6 and 8, after which cells were stored at −80 °C until determination of haem and PPIX content. On days 2, 4 and 6, the cells were also subcultured at a concentration of 1×10^5 cells/mL. Concentrations of haem and PPIX were analysed using LC/UV and LC/MS, respectively. The effect of flumioxazin treatment on REL cells in the absence of HMBA was not reported. No positive control substances were included in the investigations.

There was no effect on cell proliferation up to the highest dose of 5 µmol/L. PPIX accumulated in cells at 0.1 µmol/L and above in a dose-dependent manner from day 2. The accumulation of PPIX reached a maximum at day 4. Haem synthesis was inhibited in REL cells at 0.01 µmol/L and above in a dose-dependent manner from day 4. The inhibition of haem synthesis reached a maximum at day 6 (Kawamura, 2013a).

Effects of flumioxazin on haem synthetic pathway and cell proliferation in human CD36+ cells. The inhibition of haem synthesis by flumioxazin and accumulation of PPIX were investigated in a study with human CD36+ cells, which were derived from human cord CD34+ cells in culture. The study was not conducted according to GLP principles. Human CD36+ cells were cultured in haematopoietic progenitor growth medium supplemented with stem cell factor, erythropoietin,

interleukin-3 (IL-3) and IL-6 to induce differentiation into erythroid cells, which were then treated with flumioxazin (at 5, 1, 0.1, 0.01 or 0 µmol/L, dissolved in DMSO), plated onto 30 mm dishes at 6 × 10^5 cells/3 mL per dish. The seeding day was defined as day 0. Control cell suspension at day 0 was analysed for haem and PPIX content. The cells were counted on days 2, 4, 6 and 8, after which cells were stored at −80 °C until measurement. On days 2, 4 and 6, the cells were also subcultured at a concentration of 2 × 10^5 cells/mL. Concentrations of haem and PPIX were analysed using LC/UV and LC/MS, respectively. The effect of flumioxazin treatment on cells in the absence of the differentiation-inducing agents was not reported. No positive control substances were included in the investigations.

PPIX was accumulated slightly in human erythroidal CD36+ cells at 0.1 µmol/L and clearly at 1 µmol/L and above in a dose- and time-dependent manner. There was no effect on cell proliferation and haem synthesis up to the highest dose of 5 µmol/L (Kawamura, 2013b).

Inhibition of PPO activity by flumioxazin and its major metabolites in human liver mitochondria. The PPO IC_{50} was determined for flumioxazin and selected metabolites 3-OH-flumioxazin, 4-OH-flumioxazin and APF in human liver mitochondria in a non-GLP study. Human microsomes were a mix derived from five donors, but no further details (e.g. age, sex or genetic background/race) were included in the report. The assay was conducted in a 96-well format. Serial dilutions of the test substances were made in DMSO (or DMSO alone as a vehicle control, 10-fold spacing) and were aliquoted in duplicate along with diluted mitochondrial solution into wells. To correct for the auto-oxidation rate of PPIX, heat-inactivated mitochondria (denatured at 80 °C for 15 minutes) were also assayed in parallel with the samples. Following preincubation and the addition of protoporphyrinogen IX, the 96-well plate was loaded into a plate reader, and changes in fluorescence (excitation wavelength, 410 nm; emission wavelength, 630 nm) derived from the produced PPIX were measured automatically for 60 minutes at 1-minute intervals.

The results suggest that the PPO inhibitory activity of the two metabolites (3-OH-flumioxazin and 4-OH-flumioxazin) was weaker than that of the parent compound, flumioxazin (Table 14). An IC_{50} value for APF was not obtained in this study. APF inhibited PPO activity by approximately 40% up to 100 µmol/L. Only transformed values were given in the report (Abe, 2014).

Table 14. IC_{50} values for the inhibition of human liver mitochondrial PPO by flumioxazin and selected metabolites

	Flumioxazin	3-OH-flumioxazin	4-OH-flumioxazin	APF
IC_{50} (µmol/L)	0.021 ± 0.003 7	0.104 ± 0.019 4	0.893 ± 0.402 4	> 100

Source: Abe (2014)

Comparative effects of flumioxazin and dihydroartemisinin on the haem synthetic pathway and cell proliferation in K562 cells. The objective of this study was to investigate the effects of flumioxazin and dihydroartemisinin (DHA), which causes fetal anaemia, leading to a similar pattern of developmental toxicity in rats, on the haem synthetic pathway in K562 cells. The study was not conducted according to GLP principles. K562 cells were plated onto 6 cm dishes at 5 × 10^5 cells/5 mL per dish in RPMI medium containing flumioxazin (5 µmol/L) or DHA (2, 0.5, 0.125 or 0 µmol/L) and sodium butyrate. Test substances were dissolved in DMSO. Cell densities were determined on days 2, 4, 6 and 8, with PPIX and haem content determined at the same intervals using LC/MS or LC/UV detection, respectively.

PPIX accumulation in K562 cells was observed with flumioxazin; however, there was no effect on cell proliferation or haem content per cell. DHA caused no accumulation of PPIX in K562 cells, but caused a reduction of haem content and a severe inhibition of cell proliferation at the highest concentration of 2 µmol/L, but not at lower concentrations. The impact of inhibition of cell proliferation on the results is unclear (Kawamura, 2015b).

Comparative effects of flumioxazin and dihydroartemisinin on the haem synthetic pathway and cell proliferation in rat erythroleukaemia cells. The objective of this non-GLP study was to investigate the effects of flumioxazin and DHA on the haem synthetic pathway in REL cells. REL cells were induced to differentiate into erythroid cells using HMBA. REL cells were plated onto 6 cm dishes at 5×10^5 cells/5 mL per dish in RPMI medium containing flumioxazin (5 µmol/L) or DHA (2, 0.5, 0.125 or 0 µmol/L) and HMBA. Cell densities were determined on days 2, 4, 6 and 8. On days 2, 4 and 6, the cells were subsequently subcultured at a concentration of 1×10^5 cells/mL in new dishes at 5 mL/dish. At 2 µmol/L DHA, the number of cells was too low to subculture at a concentration of 1×10^5 cells/mL on days 4 and 6, so 1 mL of test medium was added after 1 mL of sampling. PPIX and haem content were determined using LC/MS or LC/UV detection, respectively.

PPIX accumulation and reduction of haem content per cell were observed with flumioxazin; however, there was no effect on cell proliferation. DHA caused no accumulation of PPIX in REL cells, but caused a reduction of haem content and inhibition of cell proliferation at concentrations of 0.5 and 2 µmol/L. At the concentration of 2 µmol/L, cell proliferation was practically completely inhibited. The impact of this high level of inhibition of cell proliferation on the results is unclear (Kawamura, 2015a).

Comparative effects of flumioxazin and dihydroartemisinin on the haem synthetic pathway and cell proliferation in human CD36+ cells. The inhibition of haem synthesis by flumioxazin and DHA and accumulation of PPIX were investigated in a study with human CD36+ cells. The study was not conducted according to GLP principles. Human CD36+ cells were cultured in haematopoietic progenitor growth medium supplemented with stem cell factor, erythropoietin, IL-3 and IL-6 to induce differentiation into erythroid cells, which were then treated with flumioxazin (5 µmol/L) or DHA (2, 0.5, 0.125 or 0 µmol/L, both dissolved in DMSO), plated into 30 mm dishes at 6×10^5 cells/3 mL per dish. The seeding day was defined as day 0. Control cell suspension at day 0 was analysed for haem and PPIX content. The cells were counted on days 2, 4, 6 and 8, after which cells were stored at −80 °C until measurement. On days 2, 4 and 6, the cells were also subcultured at a concentration of 2×10^5 cells/mL. Concentrations of haem and PPIX were analysed using LC/MS and LC/UV, respectively.

PPIX accumulation in human CD36+ cells was observed with flumioxazin; however, there was no effect on cell proliferation or haem content per cell. DHA caused no accumulation of PPIX in human CD36+ cells, but caused a reduction of haem content and inhibition of cell proliferation at ≥ 0.125 µmol/L and ≥ 0.5 µmol/L, respectively. At the concentrations of 0.5 and 2 µmol/L, cell proliferation was practically completely inhibited. The impact of such high levels of inhibition of cell proliferation on the results is unclear (Kawamura, 2015c).

Haematological investigations in developing rats

Additional study to evaluate the potential of flumioxazin to cause fetal anaemia at developmentally toxic doses in rats. A study was designed to investigate the potential of flumioxazin to cause fetal anaemia at developmentally toxic doses in rats. A previous study demonstrated that flumioxazin induced embryo/fetal lethality and ventricular septal defects when administered at 30 mg/kg bw per day on GDs 6–15. This study was conducted to assess whether anaemia also occurs in the embryo at this dose. The study was not conducted according to GLP principles. Mated female Sprague-Dawley rats (20 per dose) were gavaged with flumioxazin at 0 (vehicle, 0.5% aqueous methyl cellulose), 15, 30 or 60 mg/kg bw per day on days 6–15 of gestation. Embryos from 10 dams per group were examined on day 14 of gestation to identify embryonic parameters related to anaemia; fetuses from another 10 dams per group were examined on day 20 of gestation to confirm fetal lethality and ventricular septal defects. Dams showed lower body weight and body weight gain at 60 mg/kg bw per day on GD 20, but not at earlier time points. Dose-dependent anaemia was confirmed in GD 14 embryos from dams dosed at 15, 30 and 60 mg/kg bw per day. Anaemia was characterized by pale yolk sacs and pale embryos, histopathologically decreased erythroblast content in the heart, iron deposits in erythroblasts and degenerative erythroblasts. Most of these findings showed a dose–

response increase in incidence and severity. Embryo/fetal lethality (and fewer live fetuses) was observed at 60 mg/kg bw per day (increased rate of late postimplantation loss at GD 14 and of early postimplantation loss at GD 20). Slightly increased incidences of ventricular septal defects were reported at 15 mg/kg bw per day and clearly at 30 and 60 mg/kg bw per day. Increased incidence and severity of thinning of ventricular walls and of atrium dilatation were reported in fetuses at 30 and 60 mg/kg bw per day. Fetal livers showed dilatation of sinusoidal vessels and hepatocytic necrosis in the peripheral region of moderate severity at 60 mg/kg bw per day. Lower fetal body weights were reported at 60 mg/kg bw per day and to a slight extent also at 30 mg/kg bw per day.

In summary, adverse findings were reported in fetuses in all dose groups starting at the lowest dose of 15 mg/kg bw per day (Hosokawa, 2015).

Chronological changes of morphology and population of circulating erythroblasts in rat embryos during yolk sac haematopoiesis. The differentiation of circulating erythroblasts in rat embryos (Crl:CD(SD)) was investigated at daily intervals from embryonic development day 11 through day 14 in a non-GLP study. Timed pregnant females (three per time point) were anaesthetized in the morning of the scheduled necropsy, and uteri and embryos were removed. Embryos were exsanguinated from severed umbilical cord, and embryonic blood cells from the same litter were pooled. On day 11 of embryonic development, more than 95% of circulating erythroblasts were basophilic erythroblasts. From day 12 to 13 of embryonic development, the predominant cell population was the polychromatophilic erythroblast (approximately 95%). At day 14, the population of polychromatophilic erythroblasts was markedly reduced (approximately 8%), with the orthochromatophilic erythroblasts as the predominant cell type (approximately 90%). Additionally, enucleated reticulocyte populations were detected.

The results of this study show that differentiation of circulating erythroblasts in rat embryos from embryonic days 11 to 14 was synchronized (Ihara, 2011).

Placental transfer studies

The placental transfer of flumioxazin was investigated in rats and mice, and in rats and rabbits, in two GLP-compliant studies. Data indicate that flumioxazin does cross the placenta and that both parent and various metabolites are present in measurable quantities in the fetus (Isobe, 1992, 1993). At the same dose level, the concentrations of ^{14}C and of flumioxazin are higher in rat fetuses than in rabbit fetuses, but lower than in the mouse (Isobe, 1993). No clear pattern of absorption, distribution, metabolism or excretion was evident that could account for the species differences in developmental toxicity observed in rats and rabbits.

Rats at day 12 of gestation and mice at day 10 of gestation were given a single oral dose of [phenyl-^{14}C]flumioxazin (for chemical structure, see Fig. 1) at 30 mg/kg bw (dissolved in corn oil). Concentrations of ^{14}C in maternal tissues and the fetus were examined at 1, 2, 4, 8, 24 and 72 hours after administration, and the excretion of ^{14}C into faeces and urine during 24 hours (mice) or 72 hours (rats) after administration was examined. Groups of four animals per time point were treated. Metabolites in excreta, blood cells, plasma, liver and fetus (mice only) were analysed. Metabolites were separated by thin-layer chromatography using several different mobile phases, the retention times were compared with synthetic reference standards and spots were detected using autoradiography. The metabolites were quantified by liquid scintillation counting.

Concentrations of ^{14}C in maternal tissues reached maxima more rapidly and decreased more rapidly in mice than in rats. Concentrations of ^{14}C in the fetus reached maxima 1 hour after administration for both species and decreased rapidly thereafter, with half-lives of 14 and 5 hours for rats and mice, respectively. Maximum ^{14}C concentrations were 1.05 and 1.72 ppm for rats and mice, respectively. ^{14}C was almost completely excreted within 3 and 1 days for rats and mice, respectively. The total ^{14}C excretion was 95.7% (faeces, 74.7%; urine, 21.0%) for rats and 95.8% (faeces, 72.9%; urine, 22.9%) for mice. The metabolism of flumioxazin seemed to be qualitatively the same in pregnant rats and mice. However, it was suggested that 3-hydroxylation activity might be higher in

pregnant rats than in pregnant mice. The major metabolite in the fetus of mice at 1 hour after administration was 4-OH-flumioxazin (Isobe, 1992).

A single oral dose of [phenyl-^{14}C]flumioxazin at 30 mg/kg bw was administered to pregnant rats (four animals per time point) and rabbits (two animals per time point) on day 12 of gestation. Maternal tissues and fetuses were obtained at 1, 2, 4 and 24 hours after dosing for analysis of ^{14}C concentrations and identification of metabolites in bone marrow, fat, fetus, kidney, liver, ovary, placenta, spleen, uterus and blood. After 24 hours, the ^{14}C excretion was found to be much slower in rabbits (30.2%: urine, 12%; faeces, 18.3%) than in rats (76.6%: urine, 21.7%; faeces, 54.9%). Concentrations of ^{14}C in the fetus, amniotic fluid and maternal tissues were higher in the rat than in the rabbit, with liver and kidney accounting for the highest concentrations in each species. Maximum ^{14}C concentrations in maternal tissues were higher than those observed in the fetus or amniotic fluid in both species. Maximum ^{14}C concentrations in the fetus were 0.782 and 0.2 ppm for rat and rabbit, respectively, observed at 4 hours after dosing for both species. For those time points, where sufficient fetal tissue was available for metabolite identification (1 and 24 hours for rats and 2 and 24 hours for rabbits), the highest concentration of parent flumioxazin was 0.06 ppm (at 1 hour after dosing) and 0.02 ppm (at 2 hours after dosing) in fetal tissue for rats and rabbits, respectively. Ratios between the ^{14}C concentrations in fetuses and those in maternal plasma were 0.21–0.26 in rats and 0.09–0.14 in rabbits (Isobe, 1993).

Pharmacokinetics and metabolism in rats and rabbits, and human PBPK model development

The pharmacokinetics of ^{14}C-labelled flumioxazin was investigated in groups of three pregnant rats and pregnant rabbits after repeated oral administration. Non-fasted pregnant rats and pregnant rabbits were administered ^{14}C-labelled flumioxazin at 30 mg/kg bw per day for 7 days by oral gavage from GDs 6 to 12. The study was not conducted according to GLP and was not quality assured. Concentrations of radioactivity in blood and plasma, excretion in urine and faeces, tissue concentrations, tissue distribution and transfer to female reproductive tissues were investigated. Blood, plasma and excreta were obtained daily 2 and 24 hours post-dosing. To complete a time course, blood and plasma were also collected 2, 4, 6, 8 and 24 hours after the final administration. Tissues were also collected at 7 and 24 hours (rats) or 3 and 24 hours (rabbits) after the final dose. Tissues collected included kidney, liver, spleen, fat, ovaries, placenta, uterus and fetus, along with amniotic fluid, to determine the concentration of radioactivity. Concentrations of flumioxazin and its metabolites were determined in pooled samples of urine, faeces, plasma, blood cells, liver, fetus and amniotic fluid.

Flumioxazin was excreted rapidly in pregnant rabbits (urine and faeces) and pregnant rats (faeces).

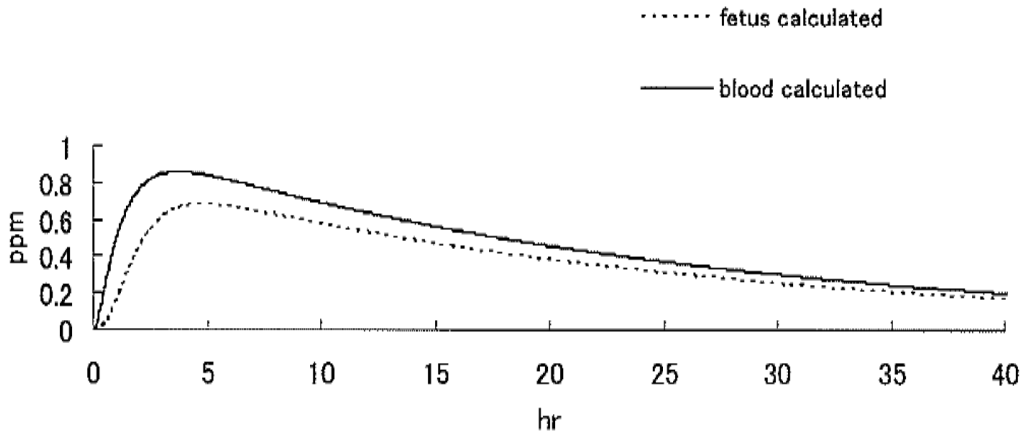
The concentration of radioactivity in blood and plasma almost reached steady state on day 4 and day 2, respectively, for rats. After the final administration to rats (day 7), the concentration of radioactivity in plasma increased gradually to reach 4.49 µg eq/mL at 8 hours after administration and then decreased to 1.71 µg eq/mL at 24 hours after administration, whereas the concentrations in both blood and plasma of rabbits did not reach steady state within the 7-day dosing period. After the final administration to rabbits on day 7, the concentration of radioactivity in plasma increased gradually to reach 4.19 µg eq/mL at 4 hours after administration, and then decreased gradually to 2.24 µg eq/mL 24 hours after administration. In rats, flumioxazin was distributed at higher concentrations in all tissues, except for the uterus, amniotic fluid and fetus, compared with plasma. In rabbits, flumioxazin was distributed at lower concentrations in all tissues, except for liver and kidney, than in plasma when compared with rats. Elimination of radioactivity from female reproductive tissue of both species was slower than that from plasma. Flumioxazin, APF, 3-OH-flumioxazin, 4-OH-flumioxazin, 3-OH-flumioxazin-SA, 4-OH-flumioxazin-SA and Ac-APFA were detected in excreta and tissues of pregnant animals and in fetuses. (An exception to this was for 3-OH-flumioxazin-SA and 4-OH-flumioxazin-SA in the fetuses of pregnant rabbits.) Concentrations of flumioxazin in the plasma and

blood cells of pregnant rats were generally similar to the concentrations in fetuses and amniotic fluid. In rat fetuses, 1.14 and 0.73 µg/g of radioactivity were detected after 7 and 24 hours, respectively. The concentration of flumioxazin in fetuses of rabbits was half that in rat fetuses: 0.32 and 0.20 µg/g of radioactivity were detected after 3 and 24 hours, respectively. When amniotic fluid of rabbits was analysed, no flumioxazin was detected. Tissue concentrations of flumioxazin and most metabolites were highest in the liver of pregnant rats compared with other tissues in the rat and compared with tissue concentrations in the rabbit. Whereas APF was identified at higher concentrations than other metabolites in most tissues of rats and in rabbit liver, it should be noted that most of the radioactivity in the evaluated rat and rabbit samples could not be identified (Shirai, 2009).

The biliary excretion of [phenyl-^{14}C]flumioxazin was investigated in female rats to determine oral absorption rate. Five bile duct–cannulated female rats received a single oral dose of [phenyl-^{14}C]flumioxazin at 1000 mg/kg bw. Recovery of ^{14}C in the bile, urine and faeces was measured during 0–72 hours. The study was not conducted according to GLP and was not quality assured. At termination, ^{14}C remaining in the gastrointestinal tract and the carcass was measured. Data from the three rats surviving to study termination were analysed (two rats died before the scheduled kill). The results demonstrated that most of the dosed radiocarbon was eliminated in the faeces and accounted for 84.7% of the dose by 72 hours post-dosing. The ^{14}C excretion in urine and bile accounted for 6.8 ± 1.9% and 5.2 ± 4.1%, respectively. In addition, residues remaining in the contents of the gastrointestinal tract and the residual carcass accounted for 0.6% and 0.3%, respectively. Total ^{14}C recoveries accounted for 97.6% of the dose. The majority of the administered compound was excreted within 24–48 hours. The absorption of [phenyl-^{14}C]flumioxazin following a single oral dose of 1000 mg/kg bw was calculated to be 12.3 ± 2.6% (amount in urine, bile and carcass; Takaku, 2012a).

A physiologically based pharmacokinetic (PBPK) model was developed to predict parent flumioxazin concentrations in the blood and fetus of pregnant humans based on data obtained in the rat. This work was not conducted according to GLP and was not quality assured. An in vitro metabolism study using rat and human liver microsomes was carried out to determine any species differences in the metabolism of flumioxazin between rat and human. Human microsomes were pooled from eight males and seven females, but no further details were included in the report (e.g. age, sex or genetic background/race). Physiological data for humans were cited from the literature, and the human model was developed to predict the pharmacokinetics in humans in several tissues. Although it is impossible, experimentally, to measure human fetal concentrations of flumioxazin, development of the PBPK model in the pregnant rat was scaled to humans to provide an estimate of the disposition of flumioxazin in pregnant humans. The metabolites produced by human microsomes were nearly identical to those produced by female rat liver microsomes; it was therefore concluded that there was no species difference in the metabolism of [phenyl-^{14}C]flumioxazin. The parameters for the rat PBPK model were manually optimized to reproduce the blood (predicted maximum concentration: approximately 0.08 ppm), liver (predicted maximum concentration: approximately 2.5 ppm) and fetus (predicted maximum concentration: approximately 0.06 ppm) concentrations of flumioxazin after a single oral dose of 30 mg/kg bw reported by Isobe (1992) and using 50% fraction absorbed (the values needed to be estimated from the figures in the study report, because the respective data were not reported). As no other experimental data were available for flumioxazin, the model could not be tested. The rat model was extrapolated to the pregnant human based on human physiological data and the in vitro metabolism data. The fraction of dose absorbed at 1000 mg/kg bw in rats was 12%. These values were then combined to develop the PBPK model at a dose of 1000 mg/kg bw. Simulated blood and fetal concentrations of flumioxazin at an oral dose of 1000 mg/kg bw in pregnant women were estimated to be 0.86 ppm and 0.68 ppm (maximum concentrations), respectively (Fig. 5). The predicted concentration in human liver was not reported. The part describing the in vitro metabolism results in the study report is too short to allow a proper evaluation of the results. AUC as a further toxicokinetic parameter was not reported (Takaku, 2012b).

Fig. 5. Predicted blood and fetal concentrations of flumioxazin at the dose of 1000 mg/kg bw in pregnant human



Source: Takaku (2012b)

Apparently, parts of the results of the study by Takaku (2012b) were also published in the open literature (Takaku, Nagahori & Sogame, 2014).

Discussion on human relevance of the developmental effects induced by flumioxazin in rats

Flumioxazin caused embryolethality, teratogenicity (especially ventricular septal defects) and growth retardation in rats at 30 mg/kg bw per day without maternal toxicity, but not in rabbits at the maternally toxic dose of 3000 mg/kg bw per day. The sponsor carried out a programme of research with flumioxazin to elucidate the mechanism of the developmental toxicity observed in rats. The assessment of the proposed mode of action for the induction of ventricular septal defects and of its relevance for humans is presented in Appendix 1.

(d) Literature review

A literature review was commissioned by the sponsor to assess published information on flumioxazin to support the European Union Plant Protection Product Regulation renewal procedure. The review focused on literature published between 1999 (the date of first inclusion into Annex I of the previous Plant Protection Product Directive) and the end of 2011, when the review was conducted. Information was sought that was relevant to the OECD data requirements for toxicological and toxicokinetic studies, residues in or on treated products (food and feed), fate and behaviour in the environment, and ecotoxicological studies. Where information with possible pertinence to other data requirements within the dossier was brought up as part of this search, this too was reviewed. A number of search terms were used separately or in conjunction across a range of databases sufficient to assess the entirety of literature available. Following a rapid assessment of the results that were obtained, 15 papers were identified as requiring detailed assessment (two for toxicology, four for environmental fate and nine for ecotoxicology). Following a detailed assessment, it was concluded that none of the papers had an impact on the human health risk assessment for flumioxazin (Hall, 2012).

A further literature search was commissioned by the sponsor to identify information published after 1 January 2005 on 1) substances inducing ventricular septal defects and 2) reports on the relationship between PPO (or porphyrinogen or porphyrin) and ventricular septal defects (Pharis, 2015). This was accomplished by four different search statements, which were used to query several relevant databases (e.g. Medline, CAplus and Biosis previews). In total, two articles were identified. One was not relevant for the intended question (a case report about a calf with ventricular septal

defect). The other article (Aasa et al., 2014) was included in Appendix 1 in the discussion of the human relevance of ventricular septal defects.

(e) Metabolites reported in residues

Some metabolites were reported to occur in residues in plant or animal metabolism studies. The metabolites 4-OH-flumioxazin and 3-OH-flumioxazin were reported to be major residues in goat and poultry matrices when animals were evaluated with a relatively short post-administration period.

Besides limited mechanistic in vitro data, no toxicological data on these metabolites were submitted. The metabolites 4-OH-flumioxazin and 3-OH-flumioxazin were observed in rats in amounts above 10%, when further downstream metabolites observed in toxicokinetic studies are included.

Flumioxazin and the metabolites 4-OH-flumioxazin and 3-OH-flumioxazin were reported as Cramer class III (modules: Cramer rules; Cramer rules, with extensions), when evaluated with Toxtree (v. 2.6.13; Patlewicz et al., 2008).

When evaluated with the module "in vitro mutagenicity (Ames test) alerts by ISS" (Benigni & Bossa, 2011; Benigni, Bossa & Tcheremenskaia, 2013), flumioxazin and the metabolites 4-OH-flumioxazin and 3-OH-flumioxazin raised alerts for *Salmonella typhimurium* mutagenicity. It is noted that the same groups led to the alerts in all three chemicals; in the available studies, however, flumioxazin was unlikely to be genotoxic in vivo.

3. Observations in humans

3.1 Medical surveillance on manufacturing plant personnel

Manufacturing records of flumioxazin technical material and the health status of workers engaged in the manufacturing operations were reviewed. Flumioxazin technical material was manufactured at factories in Japan during 2000–2010. During this period of operation, production volume rose from 50 to approximately 700 tonnes per year. During this time, 14–15 workers were engaged in the manufacture of flumioxazin. Under Japanese law, every company employee is required to undergo annual medical examinations. These examinations include investigation of medical, work, smoking and medication histories; presence/absence of subjective and/or objective symptoms; body measurements (height, weight, abdominal circumference, body mass index); eyesight and audibility; chest X-ray examination; blood pressure measurement; haematology (red and white blood cell counts, haematocrit, haemoglobin content, platelet count); liver function test (aspartate transaminase, alanine transaminase, gamma-glutamyltranspeptidase); blood lipid examination (total cholesterol, low-density/high-density lipoprotein cholesterol, triglyceride); blood sugar and haemoglobin A1c; electrocardiogram; urine analysis (sugar, protein, occult blood, urobilinogen); and renal function test (urea nitrogen, creatinine, uric acid). At the manufacturing site of flumioxazin technical material, no adverse health effects have been reported from the results of the annual medical examinations (Nishioka, 2011).

3.2 Variegate porphyria

The disease variegate porphyria is a disorder of hepatic haem biosynthesis, which is seen in humans carrying autosomal dominant mutations in the gene for PPO (Gross, Hoffmann & Doss, 2000; Nordmann & Puy, 2002; Ajioka, Phillips & Kushner, 2006; Phillips & Anderson, 2010). At least 100 mutations have been identified. These mutations can inhibit the function of the enzyme, which catalyses one of the last steps in the haem biosynthesis pathway of protoporphyrinogen IX to PPIX (see Fig. 3 above). Most haem synthesized in liver is required for cytochrome P450 synthesis. Patients with this disease present with neurovisceral symptoms and/or blistering skin lesions induced by photosensitivity. In faeces, coproporphyrin III and protoporphyrin levels are increased. A characteristic marker for variegate porphyria would be fluorescence maximum in plasma at

approximately 626 nm. The risks for chronic hypertension, renal disease and hepatocellular carcinoma are increased. Unrelated individuals with the same mutation may present with different clinical phenotypes.

According to published literature, there are several cases with heterozygous variegate porphyria, but only a few cases with the homozygous type. The latter cases are often of higher severity.

A 6-year-old boy and his 8-month-old sister were diagnosed with homozygous variegate porphyria based on analysis of plasma, urine and faeces for porphyrins and precursors and measurement of PPO activity. They had serious photodermatosis and ocular nystagmus. Both children were mentally retarded (Korda et al., 1984).

According to a case report, homozygous variegate porphyria might be associated with retarded growth and/or dwarfism. One adult patient had marked retarded bone age in X-rays of the hands (Murphy et al., 1986).

According to another case report, a boy diagnosed with homozygous variegate porphyria showed, in addition to severe skin lesions, unusually short fingers and toes (Mustajoki et al., 1987):

> Motor development was slower than usual: the patient learnt to sit at 10 months and to walk at 18 months, and was clumsy in his movements. His EEG [electroencephalogram] at the age of three years was slightly abnormal, with occasional slow waves and irregularity but without focal findings. In psychological tests mental development and speech were found to be normal. His rate of growth has followed a curve 1 SD [standard deviation] below the mean values for weight and height.

A 14-year-old girl was described as mentally subnormal and attending a special school. Her "skin of face, backs of hands and knees was dry with crusts, erosions, milia and multiple superficial scars.…Perioral radial scarring was present, as was clinodactyly and brachydactyly of the fifth fingers and flexion deformities of other fingers." She was diagnosed with homozygous variegate porphyria (Norris, Elder & Hawk, 1990).

In a further report (Hift et al., 1993), the case of a 6-year-old South African girl diagnosed with homozygous variegate porphyria was described:

> born to non-consanguineous parents, developed severe blistering of the face and hands within days of birth.…She has never developed symptoms of the acute porphyric attack. She has markedly foreshortened, stubby fingers and toes, resulting in severe functional impairment such that she cannot grasp a pencil or use a pair of scissors. Radiographs demonstrate brachydactyly with broad, short metacarpals and phalanges, and a delayed bone age. She has severe skin disease, manifested by blistering, erosions, crusting, pigmentation and milia. The lesions have a wider distribution than those of heterozygous VP [variegate porphyria]. In addition to typical blistering and scarring of sun-exposed areas, the skin in non-exposed areas of the arms, legs and trunk is thickened and infiltrated, and clearly abnormal.…She has severe myopia and a pendular nystagmus. Neurological development is delayed. Her perceptual and motor development are about one year behind the expected stage of development. Verbal skills are appropriate for age and she appears to be of normal intelligence. Since the age of 3 she has suffered infrequent complex partial seizures; an electroencephalogram was, however, normal. Over the [previous] 2 years she has developed a gross sensory neuropathy of the hands and feet, manifesting with painless trauma to the hands. Electrophysiological studies of nerve conduction have demonstrated a reduced amplitude of conduction in the right and left median sensory nerves in keeping with a sensory neuropathy. No motor abnormalities were demonstrable. Sural nerve conduction studies were normal, yet abnormalities were shown on histological examination of a sural nerve biopsy specimen.

Regarding another case, it was stated (Hift et al., 1993) that a

> male infant, born to consanguineous parents, developed epilepsy at 5 months and showed developmental delay, nystagmus and clinodactyly. Skin manifestations were noted from the age of 6 months. He presented to a dermatology clinic at the age of 6 with blistering and fragility of the skin of his hands, face and ears. Examination revealed hypertrichosis, hyperpigmentation, scarring and milia of these areas.

A 7-year-old Chilean boy had severe photosensitivity at the age of 6 months and also blisters and erosions developing recurrently. At the age of 7 years, pronounced scarring in sun-exposed body areas was additionally described, as well as a short stature (2 standard deviations below reference height) and shortening of fingers. He attended school regularly and did not reveal mental retardation or any neurological disturbances. Via polymerase chain reaction analysis, he was diagnosed with homozygous variegate porphyria (Poblete-Gutiérrez et al., 2006).

When the genes for PPO in five patients with homozygous variegate porphyria were sequenced, two had homoallelic mutations (from consanguineous parents), and three had heteroallelic mutations (from unrelated parents) (Roberts et al., 1998).

Porteous (1963) described a women with idiopathic porphyria delivering a live girl but developing a psychotic state with transfer to a mental hospital afterwards. She showed signs of severe peripheral neuritis and renal failure. Six years later, she was pregnant a second time, delivering in week 36 a live boy who died after 1 day due to atelectasis. The third pregnancy resulted in early delivery of a healthy girl in week 37. Following each pregnancy/delivery, the mother's condition was aggravated, but could be treated.

Porteous (1963) summarized the knowledge on pregnancy in patients with porphyria:

> Neilson and Neilson (1958) analyzed 40 cases of porphyria complicated by pregnancy which were reported in the world literature. Ninety-five per cent of these deteriorated during pregnancy and the overall maternal mortality was 42.5 per cent. Only 60.5 per cent of the pregnancies proceeded to foetal viability and in those which did there was a foetal mortality of 63 per cent. One of the 40 patients had the erythropoietic or congenital form of porphyria where the defect is in the bone marrow. The other 39 patients suffered from the hepatic form of porphyria. Most of the latter were affected by the acute intermittent type of hepatic porphyria but one patient had the chronic cutaneous type of hepatic porphyria, and four patients suffered from the mixed type of hepatic porphyria.

Similarly, Dean (1953) described "during pregnancy the patient is usually much worse, perhaps partly because she is then given sedatives. There is often a history that previous pregnancies were terminated because of pains, vomiting, and hysteria." However, it is not totally clear to which types of porphyria the findings relate, but a relevance for variegate porphyria cannot be excluded. Other authors concluded that pregnancy does not influence the course of variegate porphyria, nor would variegate porphyria influence pregnancy (Bloch, 1965).

Hift et al. (1993) summarized the symptoms in the known cases of patients with homozygous variegate porphyria:

> It is an interesting observation that all the parents of the cases described thus far appear to have been latent carriers as none are reported to have expressed VP clinically. A review of the eight reported cases shows them, with one exception, to be remarkably similar. Both sexes are affected and the condition presents with photosensitivity within days to months of birth. Many have been mentally retarded. Delayed development is common and most will suffer seizures. Nystagmus may be noted. Structural abnormalities of the hands are frequently reported, ranging from clinodactyly to severe deformity. Growth retardation and a retarded bone age are frequent and perhaps invariable.

The inhibition of PPO would be similar to the herbicidal mechanism of action proposed by the sponsor.

Comments

Biochemical aspects

Experiments in bile duct–cannulated rats given 1 mg/kg bw orally showed that absorption of flumioxazin was at least 80%, with similar amounts in urine and bile (Gibson et al., 1997). Radioactivity in the urine of rats given [^{14}C]flumioxazin was 31–43% at the low dose (1 mg/kg bw) and 13–23% at the high dose (100 mg/kg bw). Flumioxazin was widely distributed. Radioactivity was detected mainly in excretory organs and was eliminated rapidly, showing that flumioxazin and its

metabolites do not accumulate in tissues. Radioactivity in faeces of rats given [^{14}C]flumioxazin was 56–72% at the low dose and 78–88% at the high dose. Excretion was rapid, with 69–87% of the dose being eliminated in urine and faeces within 24 hours of dosing. Urinary excretion of radioactivity was higher in females than in males in all groups, and pretreatment with unlabelled flumioxazin had little effect on the route or rate of excretion (Matsunaga, 1993; Shiba, 1994).

Flumioxazin was metabolized extensively. Seven of the 35 metabolites detected and quantified when dosing with [phenyl-^{14}C]flumioxazin were identified, and 10 of the 29 metabolites detected and quantified when dosing with [phthalimide-^{14}C]flumioxazin were identified. The main metabolic reactions were hydroxylation of the cyclohexene ring of the tetrahydrophthalimide moiety, cleavage of the imide linkage, cleavage of the amide linkage in the benzoxazine ring, reduction of the double bond in the tetrahydrophthalimide ring, acetylation of the amino group of the aniline derivative and incorporation of a sulfonic acid group in the tetrahydrophthalimide ring (Matsunaga, 1993; Shiba 1994).

Toxicological data

Flumioxazin was not acutely toxic to rats, with no mortality at limit doses after oral (LD_{50} > 5000 mg/kg bw; Hiromori, 1990a) or dermal (LD_{50} > 2000 mg/kg bw; Hiromori, 1990b) exposure. In rats exposed to flumioxazin via inhalation, an LC_{50} greater than 3.93 mg/L was reported (Kawaguchi, 1990). Flumioxazin was not irritating to the skin (Nakanishi, 1989) and caused only minimal eye irritation in rabbits (Nakanishi, 1989). It was not a skin sensitizer in a maximization test (Nakanishi, 1990).

Short-term toxicity studies were conducted in the mouse, rat and dog. In all three species, liver was a target organ. Anaemia was also observed in the rat.

In a 13-week study, mice received flumioxazin in the diet at a concentration of 0, 100, 1000, 3000 or 10 000 ppm (equal to 0, 16.3, 164, 459 and 1062 mg/kg bw per day for males and 0, 18.6, 202, 595 and 2163 mg/kg bw per day for females, respectively). The NOAEL was 3000 ppm (equal to 459 mg/kg bw per day), based on increased liver weight and decreased ovary weight at 10 000 ppm (equal to 1062 mg/kg bw per day) (Hagiwara, 1990).

In a 90-day study in rats, flumioxazin was offered to groups of rats at a dietary concentration of 0, 30, 300, 1000 or 3000 ppm (equal to 0, 2.28, 20.7, 69.7 and 244 mg/kg bw per day for males and 0, 2.21, 21.7, 71.5 and 230 mg/kg bw per day for females, respectively). The NOAEL was 300 ppm (equal to 20.7 mg/kg bw per day), based on changes in haematological and clinical chemistry parameters at 1000 ppm (equal to 69.7 mg/kg bw per day) (Hagiwara, 1989).

In a second 90-day study in rats, flumioxazin was offered to animals at a dietary concentration of 0, 30, 300, 1000 or 3000 ppm (equal to 0, 1.9, 19.3, 65.0 and 197 mg/kg bw per day for males and 0, 2.2, 22.4, 72.9 and 218 mg/kg bw per day for females, respectively). The NOAEL was 30 ppm (equal to 2.2 mg/kg bw per day), based on haematological changes (including anaemia and extramedullary haematopoiesis) in females at 300 ppm (equal to 22.4 mg/kg bw per day), although the effects were marginal. For males, the NOAEL was 300 ppm (equal to 19.3 mg/kg bw per day), based on increases in liver, heart, kidney and thyroid weights at 1000 ppm (equal to 65.0 mg/kg bw per day) (Adachi, 1991).

In dog studies, flumioxazin was administered in gelatine capsules to groups of dogs of both sexes at a dose of 0, 10, 100 or 1000 mg/kg bw per day for 90 days (Nakano, 1993) or for 1 year (Nakano, 1992). The NOAELs for both the 90-day and 12-month studies were 10 mg/kg bw per day, based on increases in total cholesterol and phospholipid levels and elevated alkaline phosphatase activity at 100 mg/kg bw per day in the 90-day study and similar changes (elevated alkaline phosphatase activity and increased liver weights) at 100 mg/kg bw per day in the 12-month study.

In a 78-week study in mice, flumioxazin was administered at a dietary concentration of 0, 300, 3000 or 7000 ppm (equal to 0, 31, 315 and 754 mg/kg bw per day for males and 0, 37, 346 and

859 mg/kg bw per day for females, respectively). The incidence of centrilobular hepatocyte hypertrophy was increased in males receiving 3000 and 7000 ppm; at these doses, there was an increase in diffuse hypertrophy and single-cell necrosis of hepatocytes in females. The NOAEL was 300 ppm (equal to 37 mg/kg bw per day), based on non-neoplastic changes in the liver in females. In this study, no increases in tumour incidence were reported (Seki, 1993a).

In a 24-month rat study, flumioxazin was administered at a dietary concentration of 0, 50, 500 or 1000 ppm (equal to 0, 1.8, 18 and 36.5 mg/kg bw per day for males and 0, 2.2, 21.8 and 43.6 mg/kg bw per day for females, respectively). At the highest dose tested (1000 ppm), anaemia was the most significant toxicological finding; it was also apparent in rats receiving 500 ppm flumioxazin. The NOAEL was 50 ppm (equal to 1.8 mg/kg bw per day), based on an increase in anaemia. Under the conditions of the study, tumour incidence was unaffected by treatment (Seki, 1993b).

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

Flumioxazin was tested for genotoxicity in an adequate range of assays, both in vitro and in vivo. Flumioxazin was negative in in vitro studies (Kogiso, 1989; Wollny, 2011), but resulted in an increase in chromosomal aberrations (Kogiso, 1988). However, there was no genotoxic activity in follow-up in vivo studies (Hara & Kogiso, 1988; Hara, 1990; Kogiso, 1990).

The Meeting concluded that flumioxazin is unlikely to be genotoxic in vivo.

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

A two-generation reproductive toxicity study in rats was conducted with flumioxazin using dietary concentrations of 0, 50, 100, 200 and 300 ppm (during the pre-mating period, equal to 0, 3.2, 6.3, 12.7 and 18.9 mg/kg bw per day for males and 0, 3.8, 7.6, 15.1 and 22.7 mg/kg bw per day for females, respectively). The NOAEL for parental toxicity was 200 ppm (equal to 12.7 mg/kg bw per day), based on clinical signs of toxicity and reductions in body weight, body weight gain, feed consumption and organ weights. The reproductive NOAEL was also 200 ppm (equal to 12.7 mg/kg bw per day), based on reduced gestation index in both the F_0 and F_1 generations and on an increase in the number of F_0 dams that did not deliver a litter. The NOAEL for offspring toxicity was 50 ppm (equal to 3.2 mg/kg bw per day), based on increased postnatal pup mortality in the F_1 generation (Hoberman, 1992).

Flumioxazin was administered by gavage to groups of pregnant rats at a dose of 0, 1, 3, 10 or 30 mg/kg bw per day during organogenesis (days 6 through 15). The NOAEL for embryo and fetal toxicity in rats was 3 mg/kg bw per day, based on an increased incidence of malformations (cardiac ventricular septal defects) in the fetuses at 10 mg/kg bw per day. The NOAEL for maternal toxicity was 30 mg/kg bw per day, the highest dose tested (Kawamura, 1990a).

Studies have been conducted to elucidate the mechanism of formation of ventricular septal defects reported in rats as a possible consequence of PPO inhibition. GD 12 was determined to be the most sensitive day, based on the incidence of embryonic deaths, reductions in fetal weight and the incidence of ventricular septal defects (Kawamura, 1993). Effects were seen in offspring within hours after administration (Kawamura, 1996a, 1997b; Kawamura & Yoshioka, 1997; Hosokawa, 2015) and progressed through the development of the pups/fetuses (Kawamura, 1997a). In studies comparing effects seen in rats and in rabbits, fewer or no effects were seen in rabbits (Kawamura, 1996a, 1997b; Kawamura & Yoshioka, 1997).

Flumioxazin inhibits a key enzyme, PPO, in the rat, thereby interfering with normal haem synthesis (Green & Dabbs, 1993, 1996; Noda, 1995; Abe, 2011a, 2014). Available in vitro investigations in specimens and cell lines from different species indicated (slightly) higher sensitivity of rats compared with rabbits or humans (Green & Dabbs, 1993, 1996; Noda, 1995; Abe, 2011a; Kawamura, 2012a, 2013a,b, 2015a,b,c).

The disease variegate porphyria is a disorder of hepatic haem biosynthesis, which is seen in humans carrying autosomal dominant mutations in the gene for PPO (Gross, Hoffmann & Doss, 2000; Nordmann & Puy, 2002; Ajioka, Phillips & Kushner, 2006; Phillips & Anderson, 2010). Children with homozygous variegate porphyria were reported to exhibit various findings, such as photosensitivity, retarded mental development, delayed development, seizures, nystagmus or structural abnormalities of the hands (Hift et al., 1993).

The sponsor proposed a case that the induction of ventricular septal defects in rats is not relevant for humans. However, the Meeting considered that the case is not robust enough to support the mode of action in rats and to demonstrate the non-relevance for humans.

Pregnant rabbits were administered flumioxazin by oral gavage at 0, 300, 1000 or 3000 mg/kg bw per day on days 7–19 of gestation. The NOAEL for embryo and fetal toxicity was 3000 mg/kg bw per day, the highest dose tested. The NOAEL for maternal toxicity was 1000 mg/kg bw per day, based on reductions in maternal body weight gains and feed consumption (Hoberman, 1991d).

The Meeting concluded that flumioxazin is teratogenic in rats, but not in rabbits.

In an acute neurotoxicity study, flumioxazin was administered once via gavage at a dose of 0, 200, 700 or 2000 mg/kg bw to groups of rats of both sexes. The NOAEL for acute neurotoxicity was 2000 mg/kg bw, the highest dose tested, based on the absence of specific neurotoxic effects (Herberth, 2011a).

In a subchronic neurotoxicity study, flumioxazin was administered to groups of rats via the diet at a dose of 0, 500, 1500 or 4500 ppm (equal to 0, 37, 110 and 323 mg/kg bw per day for males and 0, 41, 124 and 358 mg/kg bw per day for females, respectively) for 90 days. The NOAEL for subchronic neurotoxicity was 4500 ppm (equal to 323 mg/kg bw per day), the highest dose tested. Haematological changes were reported for all dose groups from 500 ppm (equal to 37 mg/kg bw per day) (Herberth, 2011b).

The Meeting concluded that flumioxazin is not neurotoxic.

In a 28-day immunotoxicity study in female rats, groups of animals were treated with diet containing 0, 500, 1500 or 4500 ppm flumioxazin (equal to 0, 42, 126 and 371 mg/kg bw per day, respectively). No adverse effects on antibody-forming cell response were reported up to 4500 ppm (equal to 371 mg/kg bw per day), the highest dose tested. Haematological changes were reported from 1500 ppm (equal to 126 mg/kg bw per day) (Crittenden, 2011).

The Meeting concluded that flumioxazin is not immunotoxic.

Toxicological data on metabolites and/or degradates

Some metabolites were reported to occur as residues in plant metabolism studies. The metabolites 4-OH-flumioxazin and 3-OH-flumioxazin were reported to be major residues in goat and poultry matrices when animals were evaluated after a relatively short post-administration period.

Besides mechanistic in vitro data on PPO inhibition, no toxicological data were submitted on some of these metabolites. The metabolites 4-OH-flumioxazin and 3-OH-flumioxazin were observed in rats in amounts above 10%, when further downstream metabolites observed in toxicokinetic studies are included. Flumioxazin and the metabolites 4-OH-flumioxazin and 3-OH-flumioxazin were reported as Cramer class III (modules: Cramer rules; Cramer rules, with extensions), when evaluated with Toxtree (v. 2.6.13; Patlewicz et al., 2008).

Acute and chronic exposures to 3-OH-flumioxazin and 4-OH-flumioxazin (up to 9 µg/day) are below the threshold of toxicological concern (TTC) for Cramer class III compounds (90 µg/day). Hence, no safety concern is anticipated.

Human data

In reports on manufacturing plant personnel, no adverse health effects were noted (Nishioka, 2011). No information on accidental or intentional poisoning in humans is available.

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

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.02 mg/kg bw on the basis of a NOAEL of 1.8 mg/kg bw per day for anaemia in a long-term study in rats, with application of a safety factor of 100.

The Meeting established an acute reference dose (ARfD) of 0.03 mg/kg bw on the basis of a NOAEL of 3 mg/kg bw per day for malformations in a developmental toxicity study in rats, with application of a safety factor of 100. This ARfD applies to women of childbearing age only.

The Meeting concluded that it is not necessary to establish an ARfD for the remainder of the population in view of the low acute oral toxicity of flumioxazin and the absence of other toxicological effects that would be likely to be elicited by a single dose.

Levels relevant to risk assessment of flumioxazin

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of toxicity and carcinogenicity[a]	Toxicity	300 ppm, equal to 37 mg/kg bw per day	3 000 ppm, equal to 346 mg/kg bw per day
		Carcinogenicity	7 000 ppm, equal to 754 mg/kg bw per day[b]	–
Rat	Two-year study of toxicity and carcinogenicity[a]	Toxicity	50 ppm, equal to 1.8 mg/kg bw per day	500 ppm, equal to 18 mg/kg bw per day
		Carcinogenicity	1 000 ppm, equal to 36.5 mg/kg bw per day[b]	–
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	200 ppm, equal to 12.7 mg/kg bw per day	300 ppm, equal to 18.9 mg/kg bw per day
		Parental toxicity	200 ppm, equal to 12.7 mg/kg bw per day	300 ppm, equal to 18.9 mg/kg bw per day
		Offspring toxicity	50 ppm, equal to 3.2 mg/kg bw per day	100 ppm, equal to 6.3 mg/kg bw per day
	Developmental toxicity study[c]	Maternal toxicity	30 mg/kg bw per day[b]	–
		Embryo and fetal toxicity	3 mg/kg bw per day	10 mg/kg bw per day
Rabbit	Developmental toxicity study[c]	Maternal toxicity	1 000 mg/kg bw per day	3 000 mg/kg bw per day
		Embryo and fetal toxicity	3 000 mg/kg bw per day[b]	–
Dog	Ninety-day and 1-year studies of toxicity[d,e]	Toxicity	10 mg/kg bw per day	100 mg/kg bw per day

[a] Dietary administration.

[b] Highest dose tested.
[c] Gavage administration.
[d] Gelatine capsule administration.
[e] Two or more studies combined.

Estimate of acceptable daily intake (ADI)

0–0.02 mg/kg bw

Estimate of acute reference dose (ARfD)

0.03 mg/kg bw (applies to women of childbearing age)

Unnecessary (for the general population)

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

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

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

Absorption, distribution, excretion, and metabolism in mammals	
Rate and extent of oral absorption	> 80% in the rat
Dermal absorption	Not given
Distribution	Widely distributed
Potential for accumulation	No potential for accumulation
Rate and extent of excretion	Rapidly excreted, 30–40% via urine and 60% via faeces within 7 days
Metabolism in animals	Extensively metabolized; hydroxylation of cyclohexene ring and cleavage of the imide linkage
Toxicologically significant compounds in animals and plants	Parent compound
Acute toxicity	
Rat, LD_{50}, oral	> 5 000 mg/kg bw
Rat, LD_{50}, dermal	> 2 000 mg/kg bw
Rat, LC_{50}, inhalation	> 3.93 mg/L air (maximal attainable concentration)
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Minimally irritating
Guinea-pig, dermal sensitization	Not sensitizing (Magnusson & Kligman test)
Short-term studies of toxicity	
Target/critical effect	Haematotoxicity (rat), liver (mouse, rat)
Lowest relevant oral NOAEL	2.2 mg/kg bw per day (90 d rat)
Lowest relevant dermal NOAEL	300 mg/kg bw (rat)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Haematotoxicity (rat), liver (mouse)

Lowest relevant oral NOAEL	1.8 mg/kg bw per day (2-year rat)
Carcinogenicity	Not carcinogenic in mice or rats[a]
Genotoxicity	
	Unlikely to be genotoxic in vivo[a]
Reproductive toxicity	
Target/critical effect	Impairment of reproductive capacity at dose levels associated with systemic toxicity; increased postnatal pup mortality in the absence of parental toxicity
Lowest relevant parental NOAEL	12.7 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	3.2 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	12.7 mg/kg bw per day (rat)
Developmental toxicity	
Target/critical effect	Malformations (ventricular septum defects)
Lowest relevant maternal NOAEL	30 mg/kg bw per day (rat)
Lowest relevant embryo/fetal NOAEL	3 mg/kg bw per day (rat)
Neurotoxicity	
Acute neurotoxicity NOAEL	2 000 mg/kg bw (highest dose tested; rat)
Subchronic neurotoxicity NOAEL	323 mg/kg bw per day (highest dose tested; rat)
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Mechanism studies	Mechanistic studies related to developmental toxicity in rats
Studies performed on metabolites or impurities	In vitro studies with metabolites were performed within the mechanistic studies for the proposed mode of action for developmental toxicity in rats
Immunotoxicity NOAEL	371 mg/kg bw per day (highest dose tested; rat)
Medical data	
	No evidence of adverse health effects attributed to flumioxazin during manufacturing operations reported

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

Summary

	Value	Study	Safety factor
ADI	0–0.02 mg/kg bw	Two-year toxicity study (rat)	100
ARfD[a]	0.03 mg/kg bw	Developmental toxicity study (rat)	100

[a] Applies to women of childbearing age only; no ARfD necessary for the general population.

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Appendix 1. Mode of action analysis using the WHO/IPCS framework

When the sponsor was asked to provide an assessment of the human relevance of the malformations observed in rats based on the World Health Organization (WHO)/International Programme on Chemical Safety (IPCS) human relevance framework (Meek et al., 2014), a statement was provided, which is taken into account and analysed in the following.

Problem formulation

In rat developmental toxicity studies with flumioxazin, increases of ventricular septal defects were reported in offspring. The mode of action and the possible relevance of this effect for humans are analysed below.

Hypothesized mode of action statement

The sponsor (C. Harris, personal communication, 2015) proposed the following mode of action:

> Mechanistic research has established that toxic effects observed in the developmental studies and to a lesser extent the repeat dose studies (haematotoxicity) result from inhibition of the enzyme protoporphyrinogen oxidase (PPO) (PPO is responsible for the 7th step in haem production, by removing hydrogen atoms from protoporphyrinogen IX to form protoporphyrin IX). The effects reported in the rat developmental study were observed in the absence of maternal toxicity. In the rabbit developmental study, whilst the administered dose was 100-fold greater than in the rat study and maternal toxicity was observed, no embryolethal or teratogenic effects were observed. There is convincing evidence for a single mode of action causing the developmental toxicity in the rat. The sequence of key biological events in the proposed mode of action has been elucidated. Inhibition of PPO interferes with normal haem synthesis, which causes loss of blood cells leading to embryo-fetal anaemia, embryolethality and the development of malformations. Rats are particularly sensitive to the effects of PPO inhibition induced by flumioxazin in erythroblasts. This leads to anaemia that is a critical precursor of the developmental toxicity resulting from flumioxazin exposure.

Summary of data for use in mode of action analysis

In Table A1, the data used in the mode of action analysis are summarized.

Table A1. Summary of data for use in mode of action analysis

Species	Route	Route/dose (mg/kg bw per day)	Incidence of VSD (affected fetuses/ litters)	Comments	Reference
Rat	Oral	0	2/2	–	Kawamura (1990)
		1	1/1		
		2	2/2		
		10	6/5		
		30	26**/12##		
Rat	Oral	0	0/0	Range-finding study	Kawamura (1989)
		30	11**/5##		
		100, 200, 500	Not analysable	Dose groups 100, 200 and 500 mg/kg bw per day had too few offspring to analyse	
Rat	Dermal	0	1/1	–	Kawamura (1991)
		30	1/1		
		100	2/2		
		300	13*/9##		
Rat	Oral	400	GD 11: 5/2	One treatment on different GDs only, 5 dams per group	Kawamura (1993)
			GD 12: 6/3		
			GD 13: 3/2		
			GD 14: 3/2		
			GD 15: 1/1		

bw: body weight; GD: gestation day; VSD: ventricular septal defects; *: $P < 0.05$; **: $P < 0.01$ (Wilcoxon's rank sum test); ##: $P < 0.01$ (Fisher's exact test)

Listing of key events identified for a specific mode of action

According to the WHO/IPCS mode of action framework, key events are measurable events that are critical to the induction of the (adverse) effect. The key events are listed in order from earliest to latest key event; the last key event should be the (adverse) effect.

The identified key events for flumioxazin's mode of action are listed in Table A2.

Table A2. Listing of key events identified for a specific mode of action

Key event	Description
1	Inhibition of PPO
2	Anaemia

PPO: protoporphyrinogen oxidase

Bradford Hill considerations for weight of evidence analysis of available data/information for mode of action analysis in experimental species

Dose–response relationships and temporal association

In this section, concordance of dose–response relationships together with temporal association are presented.

Dose–response relationship

Only doses inducing malformations were tested for accumulation of PPIX, but not doses below this dose level. Additionally, mainly single dose levels were used in the in vivo mechanistic studies (Kawamura, 1996a,b, 1997a).

A study was designed to investigate the potential of flumioxazin to cause fetal anaemia at developmentally toxic doses in rats. Mated female Sprague-Dawley rats were gavaged with flumioxazin at 0 (vehicle, 0.5% aqueous methyl cellulose), 15, 30 or 60 mg/kg bw per day on days 6–15 of gestation. Dams showed lower body weight and body weight gain at 60 mg/kg bw per day on GD 20, but not at earlier time points. Dose-dependent anaemia was confirmed in GD 14 embryos from dams dosed at 15, 30 or 60 mg/kg bw per day. Anaemia was characterized by pale yolk sacs and pale embryos, histopathologically decreased erythroblast content in the heart, iron deposits in erythroblasts and degenerative erythroblasts. Embryo/fetal lethality (and fewer live fetuses) was observed at 60 mg/kg bw per day (increased rate of late postimplantation loss at GD 14 and of early postimplantation loss at GD 20). Slightly increased incidences of ventricular septal defects were reported at 15 mg/kg bw per day and clearly at 30 and 60 mg/kg bw per day. Increased incidence and severity of thinning of ventricular walls and of atrium dilatation were reported in fetuses at 30 and 60 mg/kg bw per day. Fetal livers showed dilatation of sinusoidal vessels and hepatocytic necrosis in the peripheral region of moderate severity at 60 mg/kg bw per day. Lower fetal weights were reported at 60 mg/kg bw per day and, to a slight extent, also at 30 mg/kg bw per day. Adverse findings were reported in fetuses in all dose groups starting at the lowest dose of 15 mg/kg bw per day (Hosokawa, 2015).

Hence, a dose–response relationship for an increase in PPIX concentration cannot be established, whereas it can be seen for the induction of anaemia.

Temporal association

To examine the accumulation of PPIX in rat and rabbit embryos, pregnant rats and rabbits were administered a single oral dose of flumioxazin in 0.5% methyl cellulose at 1000 mg/kg bw, and

the concentrations of PPIX in embryos and maternal livers were measured at 2, 6, 12, 18 and 24 hours after administration for rats and at 2, 6, 12, 24 and 48 hours after administration for rabbits. The concentrations of PPIX in rat embryos and maternal livers were much higher than in the corresponding tissues of the rabbit, with peak concentrations observed at 12 hours for embryos and 6–12 hours for maternal livers (Kawamura, 1996a).

In a follow-up study, the development of fetuses was examined 6, 12, 24, 36 or 48 hours after treatment with flumioxazin at 1000 mg/kg bw per day in rat dams and after 6, 24 or 48 hours in rabbit does. No embryonic deaths were observed in rats at 24 hours after treatment. The first intrauterine deaths were observed at 36 hours, and embryonic mortality increased to 93.2% at 48 hours after treatment. A primary effect was observed in circulating erythroblasts. Observations included mitochondrial iron deposits in polychromatophilic erythroblasts, dilatation of the mitochondrial matrix in polychromatophilic erythroblasts at 6 hours post-dosing and erythroblastic cell death evident after the appearance of the mitochondrial lesions. These findings were suggested to reflect the observed anaemia in rat embryos. No histopathological signs in the embryonic rat heart were observed up to 24 hours after treatment, and no cell death in the embryonic rat heart was observed up to 48 hours after treatment, suggesting that no primary injury to the embryonic heart was produced by flumioxazin. Histological changes in the rat embryonic hearts at 36 or 48 hours after treatment included thin ventricular wall, poorly developed ventricular trabeculae and hypoplasia of the muscular septum and endocardial cushions of the atrioventricular canal. In rat fetuses, dilatation of hepatic sinusoidal vessels was observed at 24 and 36 hours; at 48 hours, hepatic necrosis was reported. No treatment-related changes in the external appearance of embryos or intrauterine deaths were reported in rabbits. Likewise, neither iron deposits in erythroblasts of rabbit embryos nor histopathological changes similar to those produced in rat embryos were seen in the rabbit embryo (Kawamura & Yoshioka, 1997).

Flumioxazin was administered to pregnant rats on day 12 of gestation at 400 mg/kg bw by gavage, and embryos/fetuses were removed from the uterus on days 13 through 20 of gestation and examined. Developmental toxicity was noted remarkably on day 14, when treated embryos were observed to have enlarged heart, oedema and anaemia (decreased red blood cell count and haemoglobin). These effects were also observed on days 15 and 16 of gestation, after which the values for treated litters were similar to control values. Beginning on day 15 and continuing to day 20, mortality in the treated litters was increased. The mortality rate was relatively constant on GD 17 and GD 20, indicating that all deaths occurred during the earlier period. This is consistent with the early resorptions observed in other developmental toxicity studies with flumioxazin. Closure of interventricular foramen began on day 16 of gestation in control fetuses (72.7% closed). Closure of interventricular foramen did not occur in any treated fetuses until day 16, and even then, the percentage with closure on day 17 was well below control values (89.7% control versus 23.9% treated). On day 20, the foramen of 95.2% of the control fetuses had closed, with only 57.7% of the treated fetuses reaching this milestone (Kawamura, 1997a).

A similar observation was made for malformations induced in rats by trypan blue. During early pregnancy (GD 11.5), fetuses with malformations were seen, whose rate decreased as pregnancy progressed (up to GD 20.5); however, the rate of resorptions increased concomitantly (Beck & Lloyd, 1963).

Further information on the temporal association can be gained from the study by Hosokawa (2015), which is summarized above.

Consistency and specificity and biological plausibility

Consistency and specificity

The main developmental landmarks in cardiac morphogenesis occur within a relatively short period in rats, between GD 12 and GD 16 (Sissman, 1970). Malformations are considered to be adverse findings that can be induced by a single treatment. This was also demonstrated for

flumioxazin. GD 12 was determined to be the most sensitive day, based on the incidence of embryonic deaths, fetal weight reduction and incidence of ventricular septal defects when dams were evaluated on GD 20 (Kawamura, 1993). Treatment of rats on other days led to a lower effect size.

Fetal findings were not reported in rabbit developmental toxicity studies. Similarly, the mechanistic studies in rabbits did not indicate an effect on the evaluated parameters in fetuses (Kawamura, 1996a, 1997b; Kawamura & Yoshioka, 1997).

Biological plausibility

To support the analysis of biological plausibility, other PPO inhibitors were checked for the induction of similar developmental effects.

Other compounds inhibiting PPO in isolated corn etioplasts or potato mitochondria, yeast mitochondria or mouse liver mitochondria are acifluorfen-methyl (Matringe et al., 1989a) and oxadiazon (Matringe et al., 1989b).

Although some effects of sodium acifluorfen on blood (anaemia) were reported, no induction of ventricular septal defects in rats or rabbits was indicated in a USEPA (2002) report. However, in the two-generation study, increased pup mortality was seen (USEPA, 2002).

Similarly, although some effects of oxadiazon on blood (anaemia) were reported, no induction of malformations or postimplantation losses in rats or rabbits was indicated in the European Union monograph (Ministry of Health, Italy, 2006).

Information on nitrofen, which is also a PPO inhibitor, is summarized in a review. Nitrofen induced a high incidence of diaphragm hernia and Harderian gland alterations in mice and hydronephrosis and respiratory difficulties in rats. According to the review, the effects were mediated by alterations in maternal and fetal thyroid hormone status (Gupta, 2011).

A further review indicated that the PPO-inhibiting herbicides azafenidin, butafenacil, carfentrazone, fluazolate, flufenpyr-ethyl, fluoroglycofen, fomesafen and lactofen would exhibit no teratogenic potential, whereas flumiclorac, flumioxazin, oxyfluorfen and sulfentrazone would have teratogenic potential (Dayan & Duke, 2010).

It might be expected, if PPO is inhibited, that the metabolic substrate protoporphyrinogen IX would increase in concentration. However, in the available studies, the metabolic product PPIX increased in concentration. The sponsor speculated that “accumulating protoporphyrinogen eventually leaves the mitochondria, enters the plasma, and is oxidized nonenzymatically there to PPIX. Because of abnormal subcellular location, the resulting PPIX is beyond reach of ferrochelatase and cannot be transformed to heme” (Kawamura, 2012). However, this hypothesis is not supported by the data.

Apparently, no data on the phenotype of PPO knockout rats (or mice) are available. Such data might support the hypothesis that PPO inhibition might lead to ventricular septal defects in developing fetuses.

In summary, there is no indication that inhibition of PPO is a key event leading to ventricular septal defects in developing rat fetuses.

Qualitative and quantitative human concordance

Human concordance cannot be assessed in a situation where the mode of action in animals has not been established.

Other potential modes of action

Other compounds reported to induce ventricular septal defects in rats are the cyclooxygenase inhibitors diflunisal, ibuprofen and ketorolac. In the same study, diclofenac and diflunisal induced postimplantation losses (Cappon, Cook & Hurtt, 2003). In rabbits, ventricular septal defects and

postimplantation losses were induced by diflunisal, but to a lesser extent than reported for rats (Cappon, Cook & Hurtt, 2003).

Hypoxia (by reduced air pressure in a chamber on 1 day during period of GD 9 to GD 13) can induce ventricular septal defects in rats (Clemmer & Telford, 1966). Similarly, treatment of rats with an atmosphere enriched with carbon dioxide and with reduced oxygen also induced ventricular septal defects; the highest incidence was reported when the dams were treated on the tenth day of pregnancy (Haring, 1965).

The antimalaria drug artesunate induced embryolethality and teratogenicity, including cardiovascular and skeletal defects (White et al., 2006; Clark et al., 2008). White et al. (2006) reported that

> Paling of visceral yolk sacs was observed within 3–6 hr after treatment. Within 24 hr, marked paling and embryonic erythroblast depletion were observed macroscopically, which preceded malformations and embryo death, and persisted through Day 14 pc [post-coitus]. Histologically, embryonic erythroblasts were reduced and cells showed signs of necrosis within 24 hr, were maximally depleted by 48 hr, and had partially rebounded within 3–4 days after treatment (Days 13 and 14 pc). Iron accumulation was evident in treated erythroblasts as early as 6 hr after treatment, suggesting impairment of heme synthesis. Heart abnormalities (swollen or collapsed chambers) were observed within 24 hr in approximately 25–60% of embryos and within 48 hr in 100% of embryos, correlating with histologic signs of cardiac myopathy (thinned and underdeveloped heart walls and enlarged chambers). Delays in limb and tail development occurred by Day 13 pc. Embryos were viable through Day 13 pc, but approximately 77% of embryos had died by Day 14 pc, presumably due to hypoxia and/or cardiac abnormalities.

These observations are remarkably similar to the findings in the studies with flumioxazin.

Clark et al. (2008) compared the developmental toxicity of artesunate with that of the structurally related artemisinins dihydroartemisinin (DHA), artemether and arteether. They summarized:

> Several structurally related artemisinins cause similar developmental toxicity, suggesting an artemisinin class effect. Equally embryotoxic oral and IV [intravenous] treatments of one artemisinin compound (artesunate) produced similar systemic exposure to the artesunate metabolite, DHA, suggesting that DHA may be the proximate developmental toxicant. Embryolethal doses of artesunate only caused minor changes in maternal reticulocyte counts indicating that adult hematology parameters are not as sensitive as embryonic erythroblasts.

A further compound inducing ventricular septal defects is dimethadione (DMO), a metabolite of the anticonvulsant drug trimethadione (Aasa et al., 2014). Starting on GD 8 (19:00) and then every 12 hours thereafter, a total of six doses of 300 mg/kg bw each were administered to rat dams. Aasa et al. (2014) summarized their findings:

> Adult rats exposed to DMO in utero had an increased incidence of arrhythmia, elevated blood pressure and CO [cardiac output], greater left ventricular volume and elevated locomotor activity versus CTL [control]. The mean arterial pressure of DMO-exposed rats was more sensitive to changes in dietary salt load compared with CTL. Importantly, most treated rats had functional deficits in the absence of a persistent structural defect. It was concluded that in utero DMO exposure causes cardiovascular deficits that persist into postnatal life in the rat, despite absence of visible structural anomalies.

For DMO-induced heart defects, there are published hypotheses that DMO acts via radical oxygen species or via drug-induced arrhythmia (summarized in Azarbayjani, 2001).

Such alternative modes of action were not scrutinized sufficiently, especially as reduced oxygen availability might be a common mechanism for hypoxia and anaemia as induced by flumioxazin.

Uncertainties/inconsistencies and identification of data gaps

There is no sufficient indication that inhibition of PPO is a key event leading to ventricular septal defects in developing rat fetuses. Some of the conclusions are based on in vitro or in silico investigations.

It might be expected, if PPO is inhibited, that the metabolic substrate protoporphyrinogen IX would increase in concentration. However, in the available studies, the metabolic product PPIX increased in concentration. This discrepancy was not explained by the data.

Conclusions in relation to problem formulation

The sponsor proposed a new mode of action to explain the induction of ventricular septal defects in rat fetuses. Likewise, it was the aim to demonstrate the non-relevance of this mode of action for humans.

In summary, the Meeting concluded that the case is not robust enough to demonstrate that the proposed mode of action is plausible in rats. Hence, the relevance of this mode of action for humans cannot be assessed.

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