

FLUSILAZOLE

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Explanation

Flusilazole is the International Organization for Standardization (ISO) approved name for 1-[[bis(4-fluorophenyl)methyl]silyl]methyl]-1*H*-1,2,4-triazole (Chemical Abstracts Service, CAS No. 85509-19-9). It is a broad-spectrum fungicide that belongs to the triazole subclass of ergosterol biosynthesis inhibitors. Flusilazole was previously evaluated by the Joint Meeting in 1989 (Annex 5, references 56, 58) and in 1995. An acceptable daily intake (ADI) of 0–0.001 mg/kg bw was allocated in 1989, based on a no-observed-adverse-effect level (NOAEL) of 0.14 mg/kg bw per day (5 ppm) for liver toxicity in a 1-year feeding study in dogs. This was confirmed in 1995. The compound was re-examined by the present Meeting as part of the periodic re-evaluation programme of the Codex

Committee on Pesticide residues (CCPR). Three new studies were provided, two studies of developmental toxicity in rats (one of oral and one of dermal administration) and a 28-day mechanistic study in dogs.

Owing to the age of the database, some studies predate the establishment of good laboratory practice (GLP); however, all critical studies complied with GLP.

Evaluation for acceptable daily intake

1. Biochemical aspects

The toxicokinetics of technical-grade flusilazole have been studied after oral administration of radiolabelled test material in rats. Summaries of the relevant data are presented below.

1.1 Absorption, distribution and excretion

In a GLP-compliant study, groups of two male and two female Charles River CD rats were given [^{14}C]flusilazole (uniformly labelled on the phenyl ring) orally by one of the following three regimens: (1) a single low dose of at approximately 8 mg/kg bw; (2) a single low dose of approximately 8 mg/kg bw after pre-treatment for 21 days on a diet containing non-labelled flusilazole at 100 ppm; (3) a single high dose of approximately 200 mg/kg bw. By 96 h (regimen 1) or 168 h (regimen 2 or 3), a total of approximately 90% of the administered radioactivity was eliminated in the urine and faeces. The excretion half-life ($t_{1/2}$) was approximately 34 h. The amount of radioactivity recovered in the expired air (as CO_2 or volatile metabolites) was not significant. The main route of elimination was in the faeces and there was an apparent sex difference in the excretion pattern. In males, 87% and 8% of the administered dose was eliminated in faeces and urine, respectively, while in females, the corresponding figures were 59% and 23%. Pre-treatment with non-radiolabelled flusilazole did not affect excretion. Tissue retention of radioactivity was very low, with total residues accounting for < 2.5% of the administered dose. The highest amounts of radioactivity were found in the carcass, gastrointestinal tract and liver (mean, < 1% of administered dose, in each case). Tissue concentrations within the groups at the lower and higher dose were proportional to the amount of flusilazole administered. In the same study, an additional group of one male and one female rat was given a single oral dose of [^{14}C]flusilazole (triazole-3-labelled) at approximately 8 mg/kg bw. As with the phenyl-labelled flusilazole, 88% of the administered radioactivity was recovered in the urine and faeces within 96 h after dosing. However, urine was the predominant route of excretion. In males, urinary and faecal radioactivity accounted for 78% and 11% of the administered dose, respectively, while in females, the corresponding figures were 59% and 26%. Tissue retention of radioactivity was very low, with total residues accounting for approximately 3% of the administered dose. The highest concentrations of radioactivity were found in the carcass (approximately 2% of the administered dose), skin, gastrointestinal tract and liver (mean, < 0.5% of the administered dose, in each case) (Anderson et al., 1986).

In another GLP-compliant study, groups of five male and five female Charles River Crl:CD(SD) BR rats were given [^{14}C]flusilazole (triazole-3-labelled) orally by one of the following three regimens: (1) a single dose at approximately 8 mg/kg bw; (2) a single dose at approximately 8 mg/kg bw after pre-treatment with non-radiolabelled flusilazole at approximately 8 mg/kg bw per day by gavage for 14 days; (3) a single dose at approximately 224 mg/kg bw. At 96 h (regimen 1) or 120 h (regimen 2 or 3), total recovery of radioactivity was 92.6–99.2%, with approximately 90% of the radioactivity eliminated within the first 48 h after dosing. Urine was the primary route of excretion, accounting for approximately 72% of the administered dose, while faecal excretion accounted for approximately 17%. Sex or regimen differences were not observed in this study. Tissue retention of radioactivity was

very low. Total residues excluding the carcass (which accounted for < 3% of the administered dose) were < 0.2% (Cheng, 1986).

1.2 Biotransformation

[¹⁴C]Flusilazole was extensively metabolized when administered orally to Charles River CD rats. Recovered parent compound accounted for only 2–11% of the administered dose in all groups regardless of dose (low/high) or preconditioning (with/without), found predominantly in faeces; urinary levels were < 1% of the administered dose. After absorption, flusilazole was cleaved at the triazole ring. With phenyl-labelled test material, the major faecal metabolites identified were: (i) [bis(4-fluorophenyl)methyl] silanol (approximately 30% of the administered dose in males, approximately 19% in females); (ii) [bis(4-fluorophenyl)methylsilyl] methanol (approximately 9% of the administered dose in both sexes); (iii) the fatty acid conjugates of [bis(4-fluorophenyl)methylsilyl] methanol (19% of the administered dose in males, 10% in females); and (iv) disiloxane (approximately 11% in males, approximately 7% in females). Except for the fatty acid conjugates, the same metabolites were found in the urine. In males, all three urinary metabolites were < 1% of the administered dose; in females, [bis(4-fluorophenyl)methyl] silanol, [bis(4-fluorophenyl)methylsilyl] methanol and siloxane accounted for 7.5%, 2.2% and 1.9% of the administered dose, respectively. With triazole-labelled material, the main metabolite identified was 1*H*-1,2,4-triazole, which was found predominantly in the urine (63.8% of the administered dose in males, 51.6% in females). Faeces contained only a minor amount of the metabolite (4% of the administered dose in males, 17% in females). On the basis of these results, a metabolic pathway for flusilazole in the rat was proposed (Anderson, 1986).

2. Toxicological studies

2.1 Acute toxicity

The results of studies of acute toxicity with flusilazole technical are summarized in Table 1. All studies complied with GLP.

Flusilazole is slightly to moderately toxic in rats when given as a single dose via the oral route; and minimally toxic in rats and rabbits when administered acutely via the dermal or inhalation route. Symptoms of toxicity after oral administration included weight loss, weakness, lethargy and at higher doses, prostration, salivation, laboured breathing, convulsions and loss of righting reflex. Dermal administration resulted only in mild erythema at site of application. Inhalation effects were mainly laboured breathing and lung sounds.

Table 1. Results of studies of acute toxicity with flusilazole

Species	Strain	Sex	Route	LD ₅₀ (mg/kg bw)	LC ₅₀ (mg/l air)	Purity (%)	Reference
Rat	CrI:CD	Males	Oral	1110	—	> 92.7	Wylie et al. (1984a)
		Females		674			
	CrI:CD(SD)IGC BR	Males	Oral	1216	—	> 92.7	Finlay (2000)
		Females		672			
	CrI:CD(SD)BR	Males & females	Inhalation	—	6.8–7.7	> 92.7	Turner et al. (1985)
Rabbit	New Zealand White	Males & females	Dermal	> 2000	—	> 92.7	Gargus & Sutherland (1983)

*(a) Ocular irritation**Rabbits*

Two male New Zealand White rabbits were given undiluted flusilazole (purity, 90%) at a dose of 0.01 ml in the right conjunctival sac. The left eyes were left untreated to serve as negative controls. One rabbit had its right eye washed with 100 ml of water after 20 s of exposure. Ocular irritation was graded according to the Draize scale at 1, 4, 24, 48 and 72 h after instillation. This study did not comply with GLP, but was considered to be a conclusive study. Both rabbits showed mild conjunctival redness and chemosis at 1–4 h on day of instillation. The washed eye also showed some discharge at 1 h; but by 24 h these effects were no longer observed. The unwashed eye showed slight corneal opacity at 1–4 h and biomicroscopic examination revealed slight cloudiness at 24–48 h; the eye was normal at 72 h. Flusilazole was minimally irritating to the eyes of male New Zealand White rabbits, causing transient mild irritation. Flushing the eye with water after exposure eliminated the corneal but not the conjunctival effects (Hall et al., 1984).

Flusilazole was evaluated for potential acute eye irritation in a GLP-compliant study performed in compliance with test guideline OECD 405. Six young adult female New Zealand White rabbits were given approximately 46 mg of the test substance in a volume of approximately 0.1 ml in one eye. The eyes remained unwashed after treatment. The conjunctiva, iris, and cornea of each treated eye were evaluated and scored according to a numerical scale approximately 1, 24, 48, and 72 h after administration of the test substance. The test substance produced corneal opacity (score of 1), iritis (score of 1), conjunctival redness (score of 1 or 2), conjunctival chemosis (score of 1, 2, or 3), and discharge (score of 2 or 3) in the treated eyes of all six rabbits. The treated eye of one rabbit was normal by 48 h, and the treated eyes of the remaining five rabbits were normal by 72 h after instillation of the test substance. No adverse weight loss or clinical signs of toxicity occurred during the study. All the rabbits pawed their treated eye after instillation of the test substance. Mean values were calculated from numerical scores obtained from the quantitative evaluation of ocular response observed in all rabbits at 24, 48, and 72 h after treatment. The results of this study indicate that flusilazole is minimally irritating to the eye of rabbits (Finlay, 1998).

*(b) Dermal irritation**Rabbits*

In a GLP-compliant study of primary skin irritation in New Zealand White rabbits, six young males received flusilazole formulation (Nustar 20DF, containing 21% flusilazole technical and 79% inert ingredients that were not specified) at a concentration of approximately 0.5 g (undiluted, moistened with distilled water) applied topically to shaved skin sites on the back. The test site was covered with gauze patch and a sheet of rubber for 4 h, after which all coverings were removed and the site was washed gently with warm water. Each test site was evaluated for irritation potential according to the Draize scale at 4, 24, 28 and 72 h after initial dosing. At 4 h, erythema (grade 1) was observed in four out of six rabbits and oedema was found in two out of six rabbits (grade 1 or 2). By 24 h, only two rabbits had erythema and no oedema was observed. No signs of dermal irritation were evident in any rabbit at 72 h.

The primary irritation index was 0.167. On the basis of these results, flusilazole (as formulated for this test) was minimally irritating to the skin of rabbits (Brock, 1988).

Guinea-pigs

In young adult Hartley guinea-pigs, a range-finding GLP-compliant study indicated mild erythema 24 h after dermal application of flusilazole (purity, 90%) at a concentration of 100% (aliquots of 0.05 ml); no dermal irritation was observed at a concentration of 50%. In a study of primary skin

irritation, flusilazole (purity, 90%) was applied topically to the skin at concentrations of 5% or 50% (w/v, solution in demethyl phthalate) and the test sites (two per animal) were scored for signs of irritation at 24 h and 48 h. No dermal irritation was evident at any time in any of the 10 animals exposed. Flusilazole was found to be non-irritating to the skin of guinea-pigs (Wylie et al., 1984b).

(c) *Dermal sensitization*

In a GLP-compliant study of sensitization in young adult Hartley guinea-pigs, intradermal injections of flusilazole (purity, 90%) (1% solution, w/v; in methyl phthalate) weekly for 4 weeks caused erythema and edema with necrotic centres at sites of injection at 24 h. Challenge with topical applications of 5 or 50% solutions to the skin did not demonstrate sensitization. Flusilazole was not a dermal sensitizer in the guinea pig (Wylie et al., 1984b)

In another GLP-compliant study of sensitization, 10 male and 10 female young adult Duncan Hartley albino guinea-pigs were given three weekly dermal applications of flusilazole (purity, 97.7%) of 0.4 ml (equivalent to 0.192 g). The test material (slightly moistened with dimethyl phthalate) was applied to the shaved intact skin at the back of the guinea-pig and covered with plastic wrap for 6 h. The dermal irritation response was scored at 24 and 48 h after treatment. No signs of dermal irritation were observed at any time in any of the guinea-pigs after any of the induction applications. Two weeks after the last induction treatment, the guinea-pigs were challenged with a single dermal application of flusilazole of 0.4 ml (0.192 g) on an untreated site that was then covered for 6 h. the irritation response was scored again at 24 h and 48 h. No signs of dermal irritation were observed at any time in any of the guinea-pigs tested. A positive-control group treated with 1-chloro,-2,4-dinitrobenzene (DNCB) showed severe erythema with necrosis at 24 h after the second and third induction applications and severe erythema after the challenge dose 2 weeks later. Flusilazole was not a dermal sensitizer in guinea-pigs under the conditions of the study (Brock et al., 1988)

2.2 *Short-term studies of toxicity*

(a) *Oral administration*

Mice

Groups of 20 male and 20 female Crl:CD-1 mice were given diets containing flusilazole technical (purity, 96.7%) at a concentration of 0, 25, 75, 225, 500 or 1000 ppm (equal to 0, 4, 12, 36, 82 and 164 mg/kg bw per day in males and 0, 5, 15, 43, 92 and 222 mg/kg bw per day in females) for up to 90 days. Ten males and ten females from each group were killed after 4 weeks of dosing; the remaining ten males and ten females from each group were killed at study termination (90 days). No histopathological examination was conducted on mice killed at 4 weeks. This study was not GLP-compliant, but was performed in compliance with the United States Environmental Protection Agency (EPA) guidelines and was considered to be a conclusive study. There were no clinical symptoms of toxicity and no treatment-related effects on body weight or food consumption in mice killed at either interval. The target organs of toxicity were liver and urinary bladder; females were slightly more sensitive to the test substance than males. No treatment-related effects were observed at the lowest dietary concentration of 25 ppm. At the next higher concentration of 75 ppm, increased absolute and relative liver weights and increased incidence (1 out of 10) of hepatocellular vacuolar cytoplasmic changes were observed in females, which was considered to be an adaptive response. At 225 ppm and/or higher, elevated liver weight, dose-related increases in the incidence of hepatocellular vacuolar cytoplasmic changes, hepatocellular hypertrophy and urinary bladder urothelial-cell hyperplasia were evident in both sexes. At the highest dose of 1000 ppm, absolute and relative kidney

weights were decreased in males, but no treatment-related renal lesions were observed. In addition, slight decreases in erythroid parameters (haemoglobin, erythrocyte volume fraction and erythrocyte counts) were also noted in both sexes. The NOAEL was 75 ppm (equal to 12 mg/kg bw per day) (Pastoor et al., 1984).

In a GLP compliant study, groups of 16 male and 16 female Crl:CD-1(ICR)BR mice were fed diets containing flusilazole technical (purity, 94%) at a concentration of 0, 1000, 2500 or 5000 ppm (equal to 0, 161, 436 and 1004 mg/kg bw per day in males and 0, 239, 601 and 1414 mg/kg bw per day in females) for at least 90 days. Additional groups of six males and six females were assessed for cellular proliferation in the liver and urinary bladder, three males and three females being assessed each on days 14 and 106. At the lowest dose of 1000 ppm (equal to 161 mg/kg bw per day), treatment-related effects included: reduced mean body weight and food efficiency (males only); increased absolute and relative liver weights (both sexes); decreased absolute and relative kidney weights (females only); and hypertrophy/hyperplasia, cytoplasmic vacuolation and inflammation in the liver and urinary bladder (both sexes). At the intermediate dose of 2500 ppm, increased cellular proliferation of the urinary bladder (both sexes) was also observed. At the highest dose of 5000 ppm, severe body-weight loss occurred in males and reduced body weight and food efficiency were noted in females. The males at 5000 ppm were all killed on day 44 owing to excess mortality and moribund condition. No unscheduled death/killing occurred in females. No definitive NOAEL could be identified in this study (Keller, 1990).

Rat

In a non-GLP compliant, supplemental study, groups of six male Crl:CD rats were given flusilazole technical (purity, 95.5%) at a dose of 0 or 300 mg/kg bw per day orally by gavage (in corn oil) for 5 days per week for 2 weeks. Three rats per group were killed at the end of the treatment period; the remaining three rats per group were killed after a 2-week recovery period. All rats were examined histopathologically. One treated rat died after the fifth dose (on day 7). Clinical signs of toxicity (lower body weight, alopecia, diarrhoea, stained/wet perineal area, salivation and hypersensitivity) were observed in four out of six treated rats during the treatment period. Treatment-related histopathological changes were observed in liver, kidney, urinary bladder and testes; the lesions appeared to be less severe in rats killed after a 2-week recovery period. Histopathological findings included: hepatocellular vacuolation (six out of six treated rats); hyperplasia and vacuolation of the urinary bladder transitional epithelium (six out of six treated rats) and renal pelvis urothelium (two out of six treated rats); and necrosis and cellular degeneration of the seminiferous tubule germinal epithelium (two out of six treated rats) (Wylie et al., 1984c).

Four groups of 10 male and 10 female Charles River CD rats were fed daily diets containing flusilazole technical (purity, 96.7%) at a concentration of 0, 25, 125, 375 or 750 ppm (equal to 0, 2, 9, 27 and 55 mg/kg bw per day in males and 0, 2, 11, 31 and 70 mg/kg bw per day in females) for 90 days. This study was not GLP-compliant, but was considered to be conclusive. No treatment-related effects were observed at the lower doses of 25 and 125 ppm. At the next higher dose of 375 ppm, an increased concentration of serum cholesterol (both sexes) and increased incidence of mild bladder urothelial hyperplasia in males (1 out of 10) and females (4 out of 10) were noted. At the highest dose of 750 ppm, bladder urothelial hyperplasia was evident in 5 out of 10 males and 8 out of 10 females. Additional treatment-related changes included decreased body weight in females; increased absolute and relative liver weights (both sexes); and histopathological findings (hepatocellular hypertrophy, mild fatty changes and hepatocytolysis) in the liver of 5 out of 10 males. The NOAEL was 125 ppm, equal to 9 mg/kg bw per day (Pastoor et al., 1983).

Dogs

Groups of four male and four female beagle dogs were fed diets containing flusilazole technical (purity, 93%) at a concentration of 0, 25, 125 or 750/500 ppm (equal to 0.9, 4.3 and 13.4 mg/kg bw per day in males and 0, 0.9, 4.3 and 14.2 mg/kg bw per day in females) for 3 months. The group receiving the highest dose (750/500 ppm) received diet containing flusilazole at 750 ppm for the first 3 weeks, then control diet for 1 week and diet containing flusilazole at 500 ppm for the remainder of the study period. The dose was reduced owing to marked body-weight loss and decreased food intake at 750 ppm. This study was not GLP-compliant, but was considered to be conclusive.

At the lowest dose of 25 ppm (equal to 0.9 mg/kg bw per day), there was a treatment-related increase in the incidence of hyperplasia of the lymphoid follicles in the pyloric glandular mucosa of the stomach in males (zero out of four, three out of four, three out of four, and four out of four at 0, 25, 125 and 750/500 ppm, respectively). At the next higher dose of 125 ppm, an increased incidence of pyloric granular mucosa hyperplasia was also observed in females (zero out of four, zero out of four, three out of four, and four out of four at 0, 25, 125 and 750/500 ppm, respectively). Higher levels (relative to control values) of alanine aminotransferase activity and an increased incidence of mild urinary-bladder mucosal hyperplasia were observed in males at 125 ppm and in both sexes at the highest dose of 750/500 ppm. At 750/500 ppm (13.4 mg/kg bw per day), additional treatment-related effects included: clinical signs of toxicity (weakness or tremors) in both sexes; reduced mean body-weight gain (males); body-weight loss (females) and decreased mean food consumption (both sexes); a slight increase in leukocyte and monocyte counts (males) and lower (relative to control values) plasma concentrations of cholesterol, total protein and albumin concentrations (both sexes); and increased absolute and relative liver weights (both sexes). No NOAEL was identified in this study (Rickard et al., 1983).

In a GLP-compliant study, groups of five male and five female beagle dogs were a daily diet containing flusilazole technical (purity, 95.8%) at a concentration of 0, 5, 20 or 75 ppm (equal to 0, 0.14, 0.7 and 2.4 mg/kg bw per day in males and 0, 0.14, 0.7 and 2.6 mg/kg bw per day in females) for 1 year. No treatment-related effects were observed at the lowest dose of 5 ppm. At the next higher dose of 20 ppm, serum albumin concentration was decreased (males) and there was a dose-related increase in the incidence of centrilobular hepatocellular hypertrophy in males (zero out of five, zero out of five, four out of five, and five out of five) and females (zero out of five, zero out of five, two out of five, and five out of five). An increased severity of gastric-mucosa lymphoid hyperplasia (from minimal/mild at 0–20 ppm to moderate at 75 ppm) was also noted in both sexes. At the highest dose of 75 ppm, additional treatment-related effects included: higher leukocyte counts (relative to control values, both sexes); elevated alkaline phosphatase activity and lower cholesterol, total protein and albumin concentrations (males only); and increases in relative liver (both sexes) and relative kidney weights (females). All dogs at the highest dose exhibited a greater degree of hepatic centrilobular inflammatory infiltration; distinct centrilobular hepatocellular vacuolation was observed in three out of five males. Lymphoid hyperplasia in the gastric mucosa was of moderate severity in males and females. The NOAEL was 20 ppm (equal to 0.7 mg/kg bw per day) (O'Neal et al., 1985).

(b) Dermal application

Rabbits

Groups of five male and five female New Zealand White rabbits were treated dermally with flusilazole technical (purity, 94.9%) at a concentration of 0, 1, 5, 25 or 200 mg/kg bw per day for 21 days. The study was performed in compliance with GLP and test guideline OECD 410. The test material was applied daily as a paste in distilled water; the exposure sites were occluded for 6 h and then washed with water. No evidence of any systemic toxicity was observed. The NOAEL for dermal

toxicity was 5 mg/kg bw per day on the basis of diffuse epidermal hyperplasia/thickening (slight to mild) at 25 mg/kg bw per day and greater. At the highest dose of 200 mg/kg bw per day, mild erythema was also evident from days 6–12. The NOAEL for systemic toxicity was 200 mg/kg bw per day (Sarver et al., 1986).

2.3 Long-term studies of toxicity/carcinogenicity

Mice

In a GLP-compliant study, groups of 80 male and 80 female Crl:CD-1(ICR)BR mice were given diets containing flusilazole technical (purity, 96.5%) at a concentration of 0, 5, 25 or 200 ppm (equal to 0, 0.66, 3.4, and 27 mg/kg bw per day in males and 0, 0.92, 4.6 and 36 mg/kg bw per day in females) for 18 months. Each dose group contained a satellite group of 10 males and 10 females for interim killing at 6 months.

There were no treatment-related effects on mortality (terminal survival rates were 76–86% in males and 57–80% in females), body weight, food consumption, haematology or serum chemistry at any dose tested in the study. At the highest dose of 200 ppm, a toxicologically significant increase in incidence of hepatocellular fatty changes was observed (incidence at termination was 4 out of 80, 3 out of 80, 10 out of 80 and 40 out of 80 in males and 2 out of 80, 4 out of 80, 3 out of 80 and 24 out of 80 in females at 0, 5, 25 and 200 ppm, respectively). In addition, there was an increase in absolute and relative liver weights (both sexes), a decrease in absolute kidney weight (females) and an increase in lymphocytic infiltration in the lung and urinary bladder (males). There was no treatment-related increase in the incidence of any tumour type at any dose up to and including 200 ppm (equal to 27 mg/kg bw per day), the highest dose tested. Flusilazole technical was not oncogenic in the mouse under the conditions of the study. The NOAEL for systemic toxicity was 25 ppm, equal to 3.4 mg/kg bw per day (Brock et al., 1985).

Groups of 100 male and 100 female Crl:CD-1(ICR)BR mice were given daily diets containing flusilazole technical (purity, 94%) at a concentration of 0, 100, 500 or 1000 ppm in males (equal to 0, 14.3, 73.1 and 144 mg/kg bw per day) and 0, 100, 1000 or 2000 ppm in females (equal to 0, 19.4, 200 and 384 mg/kg bw per day) for 18 months. An additional group of 100 males and 100 females was fed a daily diet containing flusilazole technical at a concentration of 25 ppm (equal to 3.51 mg/kg bw per day in males and 4.38 mg/kg bw per day in females) and was used only for the study of cell proliferation; no other end-points of toxicity were measured in this group. Doses were selected on the basis of the results of a 90-day study in mice, which indicated a greater sensitivity of males to the test compound. The study was performed in compliance with GLP and test guideline OECD 451.

At the lowest dose tested (100 ppm, equal to 14.3 mg/kg bw per day), there was a slight decrease in absolute kidney weight, an increase in focal necrosis of the liver and urinary bladder hyperplasia in males. No remarkable changes were evident in females at 100 ppm. Females at higher doses (1000 and 2000 ppm) showed increased cellular hyperplasia in the urinary bladder and urethra, but no focal necrosis of the liver was observed. At the two higher doses (500 and 1000 ppm in males; or 1000 and 2000 ppm in females), additional treatment-related systemic effects included: increased absolute and relative liver weights; lower absolute and relative kidney weights; an increased number of foci of hepatocellular alteration and increased incidence of hepatic vesicular/vacuolar changes with cellular hypertrophy. Significantly higher mortality occurred at 1000 ppm in males and 2000 ppm in females. In addition, increased cellular proliferation in the urinary bladder (but not in the liver) was observed in the females at 2000 ppm. Flusilazole was found to be oncogenic in this study. At terminal sacrifice, an increased incidence of hepatocellular tumours (adenomas and carcinomas) was observed in males at all doses (13 out of 80, 23 out of 79, 20 out of 80, and 18 out of 78 at 0, 100, 500, and 1000 ppm,

Table 2. Incidence of liver tumours and associated histopathological lesions reported in combined studies in mice fed diets containing flusilazole

Finding	Study	Dietary concentration (ppm)										Range for historical controls (%) ^c
		Brock et al. (1985)	0	—	5	25	—	200	—	—	—	
	Keller (1992b)	—	0	—	—	100	—	500	1000	2000		
<i>Males</i>												
Dietary intake (mg/kg bw per day)		0	0	0.66	3.4	14.3	27.0	73.1	144.0	—	—	
Hepatocellular tumours (%) ^a		20	16	16	15	29	30	25	23	—	7.5–21.8	
Hepatocellular adenomas (%)		10	10	10	10	18	14	19	15	—	5.0–21.8	
Hepatocellular carcinomas		10	6	6	4	10	11	6	6	—	0–6.8	
Foci of cellular alteration (%)		4	4	5	3	5	4	12	29*	—	—	
Degeneration, centrilobular (%) ^b		5	0	4	10	1.2	50*	11.2*	11.4*	—	—	
Degeneration, focal (%)		4	3.8	3	8	12.5	3	13.8	15.0*	—	—	
Relative liver weight (% body weight)		4.9	5.4	4.8	4.7	6.4*	5.5*	7.6*	9.2*	—	—	
<i>Females</i>												
Dietary intake (mg/kg bw per day)		0	0	0.92	4.6	19.4	36.0	—	200	384	—	
Hepatocellular tumours (%) ^a		1.3	1.3	2.5	0	3.8	1.2	—	13.8*	53.8*	0–2.6	
Hepatocellular adenomas (%)		1.0	0	3.0	0	2.5	1.0	—	10.0*	14.0*	0–2.6	
Hepatocellular carcinomas		0	1.3	0	0	1.2	0	—	1.3	26.0*	0–1.3	
Foci of cellular alteration (%)		0	2.5	0	0	1.2	1.0	—	9.0*	34.0*	—	
Degeneration, centrilobular (%) ^b		3.0	1.3	5.0	4.0	0	30.0*	—	1.2	0	—	
Degeneration, focal (%)		10.0	12.7	9.0	5.0	7.5	5.0	—	10.0	12.5	—	
Relative liver weight (% body weight)		5.4	5.2	5.0*	5.3	5.3	5.5	—	8.9*	12.7*	—	

From Brock et al. (1985) and Keller (1992b)

^a Mice with at least one liver tumour.

^b Includes lesions described as fatty change.

^c Historical controls or controls from laboratory carrying out the study.

* $p < 0.05$.

respectively; range for historical controls, 7.5–21.8%) and in females at the two higher doses (1 out of 79, 3 out of 80, 11 out of 77 and 43 out of 76 at 0, 100, 1000 and 2000 ppm, respectively; range for historical control, 0–2.6%). The increase in incidence of tumours was not dose-related nor statistically significant in males, but found to be dose-dependent and statistically significant in females. On the basis of these results, no NOAEL for systemic toxicity could be identified. The overall NOAEL for oncogenicity was 200 ppm (36 mg/kg bw per day) for females and 1000 ppm (144 mg/kg bw per day) for males (Keller, 1992b).

Rats

In a GLP-compliant study, groups of 70 male and 70 female rats of strain Crl:CD(SD)BR were given diets containing flusilazole technical (purity, 95.6%) at a concentration of 0, 10, 50 or 250 ppm (equal to 0, 0.4, 2.0, and 10 mg/kg bw per day in males and 0, 0.5, 2.6 and 13 mg/kg bw per day in females) for 2 years. Each group had two satellite groups of 10 males and 10 females for interim killing at 6 months and 1 year, respectively. At approximately 100 days after the start of treatment, 20 males and 20 females from each group were mated for the reproduction phase of the study. These rats were returned to the long-term study after weaning of the second litter. The rats killed at 6 months were examined for bladder lesions only. No treatment-related effect on mortality was observed; survival rate was > 50% in all groups until week 98. No signs of systemic toxicity were evident at the lowest dose of 10 ppm. At the next higher dose of 50 ppm, there was a dose-related increase in the incidence of pyelonephritis in females at 2 years (3 out of 66, 3 out of 62, 8 out of 67 and 10 out of 65 at 0, 10, 50, and 250 ppm, respectively). Increases in relative liver weight (females) and incidence of hydronephrosis (males) were also observed at interim kill at 1 year. These effects were not considered adverse. At the highest dose of 250 ppm, additional treatment-related changes included: increased incidence of hepatic lesions in females (centrilobular hepatocellular hypertrophy and polyploidy) and hydro-nephrosis in males at 1 year; and increased incidence of acidophilic foci of hepatocellular alteration (13 out of 65 compared with 3 out of 66 in controls) and hepatic diffuse fatty changes (23 out of 65 compared with 9 out of 66 in controls) in females at 2 years. No hepatic lesions were observed in males at any time during the study and no lesions of the urinary bladder were evident in either sex at either interim (6 or 12 months) or terminal kill. There was no treatment-related increase in incidence of any tumour type at any dose tested in the study. At 250 ppm, males showed a slightly higher incidence of squamous cell carcinomas of the oral and nasal cavities (0 out of 66, 1 out of 63, 0 out of 67 and 3 out of 64, at 0, 10, 50, and 250 ppm, respectively). On the basis of data on historical controls from six 2-year feeding studies in rats (five studies with no tumours of this type and one study with an incidence of 2 out of 60), the incidence of nasal tumours in this study was judged to be incidental. Flusilazole technical was, therefore, not oncogenic in the rat under the conditions of this study. The NOAEL for systemic toxicity was 50 ppm, equal to 2.0 mg/kg bw per day. The Meeting noted, however, that the systemic effects reported in this study were relatively mild and higher doses might have been tolerated (Pastoor et al., 1986).

In a more recent study, groups of 65 male and 65 female Crl:CD(SD)BR rats were fed diets containing flusilazole technical (purity, 95%) at a concentration of 0, 125, 375, or 750 ppm (equal to 0, 5.03, 14.8, and 30.8 mg/kg bw per day in males and 0, 6.83, 20.5 and 45.6 mg/kg bw per day in females) for 2 years. Each group included a satellite group of 10 males and 10 females destined for interim kill at 1 year. The study complied with GLP and test guideline OECD 451.

At the lowest dose of 125 ppm (equal to 5.03 mg/kg bw per day), dose-related increases in incidence of hepatocellular hypertrophy were observed in both sexes at either interim (males, 0 out of 10, 4 out of 10, 8 out of 10 and 8 out of 10; females, 0 out of 10, 7 out of 10, 10 out of 10 and 10 out of 10; at 0, 125, 375 and 750 ppm, respectively) or terminal kill (males, 2 out of 53, 2 out of 51, 10 out of 53 and 19 out of 53; females, 2 out of 56, 4 out of 55, 12 out of 53 and 25 out of 54; values exclude

deaths that occurred before termination). There was an apparent sex difference in the type of hepatic lesions observed: periportal hepatocellular hypertrophy with lamellar bodies was seen in males, while females showed centrilobular hepatocellular hypertrophy with eosinophilic cytoplasm, but no lamellar bodies. At terminal kill, there was also an increased incidence of mixed foci of cellular alteration in males (6 out of 53, 14 out of 51, 17 out of 53 and 19 out of 53). At the next two higher doses of 375 and 750 ppm, there were also significant decreases in terminal body weight (females), increases in absolute and/or relative liver weights at interim and/or terminal kill (males and/or females) and an increase in the incidence of urinary-bladder transitional-cell hyperplasia at study termination (both sexes; 3 out of 46, 6 out of 45, 27 out of 47 and 42 out of 51 in males, and 5 out of 47, 3 out of 49, 15 out of 49 and 33 out of 53 in females). At 750 ppm, additional treatment-related changes included: increased incidence of hepatic fatty changes (males); and increased cellular proliferation in the liver and urinary bladder. Flusilazole technical was found to be oncogenic in this study. At the highest dose of 750 ppm, a treatment-related increase in incidence of urinary-bladder transitional-cell tumours (papillomas and carcinomas) was observed in males and females at termination (males, 0 out of 46, 0 out of 45, 1 out of 47 and 5 out of 51; and females, 0 out of 47, 1 out of 49, 0 out of 49 and 13 out of 53; at doses of 0, 125, 375 and 750 ppm, respectively). The incidence of testicular interstitial-cell (Leydig cell) tumours in the males at 750 ppm was also increased relative to that in the controls (2 out of 53, 4 out of 51, 2 out of 53 and 9 out of 53, at 0, 125, 375 and 750 ppm, respectively). On the basis of the results, no NOAEL for systemic toxicity could be identified; the NOAEL for oncogenicity was 375 ppm, equal to 14.8 mg/kg bw per day (Keller, 1992c).

Table 3. Incidence of tumours of the bladder and associated histopathological lesions in combined studies in rats fed diets containing flusilazole

Finding	Study	Dietary concentration (ppm)								Range for historical controls (%) ^a
	Pastoor et al. (1986)	0	—	10	50	—	250	—	—	
	Keller (1992a)		0	—	—	125	—	375	750	
<i>Males</i>										
Dietary intake (mg/kg bw per day)		0	0	0.4	2.0	5.0	10.0	14.8	30.8	—
Papilloma, transitional cell (%)		0	0	0	0	0	0	2.1	2.0	0–1.9/0–1.5
Carcinoma, transitional cell (%)		0	0	0	0	0	1.6	0	7.8*	0/0–2.0
Papillomas + carcinomas (%)		0	0	0	0	0	1.6	0	9.8*	—
Mucosal hyperplasia (%)		1.5	6.5	0	1.5	13.3	3.2	57.4*	82.4*	—
<i>Females</i>										
Dietary intake (mg/kg bw per day)		0	0	0.5	2.6	6.8	13.0	20.5	43.6	—
Papilloma, transitional cell (%)		0	0	0	0	0	0	0	3.8*	0–2.0/0–1.4
Carcinoma, transitional cell (%)		0	0	0	0	2.0	0	0	20.8*	0/0–1.4 ^b
Papillomas + carcinomas (%)		0	0	0	0	2.0	0	0	24.5*	—
Mucosal hyperplasia (%)		1.6	10.6	0	0	6.1	0	30.6*	62.3*	—

From Pastoor et al. (1986) and Keller (1992a)

^a Range for historical controls (%) in laboratories: DuPont Haskell laboratory / Charles River laboratory.

^b Total number of transitional cell tumours rats (papilloma + carcinoma) in females.

* $p < 0.05$.

Table 4. Incidence of Leydig-cell tumours and associated histopathological lesions in combined studies in male rats fed diets containing flusilazole

Finding	Study	Dietary concentration (ppm)								Range for historical controls ^a
	Pastoor et al. (1986)	0	—	10	50	—	250	—	—	
	Keller (1992a)	—	0	—	—	125	—	375	750	
Dietary intake (mg/kg bw per day)		0	0	0.4	2.0	5.0	10.0	14.8	30.8	0
Leydig-cell adenomas (%)		4.5	3.8	1.6	4.4	7.8	3.1	3.8	17.0*	0–17.7 / 0–10.0
Interstitial-cell hyperplasia (%)		0	0	1.6	2.9	7.8	1.6	5.7	17.0*	—

From Pastoor et al. (1986) and Keller (1992a)

^aRange for historical controls (%): DuPont Haskell laboratory/Charles River laboratory.

* $p < 0.05$.

2.4 Genotoxicity

A battery of studies of mutagenicity with flusilazole technical was conducted to assess potential for inducing gene mutation, chromosome aberration or unscheduled DNA synthesis. The study results (summarized in Table 5) were clearly negative. Flusilazole technical did not demonstrate any genotoxic potential under the conditions tested.

2.5 Reproductive toxicity

(a) Multigeneration studies

Rats

Groups of six male and six female Crl:CD(SD)BR rats from a 90-day feeding study were used in a one-generation study of reproduction. The rats were fed daily diets containing flusilazole technical (purity, 96.7%) at a concentration of 0, 25, 125, or 375 ppm (equal to 0, 2, 9, and 27 mg/kg bw per day in males and 0, 2, 11, and 31 mg/kg bw per day in females) for 90 days before mating. Males and females within the same dosing group were mated for 15 days; females were examined daily for evidence of a copulation plug. After the mating period, females were housed individually. The fertility index was low in all groups, especially the control group (67.7%); the number of pregnant females per group ranged from three to six. At the highest dose of 375 ppm (equal to a mean of 29 mg/kg bw per day), a lower (relative to controls) gestation index, lower percentage of liveborn pups and lower pup weight at day 4 were observed. The study was not GLP-compliant, and the small group sizes and absence of individual data on some parameters limited the usefulness of this study for evaluation of reproductive toxicity; the Meeting therefore considered the results of this study as supplementary information (Pastoor et al., 1983).

Groups of 20 male and 20 female Crl:CD(SD)BR rats from a 2-year study of toxicity/oncogenicity were used in a two-generation (two-litter) study of reproduction, which was GLP-compliant. The rats were fed daily diets containing flusilazole technical (purity, 95.6%) at a concentration of 0, 10, 50, or 250 ppm (equal to 0, 1, 3, and 18 mg/kg bw per day in males and 0, 1, 4, and 20 mg/kg bw per day in females; pre-mating intake data) for at least 100 days before mating. Males and females (F_0) within the same dose group were mated (1 : 1) for 15 days; the females were examined daily for evidence of a copulation plug. After the mating period, F_0 females were housed individually and allowed to give birth to F_{1a} litters. About 1 week after weaning the last F_{1a} litter, F_0 females were mated (with different F_0 males of the same group) to produce F_{1b} litters. At weaning of F_{1b} litters, 20 males and 20 females per

Table 5. Results of studies of genotoxicity with flusilazole

End-point	Test object	Concentration	Purity (%)	Results	GLP-compliant	Reference
<i>In vitro</i>						
Reverse mutation ^{a,b}	<i>S. typhimurium</i> TA98, TA100, TA1535, TA1537	1–250 µg/plate	90.0	Negative	No	Donovan & Irr (1982)
Reverse mutation ^{c,d}	<i>S. typhimurium</i> TA97, TA98, TA100, TA1535	5–250 µg/plate	97.7	Negative	Yes	Arce et al. (1988)
Reverse mutation ^{c,e}	<i>S. typhimurium</i> TA97, TA98, TA100, TA1535	10–300 µg/plate	95.0	Negative	Yes	Reynolds (1991)
Forward mutation ^{f,g}	Chinese hamster ovary cells (CHO-K1/BH4) <i>Hgpert</i> locus	0.04–0.275 mmol/l	95.5	Negative	No	McCooley et al. (1983)
Unscheduled DNA synthesis ^h	Rat primary hepatocyte cultures	1×10^{-5} – 1.1×10^2 mM	95.5	Negative	Yes	Chromey et al. (1983)
Chromosome aberration ^{f,i}	Human lymphocytes	1.7–100 µg/ml	94.9	Negative	Yes	Vlachos et al. (1986)
<i>In vivo</i>						
Chromosome aberration ^j	Rat (CrI:CD, 20 males and 20 females per dose) bone-marrow cells	50–500 mg/kg bw (single oral gavage dose in corn oil)	Not reported	Negative	Yes	Farrow et al. (1983)
Micronucleus formation ^k	Mouse (CD-1, 5 males and 5 females per dose) bone-marrow cells	375 mg/kg bw (single oral gavage dose in corn oil) ()	91.5	Negative	Yes	Sorg et al. (1984)

GLP, good laboratory practice; QA, quality assurance; S9, 9000 × g supernatant from rat livers.

^a Positive and negative controls included, ± S9.

^b Cytotoxicity (decrease in colony size) seen at 100 and 250 µg/plate.

^c Positive and negative controls included, ± S9; GLP and QA statements included.

^d Cytotoxicity observed at 500 µg/plate in TA98 ± S9.

^e Cytotoxicity observed at 500 µg/plate and slight cytotoxicity at 250 µg/plate in TA100 ± S9.

^f Test in duplicate; positive and negative controls included; ± S9.

^g Cytotoxicity observed at ≥ 10 µg/plate –S9 and ≥ 15 µg/plate +S9.

^h Test in duplicate; positive controls included.

ⁱ Cytotoxicity observed at 135 µg/ml.

^j Aberrations analysed in 20 males and females at each dose; positive and negative controls included; only 50 cells were scored instead of 100 cells/animal as required by OECD; clinical signs at the highest dose: slightly depressed, soft faeces, urine stains, red stains on nose and/or ears and/or decreased body weight. One mortality.

^k Analysed five males and five females per group; positive and negative controls included; clinical signs included: decreased body tone and activity at 4 and 24 h after administration. One mortality at 48 h. No clinical signs at 72 h.

group were selected as F₁ parents for the F₂ generation. These rats were maintained on the same diets as their F₀ parents for 90 days before mating to produce F_{2a} and F_{2b} litters. Sibling mating was avoided. Mean body weight of the F_{1b} males at 250 ppm were decreased during the pre-mating period. No other treatment-related systemic effects were observed in any F₀ or F₁ adult in the study. There was no evidence of a test material-related effect on reproductive parameters (mating and fertility). Treatment-related embryofetal- and litter toxicity were observed at the highest dose (250 ppm). An increased number of stillborn pups and decreased viability index (days 0–4) occurred in all litters (F_{1a}, F_{1b}, F_{2a}, F_{2b}). Litter survival after day 4 was similar in all groups in F₁ and F₂ generations. Mean weights of F_{1b} and F_{2a} pups (both sexes) at weaning were slightly reduced and absolute and relative liver weights of F_{2b} weanling pups (males only) were increased. An increased incidence of hydronephrosis (unilateral

and/or bilateral) was noted in F_{2b} female weanlings (1 out of 10, 4 out of 10, 3 out of 10 and 5 out of 10, at 0, 10, 50 and 250 ppm, respectively). Since neither the severity nor the incidence of the lesions showed a dose–response relationship and considering the fact that hydronephrosis is a common lesion in weanling pups (range for historical controls, 0–30% in 13 in-house studies in rats), the finding was judged to be toxicologically insignificant. The NOAEL was 50 ppm, equal to 3 mg/kg bw per day, on the basis of embryo/fetotoxicity and decreased pup viability (Pastoor et al., 1986).

In a two-generation (one to two litters per generation) GLP-compliant study of reproduction, groups of 30 male and 30 female Crl:CD(SD)BR rats were fed diets containing flusilazole technical (purity, 94%) at a concentration of 0, 5, 50 or 250 ppm (equal to 0, 0.34, 3.46, and 17.3 mg/kg bw per day in males and 0, 0.40, 4.04, and 19.6 mg/kg bw per day in females; pre-mating intake data) for 73 days (F₀ rats) or 91 days (F₁ parents) before mating. Males and females within the same dosing group were randomly paired (1 : 1) for 15 days; females were examined daily for evidence of a copulation plug. One litter (F_{1a}) was produced in the F₀ generation and two litters (F_{2a} and F_{2b}) in the F₁ generation.

No treatment-related effects were evident in either generation (F₀ or F₁) at the lowest dose of 5 ppm. At the next higher dose of 50 ppm, increased smooth endoplasmic reticulum in the hepatocytes of males and centrilobular hepatocellular hypertrophy in females was observed, which was considered to be an adaptive response. At the highest dose of 250 ppm, F₁ females showed a slightly but consistently lower body weight. Reproductive toxicity was indicated by higher mortality during parturition and increased duration of gestation (mean values, 22.9–23.2 days compared with 22.4–22.6 days for the controls) of the F₀ and F₁ dams. No treatment-related effects on the mating and fertility indices were observed. Embryofetal and litter toxicity were also evident, with reduced numbers of pups/litter and an increased number of stillborn pups/litter in F_{1a}, F_{2a} and F_{2b} litters and decreased mean pup weights on days 14 and 21 of lactation (F_{2a} litters only) were noted at 250 ppm. The NOAEL for systemic toxicity was 50 ppm, equal to 4.04 mg/kg bw per day, on the basis of decreased body-weight gain at 250 ppm, and the NOAEL for reproductive and offspring toxicity was 50 ppm, equal to 4.04 mg/kg bw per day, on the basis of treatment-related mortality during parturition and increased duration of gestation, as well as embryofetal and litter toxicity at 250 ppm (Mullin, 1990).

(b) *Developmental toxicity*

Rats

In a range-finding study of developmental toxicity, groups of seven pregnant rats were given flusilazole technical (purity, 99%) at a concentration of 0, 100 or 300 mg/kg bw per day orally by gavage on days 7–16 of gestation. The results indicated that flusilazole caused maternal and embryo toxicity at 100 and 300 mg/kg bw per day, and a dose of 300 mg/kg bw per day resulted in cleft palates in approximately 51% of the fetuses in each litter.

In the main study of developmental toxicity, groups of 25 mated female Crl:CD(SD)BR rats were given flusilazole technical (purity, 95.6%) at a dose of 0, 10, 50, or 250 mg/kg bw per day orally by gavage (in corn oil) on days 7–16 of presumed gestation (the day a copulation plug was observed was designated as day 1 of gestation). On day 21 of gestation, all surviving dams were killed and necropsied. Fetuses were delivered by caesarean section and examined for external, visceral and skeletal abnormalities. This study was not GLP-compliant, but was considered conclusive.

No treatment-related signs of maternal toxicity were evident at the lowest dose of 10 mg/kg bw per day. At the next higher dose of 50 mg/kg bw per day, slight decreases in body-weight gain and food consumption during the dosing period, and a slight increase in relative liver weight of dams were observed. At the highest dose of 250 mg/kg bw per day, additional maternal toxic effects

included: higher mortality (2 out of 25), clinical signs of toxicity (23 out of 25, effects including chromodacryorrhoea, chromorhinorrhoea, wet/stained perineal areas, red vaginal discharges/stains and focal alopecia). Fetal examinations revealed treatment-related increases in the incidence of skeletal anomalies (misaligned sternebra, extra ossification centres in ribs and delayed ossification in sternebra) at all doses including 10 mg/kg bw per day, the lowest dose tested in the study. At the next higher dose of 50 mg/kg bw per day, there was reduced mean number of liveborn fetuses per litter, a higher total number of stunted fetuses (0, 1, 4 and 3 at 0, 10, 50 and 250 mg/kg bw per day, respectively) and an increased incidence of rudimentary rib. At the highest dose of 250 mg/kg bw per day, additional signs of embryofetotoxicity were: increased mean incidence of resorptions, reduced mean fetal weight/litter and increased incidence of extra ribs in the fetuses. Flusilazole technical was found to be teratogenic in this study. An increased incidence of cleft palate (28 out of 241 compared with 0 out of 331 in controls) and absence of renal papilla (21 out of 155 compared with 0 out of 175 in controls) were observed in fetuses from dams at 250 mg/kg bw per day, but not at lower doses. An unusually high incidence of hydrocephalus and/or dilated lateral ventricles of the brain was also noted in all test groups including controls; the effect was not dose-related and not considered to be related to treatment. The NOAEL for maternal toxicity was 10 mg/kg bw per day; no NOAEL for embryo/fetotoxicity could be identified in this study; and the NOAEL for teratogenicity was 50 mg/kg bw per day (Lamontia et al., 1984a).

In a second study of developmental toxicity, groups of 24 mated female Crl:CD(SD)BR rats were given flusilazole technical (purity, 95.6%) at a dose of 0, 0.4, 2, 10, 50, or 250 (10 females only) mg/kg bw per day orally by gavage (in corn oil) on days 7–16 of gestation (the day a copulation plug was observed was designated as day 1 of gestation). On day 21 of gestation, all surviving dams were killed and necropsied. Fetuses were delivered by caesarean section and examined for external, visceral and skeletal abnormalities. This study was not GLP-compliant, but was considered to be conclusive.

No treatment-related signs of maternal toxicity were observed at doses of 10 mg/kg bw per day or less. Slightly reduced body-weight gain and food consumption during the dosing period, and increased relative liver weight of dams were evident at the next higher dose of 50 mg/kg bw per day. At the highest dose of 250 mg/kg bw per day, clinical signs of maternal toxicity (alopecia, brown stains on face/limbs and stained perineal area) were also observed. No treatment-related embryo/fetotoxic effects were evident at the lower doses of 0.4 and 2 mg/kg bw per day. At higher doses of 10 mg/kg bw per day and greater, increased number of dams with median/late resorptions, increased total number of stunted fetuses, higher incidence of visceral (large renal pelvis and small renal papilla) and skeletal (rib) anomalies, and an increased incidence of delayed ossification (sternebra and vertebral arch) were observed. Flusilazole technical was teratogenic in this study. An increased incidence of cleft palate (21 out of 116 compared with 0 out of 291 in controls) was observed in fetuses from dams at 250 mg/kg bw per day, but not at lower doses. Hydrocephalus was not observed in any group. The NOAEL for maternal toxicity was 10 mg/kg bw per day, the NOAEL for embryo/fetotoxicity was 2 mg/kg bw per day, and the NOAEL for teratogenicity was 50 mg/kg bw per day (Lamontia et al., 1984b).

In a dietary study of developmental toxicity, groups of 24 mated female rats, Crl:CD(SD)BR strain, received daily diets containing flusilazole technical (purity, 96.5%) at a concentration of 0, 50, 100, 300 or 900 ppm (equal to 0, 4.6, 9.0, 26.6, and 79.2 mg/kg bw per day) on days 7–16 of gestation (the day a copulation plug was observed was designated as day 1 of gestation). On day 21 gestation, all surviving dams were killed and necropsied. Fetuses were delivered by caesarean section; weighed, sexed and then examined for external, visceral and skeletal abnormalities. The study was performed in compliance with GLP and test guideline OECD 414.

No treatment-related signs of maternal toxicity were evident at doses of 100 ppm or less. Significant, dose-related reductions in body-weight gain and food consumption were observed during the dosing period at the two higher doses of 300 and 9000 ppm. Treatment-related embryo/fetal toxic effects were noted at 100 ppm and greater; these were an increased incidence of median/late resorptions, small litters (fewer than 10 fetuses/litter) and significant dose-related increases in skeletal variations with extra ossification of the sternebra. At the two higher doses (300 and 900 ppm), additional symptoms of fetal toxicity included an increased number of stunted fetuses; a higher incidence of rudimentary ribs, extra ossification in cervical ribs and delayed ossification in the cervical vertebral arches. Flusilazole was not teratogenic in this study. No fetuses showed cleft palate and there were no treatment-related malformations observed at any dose up to and including 900 ppm, the highest dose tested. The NOAEL for maternal toxicity was 100 ppm (9.0 mg/kg bw per day), the NOAEL for embryo/fetotoxicity was 50 ppm (4.6 mg/kg bw per day), and the NOAEL for teratogenicity was 900 ppm (79.2 mg/kg bw per day) (Alvarez et al., 1984).

In a GLP-compliant study of prenatal and postnatal toxicity in rats, groups of 24 (phase I, prenatal study) or 22 (phase II, postnatal study) mated female rats, strain CrI:CD(SD)BR, were given flusilazole technical (purity, 96.5%; in 0.5% aqueous methyl cellulose) at a dose of 0, 0.2, 0.4, 2, 10, or 100 mg/kg bw per day orally by gavage on days 7–16 of gestation (the day a copulation plug was observed was designated as day 1 of gestation). In phase I, dams were killed on day 21 of gestation for examination of the uterine contents. Rats in an additional control group and group at 100 mg/kg bw per day were killed on day 22 of gestation to determine whether the absence of renal papillae was a compound-related effect or an anomaly. In view of the fact that the first few dosing solutions used in phase I were found to be considerably below the nominal concentrations (1–19% of nominal) and the next analysis (75–110% of nominal) was done only on day 7, no definitive conclusions could be made from results of this part of the study. Maternal toxicity was evident at the highest dose of 100 mg/kg bw per day in the form of clinical signs (chin/perinasal staining and/or wet perineum); reduced body-weight gain and food consumption during the dosing period; and increased absolute and relative liver weights. Embryo/fetotoxicity was observed at 10 mg/kg bw per day and above, including increases in number of stunted fetuses and in dams with stunted fetuses; and a higher incidence of visceral anomalies (small renal papilla and distended ureter). At the highest dose of 100 mg/kg bw per day, additional embryo/fetotoxicity noted included an increased incidence of median/late resorptions and a decrease in the mean number of live fetuses per litter. Treatment-related malformations (absence of renal papilla) were observed in three fetuses (two litters) from dams in the group at 100 mg/kg bw per day.

In phase II, dams were permitted to deliver naturally and raise their litters to weaning. All dams and pups were killed on day 21 of lactation and subjected to gross necropsy. The dosing solutions were found to be adequately prepared in phase II. No treatment-related maternal toxicity was evident at doses of 10 mg/kg bw per day or less. At the next higher dose of 100 mg/kg bw per day, increased mortality (5 out of 22 compared with 0 out of 22 in controls), clinical signs of difficult parturition (pallor, hunching, weakness and/or dystocia during parturition and lactation in four dams), reduced body-weight gain and food consumption during the early part of dosing, and increased liver weight in dams were observed. No treatment-related embryo/fetotoxicity was noted at doses of 2 mg/kg bw per day or less. At 10 mg/kg bw per day and above, there were dose-related overt increases in the mean duration of gestation (22.8, 23.1, 22.9, 23.1, 23.5 and 24.7 days at 0, 0.2, 0.4, 2, 10 and 100 mg/kg bw per day, respectively), decreases in mean litter size and number of liveborn fetuses per litter, an increased number of small litters (with fewer than 10 fetuses per litter), and a higher incidence of dilated renal pelvic and/or ureter in pups at weaning. At the highest dose of 100 mg/kg bw per day, additional signs of treatment-related embryo/fetotoxicity included a reduced total number of live litters, an increased mean number of dead fetuses per litter, and a reduced viability index (days 0–4, 82% compared with

98–99% in the control group and at the lowest dose). Flusilazole technical was not teratogenic in this study. There was no evidence of any treatment-related malformations in any live pups examined. Of the 42 pups found dead (29 were in the group at 100 mg/kg bw per day), absence of renal papilla was observed in only 2 out of 42 and small papilla in 4 out of 42. On the basis of the results of phase II of the study, the NOAEL for maternal toxicity was 10 mg/kg bw per day, the NOAEL for embryo/feto-toxicity was 2 mg/kg bw per day, and the NOAEL for teratogenicity was 100 mg/kg bw per day, the highest dose tested (Alvarez et al., 1985a).

Five groups of 25 mated female Crl:CD®(SD)IGS BR rats were given flusilazole technical (purity, 94.85%; in aqueous methyl cellulose) at a dose of 0, 0.5, 2, 10 or 50 mg/kg bw per day by gavage on days 6–20 of gestation (the day a copulation plug was observed was designed as day of gestation). On day 21 of gestation, all surviving does were killed and necropsied. Two additional groups of rats were given flusilazole at a dose of 50 mg/kg bw per day on days 6–15 of gestation and killed on day 16 or 21 of gestation. A second control group was given a dose of 0 mg/kg bw per day (vehicle only) on days 6–15 of gestation and killed on day 16. On day 21 of gestation, all surviving dams were killed and necropsied. Fetuses were delivered by caesarean section; weighed, sexed and then examined for external, visceral and skeletal abnormalities. The study was performed in compliance with GLP and test guideline OECD 414.

No treatment-related signs of maternal toxicity were evident at 0.5 mg/kg bw per day. At doses of 2 mg/kg bw per day or greater, there was an increased incidence of red vaginal discharge during the latter part (days 13–21) of gestation (0, 0, 3, 12, and 22 at 0, 0.5, 2, 10 and 50 mg/kg bw per day, respectively) and an increase in placental weights. In addition, at doses of 10 mg/kg bw per day and greater, maternal body weight, body-weight gain and food consumption were reduced. Fetal examinations revealed a slight increase in the incidence of rudimentary cervical ribs at doses of 2 mg/kg bw per day and greater (3 out of 332, 4 out of 331, 9 out of 306, 27 out of 314 and 141 out of 302 at 0, 0.5, 2, 10 and 50 mg/kg bw per day, respectively) and an increase in patent ducts arteriosis at 50 mg/kg bw per day. At the highest dose tested (50 mg/kg bw per day), an increased incidence of the malformation naris atresia was observed in the two groups killed at day 21, but not in the group killed on day 16. The NOAEL for maternal toxicity, and embryo/fetotoxicity was 2.0 mg/kg bw per day in this study and the NOAEL for teratogenicity was 10 mg/kg bw per day (Munley, 2000).

In a study of developmental toxicity, groups of 25 Crl:CD®(SD)BR female rats were given flusilazole (purity, 94.85%) at a dose of 0, 2, 10, 50 or 250 mg/kg bw per day administered dermally on days 6–19 of gestation. The study was performed in compliance with GLP and test guideline OECD 414.

Placental changes consisting of enlargement with labyrinth angiectasis and trophoblast necrosis were observed starting at the lowest dose of 2 mg/kg bw per day. At doses of 10 mg/kg bw per day and greater, there was an increase in the number of total late resorptions as well as an increase in placental mineralization affecting the placental capillaries. An increase in the number of early and late resorptions and decreases in the total number of live fetuses and number of live fetuses per dam occurred at doses of 50 mg/kg bw per day or greater. There was an increase in the incidence of red vaginal discharge and/or red material found around the vagina or around the nose (250 mg/kg bw per day) or forelimbs at doses of 50 mg/kg bw per day and greater. Body-weight gains were decreased at 250 mg/kg bw per day at the end of the treatment period and food consumption was decreased sporadically in the group at 50 mg/kg bw per day and throughout the treatment period in the group at 250 mg/kg bw per day. Albumin, total protein and potassium concentrations were decreased at doses of 10 mg/kg bw per day or greater, globulin concentrations were decreased at doses of 50 mg/kg bw per day or greater and creatinine concentrations were decreased at 250 mg/kg bw per day. There were no treatment-related malformations. There were increases in the incidence of skeletal variations at 10 mg/kg bw per day, consisting of an increase in the incidence of rudimentary fourteenth ribs, and at

Table 6. Incidence of selected fetal malformations and variations in rats exposed prenatally to flusilazole

Study ^a	Dose (mg/kg bw per day)	Fetal malformation or variation			
		Cleft palate	Naris atresia	Absent renal papilla	Rudimentary cervical rib
		Total No. of fetuses (No. of litters) affected			
Alvarez et al., (1984)	0	—	—	1 (1)	3 (1)
	4.6	—	—	5 (2)	—
	9.0	—	—	1 (1)	—
	26.6	—	—	2 (2)	1 (1)
	79.2	—	—	1 (1)	—
Lamontia et al., (1984a)	0	—	—	—	—
	10	—	—	—	1 (1)
	50	—	—	—	13 (9)* ^c
	250	28 (10)* ^b	—	21 (12)* ^b	9 (7)* ^c
Lamontia et al., (1984b)	0	—	—	—	—
	0.4	—	—	5 (5)	—
	2	—	—	2 (2)	—
	10	—	—	5 (5)	—
	50	—	—	5 (4)	18 (8)* ^c
	250	21 (5)* ^c	—	4 (2)	15 (5)* ^c
Munley (2000)	0	—	—	—	3 (3)
	0.5	—	1 (1)	—	4 (4)
	2	—	—	—	9 (6)
	10	—	—	—	27 (15)* ^c
	50	—	3 (2), 3 (3) ^d	—	141 (22) ^c

—, no incidence.

^a Purity of flusilazole used in each study: Alvarez et al. (1984), 96.5%; Lamontia et al. (1984a), 95.6%; Lamontia et al. (1984b), 95.6%; Munley (2000), 94.85%.

^b Jonckheere test of statistical significance.

^c Fischer exact test of statistical significance.

^d Incidence in each of the two groups at 50 mg/kg bw per day.

* $p \leq 0.05$.

250 mg/kg bw per day, consisting of an increase in full fourteenth ribs and seventh cervical ribs and a decrease in ossification of the first cervical centra. The LOAEL for maternal and embryo/fetotoxicity was 2 mg/kg bw per day. No NOAEL could be identified; however, the study authors suggested that the data supported the fact that the lowest dose tested (2 mg/kg bw per day) was near the bottom end of the dose–response curve and was approaching a NOAEL (Schardein, 1998)

Rabbits

Four groups of 18 artificially inseminated New Zealand White female rabbits were given flusilazole technical (purity, 96.5%; in corn oil) at a nominal dose of 0, 2, 5, or 12 mg/kg bw per day (equal to 0, 1.9, 4.8, and 10.1 mg/kg bw per day based on analysis of dosing solutions) orally by gavage on days 7–19 of presumed gestation (the day of insemination was designated as day 0 of gestation). On day 29 of gestation, all surviving does were killed and necropsied. Fetuses were

delivered by caesarean section and examined for external, visceral and skeletal abnormalities. There was no treatment-related mortality, clinical signs of maternal toxicity nor disturbances of the intra-uterine development of the conceptuses at any dose up to and including 12 mg/kg bw per day, the highest dose tested in the study. All fetuses delivered showed normal development. No evidence of fetotoxicity and no treatment-related increases in malformations were observed in fetuses at any dose tested. The high incidence of hydrocephalus observed in this study—1 (1), 2 (1), 4 (2), and 4 (3) fetuses (litters) at 0, 2, 5, and 12 mg/kg bw per day, respectively, which was not confirmed in three subsequent studies (hydrocephalus being found in only one fetus at 35 mg/kg bw per day and in none at any lower dose nor in the controls), was not considered to be treatment-related. The NOAEL for maternal toxicity, embryo/fetotoxicity and teratogenicity was 12 mg/kg bw per day (or 10.1 mg/kg bw per day by analysis). This study was not GLP compliant and was considered to provide supplementary information, as the doses administered were not high enough to elicit maternal toxicity (Solomon et al., 1984).

In a second study, three groups of 20 artificially inseminated New Zealand White female rabbits were given flusilazole technical (purity, 96.5%) at a nominal dose of 0, 12, or 35 mg/kg bw per day (equal to 0, 11.2 and 31.5 mg/kg bw per day based on analysis of dosing solutions) orally by gavage on days 7–19 of presumed gestation (the day of insemination was designated as day 0 of gestation). On day 29 of gestation, all surviving does were killed and necropsied. Fetuses were delivered by caesarean section and examined for gross pathology. This study was not GLP compliant, but was considered conclusive.

No evidence of maternal toxicity nor embryo/fetotoxicity was observed at the lowest dose of 12 mg/kg bw per day and there were no increases in the incidence of any malformations or fetal variations, compared with the controls. At the highest dose of 35 mg/kg bw per day, there was an increased incidence of red vaginal discharge and stained tail, and an increased incidence of periodic anorexia. Abortion occurred in 2 out of 13 does (compared with 0 out of 16 in the control group) and total early resorption occurred in 10 out of 13 does (compared with 1 out of 16 in the control group). Teratogenicity could not be assessed at this dose because only one live litter was produced. The NOAEL for maternal and embryo/fetotoxicity was 12 mg/kg bw per day (or 11.2 mg/kg bw per day by analysis), and for teratogenicity was 12 mg/kg bw per day (or 11.2 mg/kg bw per day by analysis) and greater (Zellers et al., 1985).

In a range-finding dietary study of developmental toxicity in rabbits, four groups of seven artificially inseminated New Zealand White female rabbits were fed daily diets containing flusilazole technical (purity, 94.8%) at a concentration of 0, 500, 1000, or 2000 ppm on days 7–19 of gestation. The pregnancy rate (three out of seven per group) was low in groups at 500 and 1000 ppm. The incidence of mortality in utero was high at 2000 ppm (four out of seven females with total resorptions).

In the main study, groups of 20 artificially inseminated, New Zealand White female rabbits were given diets containing flusilazole technical (purity, 94.8%) at a concentration of 0, 300, 600, or 1200 ppm (equal to 0, 8.9, 21.2 and 37.8 mg/kg bw per day) on days 7–19 of presumed gestation (day of insemination was designated as day 0 of gestation). All surviving does were killed on day 29 of gestation and necropsied. Fetuses were delivered by caesarean section and examined for external, visceral and skeletal abnormalities. At the highest dose of 1200 ppm, maternal body weight and food consumption were decreased during dosing. Pregnancy rate was reduced in all treated groups (fertility, 9 out of 20, 10 out of 20, and 7 out of 20 at 300, 600, and 1200 ppm, respectively). The number of does with total resorption was increased in the groups at the intermediate (600 ppm) and highest (1200 ppm) dose. There were no treatment-related effects on mean number of live fetuses/litter, mean number of resorptions (in dams with live fetuses) or fetal weight. The small number of litters available at 600 and 1200 ppm (three per dose) precluded any definitive assessment of fetotoxicity

or teratogenic potential at these doses. There were no apparent treatment-related effects at the lowest dose of 300 ppm.

In a supplementary study, groups of 18 or 25 (300 ppm) inseminated, New Zealand White female rabbits were given flusilazole technical at a concentration of 0, 30, 100, or 300 ppm (equal to 0, 0.81, 2.84, and 8.32 mg/kg bw per day) on days 7–19 of presumed gestation. The pregnancy rate was again low in all groups, including the control group (fertility, 8 out of 18). Total resorption occurred at 0 and 300 ppm (25% and 29%, respectively, of the pregnant does), but not at the lowest (30 ppm) or intermediate (100 ppm) dose. Because of the low number of live litters available for examination, the data in this study did not allow adequate assessment of the embryo/fetotoxicity and teratogenic potential of the test compound. The NOAEL for maternal toxicity was 600 ppm (21.2 mg/kg bw per day) on the basis of decreased body weight and food consumption during dosing at 1200 ppm. No definitive NOAEL for embryo/fetotoxicity nor teratogenicity could be identified in this study. The study was not GLP compliant and was stated to, in general, conform to test guideline OECD 414, and was considered as supplemental information, owing to the lack of litters available for examination (Alvarez et al., 1985b).

In another study, groups of 18 artificially inseminated New Zealand White female rabbits were given flusilazole technical (purity, 93.8%; in 0.5% methylcellulose) at a dose of 0, 7, 15 or 30 mg/kg bw per day orally by gavage on days 7–19 of gestation. The study was performed in compliance with GLP and test guideline OECD 414.

The pregnancy rate was acceptable in all test groups (12 out of 18, 14 out of 18, 16 out of 18, and 16 out of 18 at 0, 7, 15 and 30 mg/kg bw per day, respectively). No treatment-related maternal nor embryo/fetal toxicity was evident at the lowest dose of 7 mg/kg bw per day. At the next two higher doses of 15 and 30 mg/kg bw per day, clinical signs of maternal toxicity (cageboard red discharge and brown/yellow-stained tail) and increased incidence of abortion (one dam/dose group) and total resorptions (4 out of 16 and 12 out of 16, respectively) were observed. At 30 mg/kg bw per day, food consumption was also decreased during the dosing period. The mean number of live fetuses per litter, mean number of dead fetuses (zero), mean fetal weight and mean male : female ratio were comparable between the control group and all treated groups. No treatment-related external, visceral or skeletal malformations/variations were observed in any of the fetuses from does at any dose, including those at 30 mg/kg bw per day, the highest dose tested in the study. However, it must be noted that assessment of fetotoxicity and teratogenic potential at 30 mg/kg bw per day was based on data from only three live litters available for that dose (compared with 11–12 live litters in the control group and at lower doses). The limited data points reduce confidence in the accuracy of any study conclusions made on the basis of observations at this dose. Therefore, the NOAEL for maternal and embryo/fetal toxicity was 7 mg/kg bw per day, and for teratogenicity was 15 mg/kg bw per day (Alvarez, 1990).

2.6 *Special studies*

(a) *Studies on mechanisms by which Leydig-cell tumours are induced*

Rats

In a GLP-compliant study in vivo, groups of 10 Crl:CD BR male rats were given flusilazole technical (purity, 94%; in corn oil) at a dose of 0, 20, 50, 150, or 250 mg/kg bw per day (given as two equal half-doses, twice per day) by subcutaneous injection for 14 days. The control group (0 mg/kg bw per day) and group at 250 mg/kg bw per day each contained an additional subgroup of 10 male rats that were treated with human chorionic gonadotropin (hCG) 1 h before killing. Ketoconazole (a known inhibitor of 17 α -hydroxylase) was used as the positive control. Groups of 10 male rats were treated with ketoconazole (in saline) at a dose of 0, 20, 50, 100 or 200 mg/kg bw

Table 7. Incidence of hydrocephaly in rabbits exposed prenatally to flusilazole

Study ^a	Dose (mg/kg bw per day)	Total No. of fetuses (litters) affected
Alvarez (1990)	0	—
	7	—
	15	1 (1)
	30	1 (1)
Alvarez et al. (1985b)	0	—
	8.9	—
	21.2	—
	37.8	—
Solomon et al. (1984)	0	1 (1)
	1.9	2 (1)
	4.8	5 (3)
	10.1	4 (3)
Zellers et al. (1985)	0	—
	11.2	—
	31.5	1 (1)

—, no incidence.

^a Purity of flusilazole: Alvarez (1990), 93.8%; Alvarez et al. (1985b), 94.8%; Solomon et al. (1984), 96.5%; Zellers et al. (1985), 96.5%.

per day (given as two equal half-doses, twice per day) for 14 days. The control group (0 mg/kg bw per day) and group at 200 mg/kg bw per day each contained an additional subgroup of 10 male rats that were treated with hCG 1 h before killing. All surviving rats were killed on day 15 of treatment and necropsied. Samples of testicular interstitial fluid and serum were collected from rats that were not treated with hCG; interstitial fluid was analysed for testosterone and serum was analysed for testosterone, estradiol, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Serum samples collected from the rats treated with hCG were analysed for testosterone, androstenedione, 17 α -hydroxyprogesterone and progesterone.

At the lowest dose of 20 mg/kg bw per day, and greater, increased absolute and relative liver weights and a dose-related inhibition of serum testosterone (statistically significant at 150 mg/kg bw per day and greater) and estradiol (statistically significant at all doses) concentrations were observed. At the two higher doses (150 and 250 mg/kg bw per day), clinical symptoms of toxicity (sores, stained/wet fur, dehydration and diarrhoea), decreased body weight and body-weight gain, and reduced food consumption were evident. Increased mortality (8 out of 10) also occurred at the highest dose of 250 mg/kg bw per day. At 250 mg/kg bw per day in the rats treated with hCG, serum testosterone concentrations were significantly lower than in the controls; no other significant differences in hormone levels were noted in rats treated with flusilazole. In the positive-control group treated with ketoconazole, rats showed significantly lower concentrations of testosterone, androstenedione and 17-hydroxyprogesterone, and higher concentrations of progesterone, indicating inhibition of 17 α -hydroxylase. On the basis of the results, no NOAEL for the portion of this study that was carried out in vivo could be identified.

In a GLP-compliant study in vitro, Leydig cells were collected from rat testes at termination of the study in vivo and cultured in microplate wells. The cells were then incubated with either flusilazole technical or ketoconazole at doses of 0.05–100 μ mol/l (in 70% ethanol) for 2 h. Culture media were sampled and analysed for testosterone, androstenedione, 17 α -hydroxyprogesterone

and progesterone. The concentration at which 50% inhibition was produced (IC_{50}) of testosterone was determined. Results of the study in vitro confirmed the hormonal changes observed in vivo. Incubation of testicular Leydig cells with flusilazole technical caused a dose-dependent lowering of testosterone and androstenedione concentrations, suggesting inhibition of enzymes involved in steroid biosynthesis. The IC_{50} for testosterone was 3.475–1.455 $\mu\text{mol/l}$ without hCG and 2.774–0.646 $\mu\text{mol/l}$ with hCG pre-treatment. In cells treated with ketoconazole (from rats in the positive-control group), the IC_{50} for testosterone was 0.97–0.83 $\mu\text{mol/l}$ without hCG and 0.154–0.065 $\mu\text{mol/l}$ with hCG (Cook, 1993).

In a GLP-compliant study, groups of 52 male and 52 female Crl:CD BR rats were fed daily diets containing flusilazole technical (purity, 95%) at a concentration of 0, 10, 125, 375, or 750 ppm (equal to 0, 0.58, 7.27, 22.1, and 44.7 mg/kg bw per day in males and 0, 0.74, 9.40, 27.6, and 59.0 mg/kg bw per day in females) for up to 91 days. Each group was divided into three subgroups in order to study possible mechanisms of action of toxicity in the liver and urinary bladder. In each group, subgroups of 20 males and 20 females were assigned for evaluation of the liver or urinary bladder; five males and five females per subgroup were killed on days 7 or 8, 14, 46 and 91 for cellular proliferation and histopathological assessment. An additional 10 males and 10 females per subgroup were used for evaluation of cytochrome P450 and peroxisome proliferation; five males and five females were killed on days 14 and 90 (males) or 15 and 91 (females). Serum concentrations of testosterone, estradiol and LH were determined in all males killed on days 14 and 90 ($n = 10$). No treatment-related adverse effects were observed at the lower doses of 10 and 125 ppm. At 125 ppm, absolute and relative liver weights were increased; in the absence of histopathological changes or any other symptoms of toxicity, the finding was not considered to be toxicologically significant. At the next two higher doses (375 and 750 ppm), liver hypertrophy was observed; the response was sex-specific: lamellar bodies and periportal hypertrophy occurred in males and centrilobular hypertrophy without lamellar bodies was evident in females. In the urinary bladder, transitional-cell necrosis, exfoliation and hyperplasia were noted. Hepatic cytochrome P450 levels were higher than control values, but there was no evidence of peroxisome proliferation in the liver of these treated rats. There was no increase in cellular proliferation: in the liver there was decreased proliferation in females at doses of 125 ppm and greater and in the urinary bladder of males at 750 ppm. No significant changes in the serum concentrations of testosterone, estradiol and LH were found in any of the males examined. The NOAEL was 125 ppm, equal to 7.27 mg/kg bw per day (Keller, 1992a)

(b) Study on the mechanism of liver effects in dogs

In order to distinguish an adaptive or toxic response at 20 ppm in a 1-year feeding study in dogs, groups of ten male, outbred, beagle dogs were fed diets that contained flusilazole (purity, 92.5%) at a concentrations of 0, 5, 20 or 75 ppm (equivalent to 0, 0.18, 0.68 and 2.65 mg/kg bw per day). Five dogs per group were killed after 28 days and the remaining five after a recovery period of 28 days. Daily observations were made for mortality and clinical signs; food consumption and body weights were measured weekly; haematology and clinical chemistry parameters were evaluated at study initiation, and at weeks 4 and 7. This study was GLP compliant and was performed in compliance with test guideline OECD 452.

There were no effects on survival, clinical signs or nutritional parameters. A slight increase in mean absolute and relative liver weight was observed after 4-weeks exposure in all treated groups. Aspartate aminotransferase (AST) activity was also minimally increased at 20 and 75 ppm, but was not clearly dose-related, and was not considered to be an adverse effect. The minimal liver-weight and AST changes had reversed during the 28-day recovery period. Cytochrome P450 isozymes were significantly induced at 20 and 75 ppm (i.e. total cytochrome P450 and CPYB1/2,

CYP3A and CYP4A isozymes). This was indicative of an adaptive response and is consistent with observations of increased liver weight in the absence of hepatotoxicity. It is also consistent with the suggestion of increased smooth endoplasmic reticulum in livers of dogs at 75 ppm. The observed effects were reversible, which further supports the conclusion that these are not adverse findings (Mertens, 1999).

Comments

Biochemical aspects

In rats, orally administered ^{14}C -labelled flusilazole was readily absorbed from the gastrointestinal tract and rapidly excreted in urine (72% of triazole label) and faeces (up to 87% of phenyl label), with little or no radioactivity recovered in the expired air. The excretion half-life was approximately 34 h and more than 90% of the administered dose was eliminated within 96 h. Tissue retention of radiolabelled material was low. Total tissue residues excluding the carcass (which accounted for approximately 2% of the administered dose) were less than 1% of the administered dose, therefore demonstrating no evidence of bioaccumulation.

^{14}C Flusilazole was extensively metabolized in rats. Recovered parent compound accounted for only 2–11% of the given dose, found predominantly in the faeces (urinary concentration, < 1%). After absorption, flusilazole was cleaved at the triazole ring. With phenyl-labelled test material, the major faecal metabolites identified were [bis(4-fluorophenyl)methyl] silanol, [bis(4-fluorophenyl)methylsilyl] methanol and its fatty acid conjugates, and disiloxane. Except for the fatty acid conjugates, the same metabolites were found in the urine. With triazole-labelled material, the main metabolite identified was 1*H*-1,2,4-triazole, which was found predominantly in the urine (63.8% of the administered dose in males, 51.6% in females); faeces contained only a small amount of the metabolite.

Toxicological data

Flusilazole is moderately to slightly toxic in rats when given as a single oral dose; and minimally toxic to rats and rabbits when administered as a single dose dermally or by inhalation. The oral LD_{50} in rats was 672–1216 mg/kg bw, the dermal LD_{50} in rabbits was > 2000 mg/kg bw and the inhalation LC_{50} in rats was 6.8–7.7 mg/l. Flusilazole was found to be minimally irritating to the eyes and the skin of New Zealand White rabbits. It was practically non-irritating to the skin and was not a dermal sensitizer in guinea-pigs in a Buehler test.

Short- and long-term studies of repeated oral doses of flusilazole in mice (90-day dietary study), rats (90-day studies of gavage and dietary administration) and dogs (90-day and 1-year dietary studies) resulted primarily in lesions of the liver (hepatocellular hypertrophy, fatty change, focal inflammation/necrosis (mouse only) and vacuolation) and urinary bladder (urothelial hyperplasia and vacuolation). In addition, the gastrointestinal tract was a target in dogs. Clinical chemistry was not assessed in the studies in mice, the only finding in the studies in rats was a decrease in cholesterol in both sexes in the 90-day study and increase in cholesterol in females only in the long-term studies. On the basis of the hepatic and/or urinary bladder histopathology, the NOAEL was 75 ppm (equal to 12 mg/kg bw per day) in mice, 125 ppm (equal to 9 mg/kg bw per day) in rats and 20 ppm (equal to 0.7 mg/kg bw per day) in dogs (1-year study). Lymphoid hyperplasia of the gastric mucosa was observed in all treated dogs in the 90-day study, but not in the controls. In the 1-year study, this finding was observed in all dogs, including controls, with severity increasing in a dose-related manner. Effects at the LOAEL in the 1-year study in dogs included hepatocellular hypertrophy, inflammatory infiltration and vacuolation (males only), decreased cholesterol, total protein and albumin, and increased alkaline phosphatase activity and leukocyte counts. A mechanistic study in male dogs at

the doses used in the 1-year study indicated that after 28 days of exposure, the effects observed on the liver were adaptive and reversible (weight, increased AST activity and cytochrome P450). The dog appeared to be the most sensitive species in these studies, with a NOAEL of 0.7 mg/kg bw per day in the 1-year study, on the basis of histopathology changes in the liver and stomach and changes in clinical chemistry.

After repeated short-term (21-day) dermal application of flusilazole, there was no evidence of any treatment-related systemic toxicity in rabbits given doses of up to 200 mg/kg bw per day.

Flusilazole was tested for genotoxicity in an adequate range of assays in vitro and in vivo. It was not genotoxic in mammalian or microbial systems. The Meeting concluded that flusilazole was unlikely to be genotoxic.

Two 18-month dietary studies with flusilazole were conducted in mice. In the first study in which flusilazole was administered at concentrations of up to 200 ppm in the diet, the target organs identified were the liver (hepatocellular fatty changes), kidney (decreased weight), and urinary bladder (histopathological change). There was no evidence of carcinogenicity in this study. Concentrations from 100 to 2000 ppm were used in the second 18-month study. Systemic toxicity was observed at all doses. At doses of 500 and 1000 ppm in males (73.1 and 144 mg/kg bw per day, respectively) or 1000 and 2000 ppm in females (200 and 384 mg/kg bw per day, respectively), overt hepatic lesions (increased foci of hepatocellular alteration and hepatocellular hypertrophy with cytoplasmic vesiculation and/or vacuolation) and cellular hyperplasia in the urinary bladder were observed. Increased incidences of liver tumours (hepatocellular adenomas and carcinomas) were observed at concentrations of more than 1000 ppm. Liver tumours occurred at doses in excess of the maximum tolerated dose (MTD) and were preceded at lower concentrations by clear histopathological changes in the liver. The overall NOAEL for systemic toxicity was 25 ppm, equal to 3.4 mg/kg bw per day, on the basis of hepatotoxicity and urinary bladder hyperplasia at 100 ppm (14.3 mg/kg bw per day) in males and hepatocellular fatty changes at 200 ppm (27 mg/kg bw per day) in both sexes. The overall NOAEL for carcinogenicity was 200 ppm (equal to 36 mg/kg bw per day) in females and 1000 ppm (equal to 144 mg/kg bw per day) for males. The incidence of tumours at the NOAEL was within the range for historical controls.

The toxicity and carcinogenicity of flusilazole were investigated in two 2-year studies in rats. The target organs identified were the liver and bladder. The overall NOAEL for systemic toxicity was 50 ppm, equal to 2.0 mg/kg bw per day, on the basis of mild nephrotoxicity (pyelonephritis in females) and hepatotoxicity (hepatocellular hypertrophy in both sexes), acidophilic foci, and diffuse fatty change (females only). There was no treatment-related increase in the incidence of any tumour type AT up to 250 ppm (the highest dose tested in the first study). Concentrations of between 125 and 750 ppm, the latter exceeding the MTD, were used in the second study. Flusilazole was found to be tumorigenic at the highest dose of 750 ppm (30.8 mg/kg bw per day) causing bladder transitional cell neoplasia in both sexes and testicular Leydig-cell tumours in males. There was no evidence of any treatment-related increase in tumour incidence at a dietary concentration of 375 ppm. The overall NOAEL for carcinogenicity was 375 ppm (14.8 mg/kg bw per day).

A special 2-week study to investigate the possible mechanism for the induction of testicular Leydig-cell tumours was conducted in rats. The results demonstrated that flusilazole caused a dose-dependent lowering of estradiol concentrations at 20 mg/kg bw per day and above, and of serum and interstitial testosterone concentrations at 150 mg/kg bw per day in vivo after subcutaneous exposure ($n = 10$) and a dose-related decrease in testosterone and androstenedione production in testicular Leydig-cell cultures by inhibition of enzymes involved in steroid biosynthesis in vitro at less than 5 $\mu\text{mol/l}$. In the 90-day mechanistic study in rats given flusilazole at doses similar to those used in the second long-term study in rats (0, 10, 125, 375 or 750 ppm), there were no changes in serum concentrations of testosterone, estradiol or LH, which would be expected for this mode of action. However, there was appreciable inter-animal variability in the hormone measurements.

Overall, the data suggested that flusilazole may induce Leydig-cell tumours via an endocrine-related mechanism—inhibition of testosterone and estradiol biosynthesis could contribute to disruption of the hypothalamus–pituitary–testis axis, resulting in overstimulation of the testicular endocrine tissues. Exposure to flusilazole at doses not causing disruption of the hypothalamus–pituitary–testis axis would, therefore, be unlikely to induce an increase in Leydig-cell tumours. Although this mode of action is relevant to humans, there was good evidence to suggest that humans are less sensitive to chemically-induced Leydig-cell tumours than are rats, owing to differences in sensitivity to LH on the basis of number of Leydig-cell receptors and control of LH-receptor expression (e.g. by prolactin in rodents but not in humans) (Foster, 2007; Cook et al., 1999).

The Meeting concluded that the weight of evidence indicated that the mode of action for bladder tumours was via cell injury and regenerative hyperplasia (Cohen, 1998).

In view of the lack of genotoxicity and the finding of hepatocellular tumours in mice and testicular and bladder transitional cell tumours in rats only at doses at which marked toxicity was observed, the Meeting concluded that flusilazole is not likely to pose a carcinogenic risk to humans at dietary levels of exposure.

The effect of flusilazole on reproduction in rats was investigated in two two-generation studies. The first was a part of a 2-year feeding study. No parental toxicity was observed at doses of up to 250 ppm. The same doses were used in the second definitive two-generation study. The NOAEL for parental systemic toxicity was 50 ppm, equal to 4.04 mg/kg bw per day, on the basis of slightly lower body-weight gain in F₁ females. The main reproductive effects at 250 ppm included increased duration of gestation and increased maternal mortality during parturition. The NOAEL for reproductive toxicity was 50 ppm, equal to 3.46 mg/kg bw per day. Toxicity observed in offspring at 250 ppm included a reduced number of live pups per litter and decreased pup growth. The NOAEL for offspring toxicity was 50 ppm, equal to 4.04 mg/kg bw per day.

Nine studies of developmental toxicity were carried out with flusilazole administered orally, of which five were in rats (one dietary study and four with gavage administration) and four (one dietary study and three with gavage administration) in rabbits to characterize potential teratogenicity observed in some studies.

In most of the studies in rats, the NOAEL for maternal toxicity was 10 mg/kg bw per day on the basis of reduced body-weight gain and decreased food consumption. In one study, the NOAEL for maternal toxicity was 2 mg/kg bw per day on the basis of increased incidence of red vaginal discharge during the latter part of gestation and an increase in placental weights at 10 mg/kg bw per day, which was not assessed in the other studies. At maternally toxic doses, specific malformations noted were cleft palate, nares atresia and absent renal papillae. An increased incidence of anomalies (extra cervical ribs, patent ductus arteriosus) was also observed. The incidence of rudimentary cervical ribs was slightly, but not statistically significantly increased at 2 mg/kg bw per day (3 out of 3, 4 out of 4, 9 out of 6, 27 out of 15, and 141 out of 22 fetuses per litter in the groups at 0, 0.5, 2, 10, 50 mg/kg bw per day, respectively). The overall NOAEL for embryo/fetotoxicity was 2 mg/kg on the basis of a higher incidence of skeletal variations (extra cervical ribs) at 10 mg/kg bw per day. No malformations were found at doses of less than 50 mg/kg bw per day.

In four studies of developmental toxicity in rabbits, the NOAEL for maternal and embryo/fetal toxicity was 7 mg/kg bw per day on the basis of clinical signs of toxicity, increased incidence of abortion and total resorption at 15 mg/kg bw per day. There was no evidence for any teratogenic potential in rabbits given flusilazole at doses of up to 15 mg/kg bw per day, the maximum tolerated dose in this study.

A major metabolite identified was 1*H*-1,2,4-triazole, which was found predominantly in the urine (63.8% of the administered dose in males, 51.6% in females). Studies with this metabolite are summarized in the evaluation of difenoconazole in the present report.

No neurotoxic effects were seen during conventional repeat-dose studies with flusilazole.

There were no reports of adverse health effects in manufacturing plant personnel or in operators and workers exposed to flusilazole formulations during their use. Also, there was no evidence or data to support any findings in relation to poisoning with flusilazole.

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

Toxicological evaluation

The Meeting established an ADI of 0–0.007 mg/kg bw based on the NOAEL of 0.7 mg/kg bw per day for lymphoid hyperplasia in the gastric mucosa, liver histopathology (hypertrophy, inflammatory infiltration in males and females, and vacuolation in males only), and clinical chemistry (decreased concentrations of cholesterol, total protein and albumin and increased alkaline phosphatase activity and leukocyte counts) in the 1-year dietary study in dogs and a safety factor of 100.

The Meeting established an acute reference dose (ARfD) of 0.02 mg/kg bw based on the NOAEL of 2 mg/kg bw per day for skeletal anomalies in the study of developmental toxicity in rats treated orally and a safety factor of 100.

Levels relevant to risk assessment

Species	Study	Effect	NOAEL	LOAEL
Mouse	Two-year studies of toxicity and carcinogenicity ^a	Toxicity	25 ppm, equal to 3.4 mg/kg bw per day	200 ppm, equal to 27 mg/kg bw per day
		Carcinogenicity ^d	200 ppm equal to 36 mg/kg bw per day (females)	1000 ppm equal to 384 mg/kg bw per day
Rat	Two-year studies of toxicity and carcinogenicity ^{a,c}	Toxicity	50 ppm, equal to 2 mg/kg bw per day	250 ppm, equal to 10 mg/kg bw per day
		Carcinogenicity	375 ppm, equal to 14.8 mg/kg bw per day	750 ppm, equal to 30.8 mg/kg bw per day
	Multigeneration reproductive toxicity ^{a,c}	Parental toxicity	50 ppm, equal to 4.04 mg/kg bw per day	250 ppm, equal to 19.6 mg/kg bw per day
		Offspring toxicity	50 ppm, equal to 4.04 mg/kg bw per day	250 ppm, equal to 19.6 mg/kg bw per day
		Reproduction	50 ppm, equal to 4.04 mg/kg bw per day	250 ppm, equal to 19.6 mg/kg bw per day
	Developmental toxicity ^{a,b,c}	Maternal toxicity	2 mg/kg bw per day	10 mg/kg bw per day
		Embryo/fetotoxicity	2 mg/kg bw per day	10 mg/kg bw per day
Rabbit	Developmental toxicity ^{a,b,c}	Maternal toxicity	7 mg/kg bw per day	15 mg/kg bw per day
		Embryo/fetotoxicity	7 mg/kg bw per day	15 mg/kg bw per day
Dog	One-year study of toxicity ^a	Toxicity	20 ppm, equal to 0.7 mg/kg bw per day	75 ppm, equal to 2.4 mg/kg bw per day

^a Dietary administration.

^b Gavage administration.

^c Two or more studies combined.

^dGreater than the maximum tolerated dose (MTD).

Estimate of acceptable daily intake for humans

0–0.007 mg/kg bw

Estimate of acute reference dose

0.02 mg/kg bw

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

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

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

<i>Absorption, distribution, excretion and metabolism in mammals</i>	
Rate and extent of oral absorption	Rapid and extensive (up to 80%)
Dermal absorption	Data not available
Distribution	Widely
Potential for accumulation	Low
Rate and extent of excretion	Rapidly excreted
Metabolism in animals	Extensively metabolized
Toxicologically significant compounds in animals, plants and the environment	Parent compound, 1,2,4-triazole
<i>Acute toxicity</i>	
Rat, LD ₅₀ , oral	674 mg/kg bw
Rat, LD ₅₀ , dermal	> 2000 mg/kg bw
Rat, LC ₅₀ , inhalation	2.7–3.7 mg/l, 4 h
Guinea-pig, skin sensitization (test method used)	Non-sensitizing (Buehler)
<i>Short-term studies of toxicity</i>	
Target/critical effect	Liver and urinary bladder
Lowest relevant oral NOAEL	0.7 mg/kg bw per day (1-year study in dogs)
Lowest relevant dermal NOAEL	5 mg/kg bw per day (21-day study in rabbits)
Lowest relevant inhalation NOAEC	No data presented
<i>Genotoxicity</i>	
	Not genotoxic
<i>Long-term studies of toxicity and carcinogenicity</i>	
Target/critical effect	Liver and bladder
Lowest relevant NOAEL	2.0 mg/kg bw per day (2-year study in rats)
Carcinogenicity	No carcinogenic concern at levels of dietary exposure
<i>Reproductive toxicity</i>	
Reproduction target/critical effect	Increased gestation length, reduced live born pups/litter and decreased pup growth

Lowest relevant reproductive NOAEL	50 ppm (4.04 mg/kg bw per day)		
Developmental target/critical effect	Skeletal anomalies, malformations at higher doses		
Lowest relevant developmental NOAEL	2 mg/kg bw per day (rats)		
<i>Neurotoxicity/delayed neurotoxicity</i>			
	No indications of neurotoxicity in studies of acute toxicity or repeated doses		
<i>Other toxicological studies</i>			
Mechanistic studies	Disruption of the hypothalamus–pituitary–testis axis		
	Necrosis and hyperplasia in the rat bladder		
<i>Medical data</i>			
	No occupational or accidental poisoning reported		
<i>Summary</i>			
	Value	Study	Safety factor
ADI	0–0.007 mg/kg bw	Dog, 1-year study	100
ARfD	0.02 mg/kg bw	Rat, study of developmental toxicity	100

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