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GENTIAN VIOLET page 3-34

Toxicological evaluation of certain veterinary drug residues in food

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GENTIAN VIOLET

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1. EXPLANATION

Gentian violet (Chemical Abstracts Service No. 548-62-9) has many common names, including CI Basic Violet 3, crystal violet and methyl violet 10B. It is a triphenylmethane dye with antibacterial, antifungal and anthelminthic properties. Gentian violet has been used for the treatment of fungal and parasitic infections in fish and topically for skin and eye infections in livestock. It was previously used in poultry feeds to inhibit the growth of mould and fungus; however, several countries have withdrawn approval or registration of this use.

In humans, gentian violet has been used as a hair dye, to treat gut parasites and for topical fungal treatment. It has also been used in human medicine to treat blood held for transfusions in order to prevent the transmission of Chagas disease caused by *Trypanosoma cruzi*. It also has activity as a topical antiviral agent.

Gentian violet is used in industrial processes for wood, leather, silk, nylon, paper and ribbon tapes and also as a biological stain. Contamination of the environment can occur, as about 10–15% of all dyes are lost directly to wastewater in the dyeing process. Gentian violet in water originating from contamination as a result of its industrial applications or from its illegal use in aquaculture is efficiently taken up from the water by fish.

Gentian violet has not previously been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). It was evaluated by the Committee at the current meeting at the request of the Twentieth Session of the Codex Committee on Residues of Veterinary Drugs in Foods (FAO/WHO, 2012), which asked for advice as to whether an acceptable daily intake (ADI) can be established and whether the continued use of gentian violet in food-producing animals is safe for humans.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

Docampo & Moreno (1990) reviewed the metabolism and mode of action of gentian violet. No data were available on skin absorption of gentian violet in food-producing animals when the drug is applied topically, although Diamante et al. (2009) considered gentian violet to be poorly absorbed through human skin.

(a) Mice

Groups of 12 male and 12 female B6C3F1 mice were housed three per metabolism cage and given 14 doses of ^{14}C-labelled gentian violet (94.8% gentian violet and 5.2% pentamethylpararosaniline) at 12-hour intervals by gavage. The total gentian violet doses were 5.6 mg/kg body weight (bw) (0.72 MBq/animal) and 7.1 mg/kg bw (0.72 MBq/animal) for males and females, respectively. The mice were killed 2 hours after receiving the final dose. Urine, faeces, liver, kidney, muscle, testes or ovaries and a fat sample were collected during the study, and radioactivity was measured (Table 1). The data show that gentian violet residues

Table 1. Disposition and excretion of multiple oral doses of ^{14}C-labelled gentian violet administered to mice

Sample	Gentian violet residues[a] (µg/g or µg/mL)	
	Males	Females
Liver	17.8 ± 2.6**	10.7 ± 3.4**
Kidney	1.6 ± 0.1**	2.7 ± 0.8**
Muscle	0.6 ± 0.4**	1.3 ± 0.7**
Gonad	0.49 ± 0.08	3.66 ± 1.08[b]
Fat	14.3 ± 3.0**	24.1 ± 7.0**
Urine	1.16 (5.9%)	1.58 (8.1%)
Faeces	12.89 (65.9%)	13.17 (67.4%)

**: $P < 0.01$ (student *t*-test for a significant sex difference)

[a] Values are means ± 1 standard deviation for 12 male and 12 female mice. Numbers in parentheses indicate the percentage of the total dose.

[b] Mean of eight mice.

Source: McDonald et al. (1984a); McDonald (1989)

were highest in adipose tissue (particularly in females), although a major portion (66–67%) was excreted in faeces (McDonald et al., 1984a; McDonald, 1989).

(b) Rats

Groups of three male and three female F344 rats were housed individually in metabolism cages and given a single dose by gavage of ^{14}C-labelled gentian violet (94.8% gentian violet and 5.2% pentamethylpararosaniline). Gentian violet doses were 4.8 mg/kg bw (0.11 MBq/animal) and 5.2 mg/kg bw (0.34 MBq/animal) for males and females, respectively. Rats were killed 2, 4, 14 or 24 hours after dosing. Urine, faeces, liver, kidney, muscle, testes or ovaries and a fat sample were collected, and radioactivity was measured (Table 2). Half-lives of 14.5 and 14.4 hours were calculated following a single dose for the liver and kidney, respectively, for males and 17.0 and 18.3 hours, respectively, for females (McDonald et al., 1984a).

Groups of eight male and eight female F344 rats were housed individually in metabolism cages and given 14 doses of ^{14}C-labelled gentian violet (94.8% gentian violet and 5.2% pentamethylpararosaniline) at 12-hour intervals by gavage. The total gentian violet doses were 3.5 mg/kg bw (5.2 MBq/animal) and 5.69 mg/kg bw (2.9 MBq/animal) for males and females, respectively. The rats were killed 2 hours after receiving the final dose. Urine, faeces, liver, kidney, muscle, testes or ovaries and a fat sample were collected, and radioactivity was measured (Table 3). As for mice, gentian violet residues concentrated in the adipose tissue of females; in males, the levels in liver and fat were similar. The percentages of administered gentian violet radioactivity excreted in the faeces of rats and mice were very similar, whereas considerably more was excreted in the urine of mice than in that of rats (McDonald et al., 1984a).

Two female bile duct–cannulated rats were administered a single dose of ^{14}C-labelled gentian violet (94.8% gentian violet and 5.2% pentamethylpara-rosaniline) by gavage at 300 µg (0.12 MBq) or 840 µg (0.34 MBq), and bile was

Table 2. Deposition and excretion of a single oral dose of ^{14}C-labelled gentian violet administered to rats[a]

Time after dose (h)	Tissue residue (µg/g or µg/mL)					Excretion (µCi)[b]	
	Liver	Kidney	Muscle	Testis/ ovary	Fat	Urine	Faeces
Males							
2	2.52 ± 0.75	0.48 ± 0.11	0.05 ± 0.01	0.03 ± 0.01	0.12 ± 0.05	0.045	0.001
4	3.51 ± 0.79	0.47 ± 0.04	0.05 ± 0.01	0.02 ± 0.02	0.12 ± 0.03	0.064	0.009
14	1.71 ± 0.15	0.22 ± 0.01	0.05 ± 0.02	0.04 ± 0.01	0.50 ± 0.1	0.25	3.76
24	0.99 ± 0.14	0.13 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.66 ± 0.07	0.33	11.10
36	0.76 ± 0.12	0.10 ± 0.01	0.03 ± 0.02	0.02 ± 0.01	0.72 ± 0.14	0.29 (2.2%)	9.55 (72.9%)
Females							
2	1.37 ± 0.28	0.48 ± 0.11	0.05 ± 0.01	0.03 ± 0.01	0.13 ± 0.01	0.025	0.001
4	2.84 ± 0.41	0.52 ± 0.11	0.15 ± 0.01	0.02 ± 0.02	0.42 ± 0.09	0.017	0.011
14	1.22 ± 0.19	0.23 ± 0.05	0.13 ± 0.05	0.04 ± 0.01	2.07 ± 0.36	0.11	4.39
24	1.11 ± 0.23	0.21 ± 0.06	0.16 ± 0.10	0.02 ± 0.01	3.30 ± 0.45	0.33	5.14
36	0.69 ± 0.15	0.14 ± 0.02	0.05 ± 0.01	0.02 ± 0.01	2.92 ± 0.77	0.20 (2.2%)	5.91 (63.8%)

[a] Values are means ± 1 standard deviation for three rats. Numbers in parentheses indicate percentage of the dose.
[b] 1 µCi = 37 kBq.
Source: McDonald et al. (1984a)

Table 3. Disposition and excretion of multiple oral doses of ^{14}C-labelled gentian violet administered to F344 rats[a]

Sample	Gentian violet residues (µg/g or µg/mL)	
	Males	Females
Liver	4.0 ± 0.6	3.7 ± 0.8
Kidney	0.7 ± 0.1**	2.9 ± 1.7**
Muscle	0.09 ± 0.03*	0.6 ± 0.5*
Gonad	0.08 ± 0.04	3.67 ± 0.76
Fat	3.2 ± 0.4**	20.2 ± 5.8**
Urine	3.18 (2.2%)	1.29 (1.6%)
Faeces	92.02 (65.5%)	58.04 (72.8%)

*: $P < 0.02$; **: $P < 0.01$ (student *t*-test for a significant sex difference)
[a] Values are means ± 1 standard deviation for seven male and eight female rats. Numbers in parentheses indicate the percentage of the total dose.
Source: McDonald et al. (1984a)

collected for 24 and 28 hours, respectively. The percentages of the oral dose collected from the two rats were 6.4% and 5.7% after 24 and 28 hours, respectively (McDonald et al., 1984a).

The authors concluded that orally administered gentian violet cation (which could be combined with a hydroxyl ion in the small intestine) with a relative molecular mass of 372 was absorbed to a greater extent than had been reported for other triphenylmethane dyes. The authors speculated that leucogentian violet, which is produced under anaerobic conditions by intestinal bacteria, may be absorbed and be preferentially taken up in the fat (McDonald et al., 1984a).

2.1.2 Biotransformation

(a) Bacteria

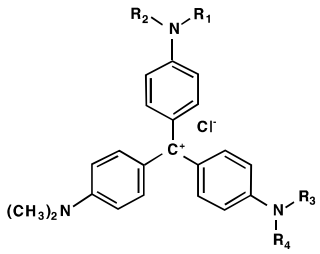
The biotransformation of gentian violet by cell suspensions of human, rat and chicken intestinal microflora and by 12 pure bacterial cultures has been studied. Incubations were carried out under anaerobic and aerobic conditions. All pure cultures and mixed intestinal microflora converted gentian violet to leucogentian violet. Gentian violet and leucogentian violet were identified in the incubation mixtures. The facultative anaerobes *Escherichia coli* and *Salmonella typhimurium* possessed little ability to reduce gentian violet under either anaerobic or aerobic conditions. Gentian violet at a concentration of 2.67 µg/mL of incubation medium was not toxic and did not inhibit bacterial growth when compared with control incubations (McDonald & Cerniglia, 1984).

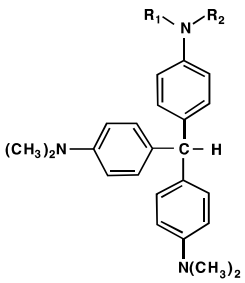
(b) In vitro

The in vitro metabolism of gentian violet (*1a* in Fig. 1, hexamethylpararosaniline chloride, purity 98%) was studied in microsomes isolated from the livers of four strains of mice, three strains of rats, hamsters, guinea-pigs and chickens. Recovery of quantified products on a molar basis was only 30–35% from incubation mixtures with active microsomes containing gentian violet at 0.01 mmol/L. The gentian violet was demethylated to pentamethylpararosaniline chloride (*1b* in Fig. 1), *N*,*N*,*N*′,*N*′-tetramethylpararosaniline chloride (*1c* in Fig. 1) and *N*,*N*,*N*′,*N*″-tetramethylpararosaniline chloride (*1d* in Fig. 1). In general, microsomes from mice produced less demethylated products compared with microsomes from the other species. Microsomes from the guinea-pig produced less of *1c* and more of *1d* compared with microsomes from the other species. Demethylation differences between males and females were not apparent among the species (McDonald et al., 1984b; McDonald, 1989). The authors made no mention of leucogentian violet (*1e* in Fig. 1).

Gentian violet was metabolized under a nitrogen atmosphere by rat liver microsomes supplemented with reduced nicotinamide adenine dinucleotide phosphate (NADPH) to give a single-line electron spin resonance spectrum, which was considered to be from the tri-(*p*-dimethylaminophenyl) methyl radical. Elimination of the NADPH-generating system, use of heat-denatured microsomes or the presence of oxygen resulted in no electron spin resonance spectrum. This one-electron reduction to produce a carbon-centred free radical was inhibited approximately 50% by metyrapone and by an atmosphere of carbon monoxide (Harrelson & Mason, 1982).

Fig. 1. Structures of substituted pararosanilines





	R_1	R_2	R_3	R_4
1a	CH_3	CH_3	CH_3	CH_3
1b	CH_3	CH_3	CH_3	H
1c	CH_3	CH_3	H	H
1d	CH_3	H	CH_3	H

	R_1	R_2
1e	CH_3	CH_3
1f	CH_3	H

1a Gentian violet
1b Pentamethylpararosaniline chloride
1c *N,N,N′,N′*-tetramethylpararosaniline chloride
1d *N,N,N′,N″*-tetramethylpararosaniline chloride
1e Leucogentian violet
1f Leucopentamethylpararosaniline

(c) Mice

McDonald (1989) analysed the metabolites in tissues and faeces. Three demethylated metabolites (*1b*, *1c* and *1d* in Fig. 1) and two reduced metabolites, leucogentian violet (*1e*) and leucopentamethylpararosaniline (*1f*), were found in tissues and faecal extracts. Reduced metabolites (*1e* and *1f*) were predominant in tissues, and the parent compound was predominant in faeces.

(d) Rats

A female Fischer 344 rat was given 0.84 mg ^{14}C-labelled gentian violet (0.21 MBq) (94.8% gentian violet and 5.2% pentamethylpararosaniline) twice daily by gavage for 3 days, and faeces were collected for identification of metabolites. Leucogentian violet accounted for 11% of the radioactivity present in the 48- to 72-hour faeces collection (McDonald & Cerniglia, 1984).

McDonald (1989) also analysed the metabolites of gentian violet. In addition to the parent compound (*1a* in Fig. 1), demethylated metabolites (*1b*, *1c* and *1d*) and reduced metabolites (*1e* and *1f*) were identified. The highest metabolite concentrations observed were for reduced metabolites (*1e* and *1f*) in fat tissue. These metabolites were also found in other tissues. All five metabolites were also detected in faecal extracts, but the parent compound was more dominant in faeces than in other tissues.

2.2 Toxicological studies

2.2.1 Acute toxicity

The acute oral toxicity of gentian violet has been reported by Hodge et al. (1972) and is summarized in Table 4.

Table 4. Results of studies of the acute oral toxicity of gentian violet

Species	Vehicle	LD_{50} (mg/kg bw)	Reference
Mouse	Water	405–570 (7-day follow-up)	Hodge et al. (1972)
Mouse	Propylene glycol	800 (7-day follow-up)	Hodge et al. (1972)
Rat	Propylene glycol	180 (7-day follow-up) 1 000 (24 h follow-up)	Hodge et al. (1972)
Guinea-pig	Propylene glycol	100–150	Hodge et al. (1972)
Rabbit	Propylene glycol	125–250	Hodge et al. (1972)
Cat	Propylene glycol	100–150	Hodge et al. (1972)
Dog	Propylene glycol	1 000	Hodge et al. (1972)

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

Hodge et al. (1972) also referred to unpublished results of Seppelin (1949), who identified an oral median lethal dose (LD_{50}) of 330 mg/kg bw in mice.

Hodge et al. (1972) reported that the most common sign of toxicity was lethargy, occurring approximately 1 hour after dosing, with anorexia in rats, rabbits, cats and dogs as the second most common sign. Ataxia occurred in mice, rats and guinea-pigs. Other signs seen, but not in all animals, were diarrhoea, excessive thirst, emesis and weight loss. Histological evidence of irritation, congestion and haemorrhage was also observed.

These animal data indicate that gentian violet is of moderate acute oral toxicity.

2.2.2 Short-term studies of toxicity

There are few published data describing short-term toxicity in laboratory animals.

(a) Rats

Littlefield et al. (1989) cited unpublished data from the United States Food and Drug Administration (USFDA, 1976) reporting on a 90-day study in rats that were fed gentian violet at levels up to 500 mg/kg in feed. Other than reporting a slight body weight loss, no other significant results were identified. No further details of the study were available.

(b) Dogs

Littlefield et al. (1989) cited the USFDA's (1976) unpublished data to report that a 90-day study in dogs was conducted by feeding gentian violet at levels up to 516 mg/kg in feed. Other than a liver weight increase, no other significant effects were identified. No other details of the study were available.

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Mice

In a study compliant with good laboratory practice (GLP), 720 male and 720 female B6C3F1 (C57BL/6 × C3H) mice (approximately 4–5 weeks old) were fed gentian violet (99% gentian violet and 1% methyl violet) at a dietary concentration of 0, 100, 300 or 600 mg/kg feed (equal to 0, 10.7–14.3, 32.1–35.7 and 64.3 mg/kg bw per day for males and 0, 14.3, 35.7–39.3 and 71.4 mg/kg bw per day for females, respectively). The diets were certified to be within ±10% of target values. The allocation of the mice to the groups and sacrifice times are presented in Table 5. The mice were housed four per cage. Body weights, feed consumption and clinical signs were recorded weekly. The mice received a complete necropsy, histopathological examination and clinical chemistry analysis at the scheduled sacrifices after 12, 18 and 24 months of continuous dosing.

There was no effect on feed consumption or body weight gain; however, a dose-related effect was noted for mortality rates. Mortality (adjusted for sacrifices) in the controls of both sexes was less than 15% at 24 months, but was approximately 64% in the females and 23% in the males given the high dose. The histopathological findings are presented in Tables 6 and 7. Few dose-related non-neoplastic lesions were reported, but there were statistically significant dose-related increases in erythropoiesis in the spleen and atrophy of the ovaries in females at 24 months. A positive dose–response relationship for hepatocellular carcinoma was noted in males at 24 months and in females at 18 and 24 months. Statistical tests for dose-related trends with respect to 1) mortality due to liver neoplasms, 2) prevalence of liver neoplasms and 3) time to onset of liver neoplasms showed positive trends in both males and females (Table 8). Other dose-related toxicological responses, particularly in the female mice, included erythropoiesis in the spleen, atrophy of the ovaries, adenoma of the Harderian gland and the presence of type A reticulum cell sarcomas in the urinary bladder, uterus, ovaries and vagina.

Table 5. Experimental design for mice lifespan study

Dietary concentration (mg/kg feed)	Total number of mice		Interim sacrifice (12 months)		Interim sacrifice (18 months)		Terminal sacrifice (24 months)	
	Males	Females	Males	Females	Males	Females	Males	Females
0	288	288	48	48	48	48	192	192
100	144	144	24	24	24	24	96	96
300	144	144	24	24	24	24	96	96
600	144	144	24	24	24	24	96	96
Total	720	720	120	120	120	120	480	480

Source: Littlefield (1984); Littlefield et al. (1985)

Table 6. Microscopic histopathology summary of female mice

Site and lesion	12-month sacrifice[a]				18-month sacrifice[a]				24-month sacrifice[a]			
	0 mg/kg feed	100 mg/kg feed	300 mg/kg feed	600 mg/kg feed	0 mg/kg feed	100 mg/kg feed	300 mg/kg feed	600 mg/kg feed	0 mg/kg feed	100 mg/kg feed	300 mg/kg feed	600 mg/kg feed
Liver: benign neoplasm	0/48 (0%)	0/24 (0%)	0/24 (0%)	0/24 (0%)	3/47 (6%)	0/22 (0%)	3/24 (13%)	8/64 (33%)	8/185 (4%)	8/93 (9%)	36/93 (39%)	20/95 (21%)
Liver: malignant neoplasm	0/48 (0%)	0/24 (0%)	0/24 (0%)	0/24 (0%)	1/47 (2%)	0/22 (0%)	1/24 (4%)	3/24 (13%)	7/185 (4%)	5/93 (5%)	30/93 (32%)	73/95 (77%)
Uterus: RCS type A	0/47 (0%)	0/23 0%)	0/24 (0%)	0/24 (0%)	0/47 (0%)	0/22 (0%)	1/24 (4%)	1/24 (4%)	0/188 (0%)	2/95 (2%)	6/90 (0%)	12/93 (13%)
Uterus: RCS total	0/47 (0%)	0/23 (0%)	0/24 (0%)	0/24 (0%)	0/47 (0%)	1/22 (4%)	1/24 (4%)	1/24 (4%)	3/188 (2%)	2/95 (2%)	8/90 (9%)	14/93 (15%)
Bladder: RCS type A	0/48 (0%)	0/23 (0%)	0/24 (0%)	0/24 (0%)	0/47 (0%)	0/22 (0%)	1/24 (4%)	0/23 (0%)	0/188 (0%)	2/92 (2%)	3/89 (3%)	5/91 (6%)
Bladder: RCS total	0/48 (0%)	0/23 (0%)	0/24 (0%)	0/24 (0%)	0/47 (0%)	1/22 (5%)	1/24 (4%)	0/23 (0%)	13/18 (7%)	5/92 (7%)	6/89 (7%)	6/91 (7%)
Spleen: erythropoiesis	0/47 (0%)	0/24 (0%)	0/24 (0%)	0/24 (0%)	2/47 (4%)	1/21 (5%)	1/24 (4%)	0/23 (0%)	13/190 (7%)	15/96 (16%)	18/92 (20%)	42/95 (44%)
Ovaries: atrophy	0/47 (0%)	0/23 (0%)	0/22 (0%)	0/24 (0%)	0/45 (0%)	0/21 (0%)	0/22 (0%)	1/21 (5%)	11/178 (6%)	13/90 (15%)	25/89 (28%)	37/89 (42%)
Harderian gland: adenoma	2/48 (0%)	0/24 (0%)	1/24 (4%)	0/24 (0%)	2/46 (4%)	2/21 (10%)	3/23 (13%)	1/23 (4%)	8/186 (4%)	11/93 (12%)	18/89 (20%)	15/94 (16%)
Ovaries: RCS type A	0/47 (0%)	0/23 (0%)	0/22 (0%)	0/24 (0%)	0/45 (0%)	0/21 (0%)	0/22 (0%)	0/21 (0%)	0/178 (0%)	1/90 (1%)	3/89 (3%)	5/89 (6%)
Vagina: RCS type A	0/45 (0%)	1/23 (4%)	0/24 (0%)	0/23 (0%)	0/46 (0%)	0/22 (0%)	1/23 (4%)	0/22 (0%)	1/182 (0.5%)	1/90 (1%)	4/88 (5%)	8/87 (9%)
Vagina: RCS total	0/45 (0%)	1/23 (4%)	0/24 (0%)	0/23 (0%)	0/46 (0%)	1/22 (4%)	1/23 (4%)	0/22 (0%)	3/182 (2%)	1/90 (1%)	5/88 (6%)	10/57 (11%)

RCS: reticulum cell sarcoma

[a] Includes dead or moribund animals that were removed from the study prior to the scheduled sacrifice dates.

Source: Littlefield (1984); Littlefield et al. (1985)

Table 7. Microscopic histopathology summary of male mice

Site and lesion	12-month sacrifice[a]				18-month sacrifice[a]				24-month sacrifice[a]			
	0 mg/kg feed	100 mg/kg feed	300 mg/kg feed	600 mg/kg feed	0 mg/kg feed	100 mg/kg feed	300 mg/kg feed	600 mg/kg feed	0 mg/kg feed	100 mg/kg feed	300 mg/kg feed	600 mg/kg feed
Liver: benign neoplasm	0/48 (0%)	2/24 (8%)	0/24 (0%)	0/24 (0%)	3/48 (6%)	0/24 (0%)	2/24 (8%)	2/22 (9%)	17/183 (10%)	14/92 (19%)	20/93 (22%)	37/93 (38%)
Liver: malignant neoplasm	0/47 (0%)	0/24 (0%)	0/24 (0%)	0/24 (0%)	5/48 (10%)	1/24 (4%)	2/24 (8%)	2/22 (9%)	27/183 (15%)	15/92 (17%)	17/93 (18%)	33/93 (35%)
Harderian gland: adenoma	1/48 (2%)	0/24 (0%)	0/24 (0%)	0/24 (0%)	2/47 (4%)	2/24 (8%)	2/23 (9%)	0/21 (0%)	7/187 (4%)	7/92 (7%)	10/94 (11%)	9/89 (10%)

[a] Includes dead or moribund animals that were removed from the study prior to the scheduled sacrifice dates.

Source: Littlefield (1984); Littlefield et al. (1985)

Table 8. Significance table for chronic toxicity and carcinogenicity study in mice[a]

	Females			Males		
	Mortality	Prevalence	Onset	Mortality	Prevalence	Onset
Mortality overall	0.000 05	–	–	0.012 88	–	–
Control vs 100 mg/kg feed	0.000 88	–	–	0.487 59	–	–
Control vs 300 mg/kg feed	0.000 79	–	–	0.100 99	–	–
Control vs 600 mg/kg feed	0.000 05	–	–	0.030 62	–	–
Liver: malignant neoplasms overall	0.000 05	0.000 05	0.000 05	0.013 54	0.000 45	0.000 05
Control vs 100 mg/kg feed	0.101 95	0.357 80	0.429 67	0.252 58	0.258 08	0.505 02
Control vs 300 mg/kg feed	0.008 18	0.000 05	0.000 05	0.245 63	0.433 69	0.306 65
Control vs 600 mg/kg feed	0.000 05	0.000 05	0.000 05	0.019 93	0.001 57	0.000 09
Liver: malignant neoplasms and benign tumours	0.000 05	0.000 05	0.000 05	0.013 54	0.000 05	0.000 05
Control vs 100 mg/kg feed	0.008 18	0.475 10	0.272 58	0.252 58	0.374 48	0.237 48

Table 8 (continued)

	Females			Males		
	Mortality	Prevalence	Onset	Mortality	Prevalence	Onset
Control vs 300 mg/kg feed	0.000 05	0.000 05	0.000 05	0.245 63	0.016 67	0.009 56
Control vs 600 mg/kg feed	0.000 05	0.000 05	0.000 05	0.019 93	0.000 05	0.000 05
Spleen: erythropoiesis	–	0.000 05	–	–	0.043 42	–
Control vs 100 mg/kg feed	–	0.017 38	–	–	0.007 43	–
Control vs 300 mg/kg feed	–	0.001 47	–	–	0.056 95	–
Control vs 600 mg/kg feed	–	0.000 05	–	–	0.022 48	–
Ovaries: atrophy	–	0.000 05	–	–	–	–
Control vs 100 mg/kg feed	–	0.014 90	–	–	–	–
Control vs 300 mg/kg feed	–	0.000 05	–	–	–	–
Control vs 600 mg/kg feed	–	0.000 05	–	–	–	–
Harderian gland: adenoma	–	0.001 10	–	–	0.061 59	–
Control vs 100 mg/kg feed	–	0.037 54	–	–	0.162 34	–
Control vs 300 mg/kg feed	–	0.000 05	–	–	0.030 70	–
Control vs 600 mg/kg feed	–	0.003 23	–	–	0.106 44	–

[a] Levels of significance (*P* values) for positive trends among dose groups for 1) mortality due to a specific disease, 2) prevalence (non-fatal) and (3) time to onset. Trend tests were performed across all dose groups and controls.

The lowest-observed-adverse-effect level (LOAEL) for non-carcinogenic effects was 14.3 mg/kg bw per day, the lowest dose tested. The authors concluded that under the conditions of the experiment, gentian violet appeared to be a carcinogen in mice at several different organ sites (Littlefield, 1984; Littlefield et al., 1985).

(b) Rats

In a GLP-compliant study, male and female weanling animals (F_0) were randomly divided into four groups and administered gentian violet (99% gentian violet and 1% methyl violet) in their feed at 0, 100, 300 or 600 mg/kg for at

least 80 days. While receiving dosed feed, the females were mated with males of the same dose level. Two males and two females were randomly selected from each litter (F_{1a} generation), and three animals per cage were allocated as weanlings to the chronic study. The F_{1a} animals continued on the same dose levels as their respective parents for the carcinogenicity studies (Littlefield et al., 1989).

In total, 570 male and 570 female F_{1a} Fischer 344 rats were fed gentian violet (99% gentian violet and 1% methyl violet) at 0, 100, 300 or 600 mg/kg diet (equal to approximately 0, 30, 80 and 160 mg/kg bw per day for males and 0, 40, 100 and 200 mg/kg bw per day for females, respectively) for 12, 18 and 24 months. The diets were certified to be within ±10% of target values. The allocation of the rats to the groups and sacrifice times are presented in Table 9. Feed consumption, body weights and clinical signs were recorded weekly. The rats received a complete necropsy, histopathological examination and clinical chemistry analysis at the scheduled sacrifices after 12, 18 and 24 months of continuous dosing.

The animals sacrificed after 12 months of dosing with gentian violet showed no dose-related pathology and are excluded from the results. Measurements of body weights, feed consumption and mortality and results of histopathological examinations were analysed statistically. Male and female rats fed 600 mg/kg feed for 24 months showed a decrease in body weight. Average feed consumption (based on grams of food per kilogram average body weight) was the same in all groups. Mortality rates at 24 months for the females were 33%, 38%, 60% and 66% for the control, low-dose, mid-dose and high-dose groups, respectively. For males, the same respective dose groups had mortality rates after 104 weeks of 33%, 33%, 48% and 39%.

The incidences of neoplastic lesions observed at the 18- and 24-month necropsies are presented in Tables 10 and 11. The majority of lesions were observed only at the 24-month necropsy, and incidences were mostly low.

Table 9. Experimental design of a chronic toxicity study carried out in Fischer 344 rats

Dietary concentration (mg/kg feed)	Total number of rats		Interim sacrifice (12 months)		Interim sacrifice (18 months)		Terminal sacrifice (24 months)	
	Males	Females	Males	Females	Males	Females	Males	Females
0	210	210	15	15	15	15	180	180
100	120	120	15	15	15	15	90	90
300	120	120	15	15	15	15	90	90
600	120	120	15	15	15	15	90	90
Total	570	570	60	60	60	60	450	450

Source: Littlefield et al. (1989)

Table 10. Incidence of neoplastic lesions in male Fischer 344 rats fed gentian violet in the diet for 18 or 24 months

Site and type of neoplastic lesion	Incidence of lesion							
	18 months				24 months			
	0 mg/kg feed	100 mg/kg feed	300 mg/kg feed	600 mg/kg feed	0 mg/kg feed	100 mg/kg feed	300 mg/kg feed	600 mg/kg feed
Liver: hepatocellular adenoma	0/15[a] (0%)[b]	1/15 (7%)	0/15 (0%)	0/14 (0%)	1/179 (0.5%)	1/90 (1%)	3/88 (3%)	4/89 (4%)
Testes: malignant mesothelioma	0/15 (0%)	0/15 (0%)	1/15 (7%)	1/15 (7%)	0/177 (0%)	0/90 (0%)	0/87 (0%)	1/90 (1%)
Thyroid: follicular cell adenocarcinoma	0/15 (0%)	0/15 (0%)	0/14 (0%)	0/13 (0%)	1/163 (1%)	4/84 (5%)	2/74 (3%)	5/79 (6%)
Thyroid: follicular cell adenoma	0/15 (0%)	0/15 (0%)	1/15 (7%)	1/15 (7%)	1/163 (1%)	0/84 (0%)	0/74 (0%)	2/79 (3%)
Thyroid: folliwcular cell adenoma and adenocarcinoma	0/15 (0%)	0/15 (0%)	1/15 (7%)	1/15 (7%)	2/163 (1%)	4/84 (5%)	2/74 (3%)	3/78 (9%)
Multiple organs: mononuclear cell leukaemia	6/15 (40%)	1/15 (7%)	3/15 (20%)	4/15 (27%)	104/180 (58%)	66/90 (77%)	69/90 (77%)	51/90 (57%)

[a] Incidence is expressed as the number of rats with the specified neoplastic lesion divided by the number of rats at risk.

[b] Values in parentheses represent the incidence of the neoplastic lesions as a percentage of the number of rats.

Source: Littlefield et al. (1989)

The incidences of follicular cell adenocarcinomas of the thyroid gland at 24 months for males in the 600 mg/kg feed group (6% versus 1% in controls) and females in the 300 and 600 mg/kg feed groups (5% and 8%, respectively, versus 1% in controls) were significantly increased compared with control group rats. The incidences of hepatocellular adenomas at 24 months were significantly increased in females in the 300 mg/kg feed group (2% versus 0% in controls), but not in the 600 mg/kg feed group, and in males in the 300 and 600 mg/kg feed groups (3% and 4%, respectively, versus 0.5% in controls), when compared with controls (Table 12). The incidence of mononuclear cell leukaemia appeared to be a time-related response; leukaemia showed a dose–response relationship in females at 18 months, but not at 24 months.

Table 11. Incidence of neoplastic lesions in female Fischer 344 rats fed gentian violet in the diet for 18 or 24 months

Site and type of neoplastic lesion	Incidence of lesion							
	18 months				24 months			
	0 mg/kg feed	100 mg/kg feed	300 mg/kg feed	600 mg/kg feed	0 mg/kg feed	100 mg/kg feed	300 mg/kg feed	600 mg/kg feed
Liver: hepatocellular adenoma	0/15[a] (0%)[b]	0/11 (0%)	0/10 (0%)	0/14 (0%)	0/170 (0%)	1/90 (1%)	2/84 (2%)	1/87 (1%)
Heart: mononuclear cell leukaemia	0/15 (0%)	0/11 (0%)	0/10 (0%)	2/14 (14%)	27/169 (16%)	16/90 (18%)	19/83 (23%)	22/87 (25%)
Thyroid: follicular cell adenocarcinoma	0/15 (0%)	1/11 (9%)	0/10 (0%)	0/14 (0%)	1/159 (1%)	1/83 (1%)	4/76 (5%)	6/77 (8%)
Thyroid: follicular cell adenoma	0/15 (0%)	0/11 (0%)	0/10 (0%)	0/14 (0%)	1/159 (1%)	2/83 (2%)	3/76 (4%)	3/77 (4%)
Thyroid: follicular cell adenoma and adenocarcinoma	0/15 (0%)	1/11 (9%)	0/10 (0%)	0/14 (0%)	2/159 (2%)	3/83 (4%)	7/76 (9%)	9/77 (12%)
Multiple organs: mononuclear cell leukaemia	0/15 (0%)	2/11 (18%)	2/10 (20%)	6/14 (43%)	77/171 (45%)	38/90 (42%)	45/87 (52%)	40/87 (46%)

[a] Incidence is expressed as the number of rats with the specified neoplastic lesion divided by the number of rats at risk.

[b] Values in parentheses represent the incidence of the neoplastic lesions as a percentage of the number of rats.

Source: Littlefield et al. (1989)

The incidences of non-neoplastic lesions are presented in Table 13. There was a statistically significant increase in liver regeneration in all dose groups and statistically significant dose-related increases in eosinophilic foci in the liver in both sexes in the mid- and high-dose groups. For liver centrilobular necrosis, there was a dose-related increase, but statistical significance was seen only in the 300 mg/kg feed group in males and in the 600 mg/kg feed group in females. Female rats appeared to be more sensitive than males.

The LOAEL for non-neoplastic effects was 30 mg/kg bw per day, the lowest dose tested, based on the increase in liver regeneration observed in all dose groups. Gentian violet was carcinogenic in rats, with thyroid follicular cell adenocarcinomas and hepatocellular adenomas observed in both sexes (Littlefield et al., 1989).

In addition, Docampo & Moreno (1990) noted a report (National Toxicology Program, 1986) that the completely demethylated derivative of gentian violet, leucopararosaniline, is carcinogenic in rats, but no information was available on its potency. The tumours seen included the same thyroid tumours seen in the carcinogenicity study of gentian violet in rats (Littlefield et al., 1989).

Table 12. Mortality and incidence of specific neoplastic lesions expressed as levels of significance (P values) in Fischer 344 rats fed gentian violet in the diet for 24 months

	Significance levels (*P* values)[a]			
	Overall	Control vs 100 mg/kg feed level	Control vs 300 mg/kg feed level	Control vs 600 mg/kg feed level
Males				
Mortality	0.067	0.45	0.005 7	0.16
Liver: hepatocellular adenoma	0.009	0.069	0.004	0.008
Thyroid: follicular cell adenocarcinoma	0.004	0.017	0.066	0.002
Females				
Mortality	0.000 05	0.34	0.000 07	0.000 05
Liver: hepatocellular adenoma	0.092	0.083[b]	0.003	0.048
Thyroid: follicular cell adenocarcinoma	0.000 05	0.092	0.002	0.000 05
Multiple organs: mononuclear cell leukaemia	0.062	0.361	0.141	0.053

[a] Significant trend at 0.05 level for overall, 0.05/3 for control versus dose comparison (Bonferroni corrected). Significant trend at 0.01 level for overall, 0.01/3 for control versus dose comparison (Bonferroni corrected). Significant trend at 0.001 level for overall, 0.001/3 for control versus dose comparison (Bonferroni corrected).

[b] This significant value arises from the small number of tumours; the result was determined using Fisher's exact test.

Source: Littlefield et al. (1989)

2.2.4 Genotoxicity

Muller & Gauthier (1975) reported the binding of gentian violet with high preference to two adjacent A–T pairs and also a second deoxyribonucleic acid (DNA) interaction, which is much weaker and nonspecific. Using more sensitive methodology, Wakelin et al. (1981) showed that gentian violet binds externally to DNA, causing severe kinking and/or bending accompanied by a coupled unwinding of the Watson–Crick helix. The authors further concluded that the binding complexes with ribonucleic acid (RNA) were different and evidently a cooperative process.

The results of the assays to assess the genotoxic and mutagenic potential of gentian violet are presented in Table 14. The strong binding affinity and cellular toxicity of gentian violet have complicated the testing and interpretation of the assay results. Au et al. (1979) suggested that low levels of gentian violet were being inactivated in the test system by the 9000 × *g* supernatant fraction of rat liver homogenate (S9), whereas high levels were toxic to the organism.

Table 13. Incidence of non-neoplastic lesions in Fischer 344 rats fed gentian violet in the diet for 24 months

Site and type of lesion	Incidence of lesion[a]			
	0 mg/kg feed	100 mg/kg feed	300 mg/kg feed	600 mg/kg feed
Males				
Liver				
Clear cell foci	6/179 (3%)	5/90 (6%)	5/88 (6%)	8/89 (9%)
Eosinophilic foci	7/179 (4%)	5/90 (6%)	20/88 (23%)	33/89 (37%)
Mixed foci	32/179 (18%)	26/90 (29%)	28/88 (26%)	47/89 (53%)
Regeneration	7/179 (4%)	11/90 (12%)	21/88 (24%)	15/89 (17%)
Centrilobular necrosis	5/179 (3%)	4/90 (4%)	8/88 (9%)	11/89 (12%)
Thyroid gland				
Follicular cysts	18/163 (11%)	7/84 (8%)	9/74 (12%)	17/97 (22%)
Spleen				
Red pulp hyperplasia	11/175 (6%)	7/88 (8%)	3/87 (3%)	15/86 (17%)
Lymph node	8/168 (5%)	9/86 (10%)	5/84 (6%)	11/81 (14%)
Females				
Liver				
Clear cell foci	1/170 (1%)	1/90 (1%)	3/84 (4%)	1/87 (1%)
Eosinophilic foci	0/170 (0%)	0/90 (0%)	6/84 (7%)	10/87 (11%)
Mixed cell foci	29/170 (17%)	32/90 (36%)	39/84 (46%)	30/87 (34%)
Regeneration	4/170 (2%)	9/90 (10%)	20/84 (24%)	18/87 (21%)
Centrilobular necrosis	7/170 (4%)	8/90 (9%)	6/84 (7%)	20/87 (23%)
Thyroid gland				
Follicular cysts	8/159 (5%)	9/83 (11%)	8/76 (11%)	7/77 (9%)

[a] Incidence is expressed as the number of rats with the identified non-neoplastic lesion divided by the number of rats at risk. Values in parentheses represent the incidence of the non-neoplastic lesions expressed as a percentage of the number of rats surviving.

Source: Littlefield et al. (1989)

Levin, Lovely & Klekowski (1982) studied the effect of light (plates containing gentian violet were irradiated at 23°C with a Sylvania tungsten/halogen lamp for 3 minutes at 20 cm) on the genotoxicity of gentian violet. In the Rosenkranz assay, a genotoxic effect was observed under conditions of dark and was enhanced by the irradiation. Harrelson & Mason (1982) reported that in the presence of NADPH and light, gentian violet was photoreduced to the same triarylmethyl free radical that is formed by enzymatic reduction. The presence of S9 had no effect on the genotoxicity of gentian violet. However, in the Ames test, where no mutagenic activity was observed but gentian violet was sufficiently toxic to sterilize the plate under conditions of dark incubation, the presence of S9 (active or thermally deactivated) virtually eliminated the toxicity of gentian violet under dark incubation and greatly decreased its toxicity under light conditions.

Table 14. Results of tests for genotoxicity and mutagenicity with gentian violet

Test system	Test object	Concentration	Results	Reference
In vitro				
Rosenkranz repairable DNA assay	*Escherichia coli* DNA polymerase-deficient strain	Not stated	Positive	Rosenkranz & Carr (1971)
Cytogenetic toxicity	CHO cells, human lymphocytes and HeLa and L cells, as well as *Peromyscus eremicus* and Indian Muntjac cell lines	0, 0.5 or 5 µg/mL	Positive (mitotic poison and clastogen)	Au et al. (1978)
Ames test[a]	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0, 1, 2 or 4 µg/plate	Negative, TA1535 equivocal	Shahin & von Borstel (1978)
	Saccharomyces cerevisiae XV185-14C	0, 2, 4, 6 or 8 µg/plate	Negative	
Chromosome breakage	CHO cells	0 or 10 µmol/L	Positive	Au & Hsu (1979)
Ames test[a]	*S. typhimurium* TA98, TA100, TA1535, TA1537	0, 0.1–50 µg	Negative, bactericidal ≥ 10 µg (no activation)	Au et al. (1979)
Rosenkranz repairable DNA assay[b]	*E. coli* W3110 pol A^+, mutant p3478 pol $A^=$	1, 10, 25 or 100 µg/plate	Positive	
Chromosome breakage[c]	CHO cells	5, 10 or 20 µg/mL	Positive, no activation	
Ames test[b]	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0, 0.1, 0.32, 1 or 3.2 µg/plate	Negative except for TA1535 only at 0.32 µg, and no activation	Bonin, Farquharson & Baker (1981)

Table 14 (continued)

Test system	Test object	Concentration	Results	Reference
Chromatid breakage	Human peripheral blood cells	20 µg/mL	Positive	Hsu, Cherry & Pathak (1982)
Ames test[b]	*S. typhimurium* TA98, TA100, TA1537	0, 1, 5, 10 or 50 µg/plate	Negative[c]	Levin, Lovely & Klekowski (1982)
Rosenkranz repairable DNA assay[b]	*E. coli* W3110 pol A^+, mutant p3478 pol $A^=$	0.1, 0.5, 1, 5, 7 or 10 µg/plate	Positive	
Ames test[b]	*S. typhimurium* TA1535	0, 0.025, 0.05, 0.1 or 0.5 µg/plate	Negative	Thomas & MacPhee (1984)
	E. coli DG1669	0, 25, 50, 75 or 100 µg/plate	Positive[d,e]	
Chromosome damage	Human lymphocytes[f,g]	1 µg/mL	Positive	Krishnaja & Sharma (1995)
Ames test	*S. typhimurium* TA97, TA98, TA100 *E. coli* WP2s	1–50 µg of metabolites/plate for *Salmonella* 5 µmol/L concentration for *E. coli*	Negative (metabolites) in *Salmonella* test Positive in *E. coli* test, maybe metabolites positive as well	Hass, Heflich & McDonald (1986)
Ames test	*S. typhimurium* TA97, TA98, TA100, TA104	0, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 µg/plate with or without S9	Positive in TA97 with and without S9, positive in TA104 with S9, negative in others	Aidoo et al. (1990)
Mammalian cell mutagenicity	CHO-K1-BH_4 and CHO-AS52 cells	0–1.5 µg/mL with or without S9	Negative for CHO-K1-BH_4 cells, equivocal results for CHO-AS52 cells	

Table 14 (continued)

Test system	Test object	Concentration	Results	Reference
Lymphocyte DNA damage assay	B6C3F1 mouse	0, 0.2, 0.4, 0.6, 0.8 or 1.0 µg/mL	Positive for DNA damage	
Gene amplification test	SV40-transformed Chinese hamster cell line C060	0.02, 0.05 or 0.125 µg/mL	Dose-related weak SV40 DNA amplification	
In vivo				
Chicken embryo assay	Chicken embryos	0.5, 2, 5, 10, 20, 100, 1 000 or 2 000 µg/embryo	Toxicity ≥ 20 µg; no increase in sister chromatid exchange	Au et al. (1979)
Bone marrow assay	Mouse	Drinking-water 20 or 40 µg/mL for 4 weeks, calculated to be 4 and 8 mg/kg bw per day, respectively	No chromosome damage, decreased mitotic index	
Lymphocyte DNA damage assay	B6C3F1 mouse	Animals treated with 0, 2, 4 or 6 mg/kg bw as a single dose	Negative for DNA damage	Aidoo et al. (1990)

bw: body weight; CHO: Chinese hamster ovary; DNA: deoxyribonucleic acid; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] Without metabolic activation.

[b] With and without metabolic activation.

[c] At concentrations above 5.0 µg/plate, gentian violet was sufficiently toxic to sterilize the plates without S9 under conditions of dark incubation. The presence of S9 virtually eliminated the toxicity of gentian violet in the dark and greatly decreased the toxicity in the light.

[d] At gentian violet concentrations of 75 and 100 µg/plate with no S9, a large number of the cells were killed.

[e] With S9, all concentrations of gentian violet resulted in similar numbers of mutants.

[f] Cultured blood lymphocytes from β-thalassaemia traits and healthy individuals.

[g] The incidence of chromatid aberrations was not statistically different between normal and β-thalassaemia trait blood samples.

Thomas & MacPhee (1984) pointed out that all of the strains used by Au et al. (1979) and Bonin, Farquharson & Baker (1981) carried *rfa* mutations and were exceptionally sensitive to the toxic effects of gentian violet and thus not the most suitable strains to assess the mutagenicity of gentian violet, other than at very low dose levels. These authors reported negative results with *Salmonella typhimurium* strain TA1535 in the Ames assay using low doses (0.025–0.5 μg/plate) of gentian violet because of the toxic effects of gentian violet and thus disagreed with the positive results by Bonin, Farquharson & Baker (1981). However, the authors concluded that the positive results with DG1669 (an *Escherichia coli* K12 derivative that carries the *lac*Z(ICR24) frameshift marker and is DNA repair proficient) indicated that gentian violet is a direct-acting mutagen causing frameshift mutations in repair-proficient bacteria. Dose levels of 75 and 100 μg/plate were toxic when S9 was omitted, but not when S9 was present in the incubation mixture.

Aidoo et al. (1990) re-evaluated the genotoxicity of gentian violet (> 96% gentian violet, with the remainder being mainly methyl violet) by conducting mutagenesis and DNA damage experiments in both bacterial and mammalian cell systems. Mutagenicity of gentian violet in *Salmonella* was strain specific; it was mutagenic in TA97 and TA104 strains, but not in TA98 and TA100 strains. S9 tended to increase its mutagenicity. *N,N,N′,N″*-Tetramethylpararosaniline, a metabolite of gentian violet, was a weak mutagen in *Salmonella*. Gentian violet was not mutagenic in Chinese hamster ovary (CHO) cell line CHO-K1-BH_4, but equivocal results were obtained with CHO-AS52 cells. Gentian violet produced DNA damage in B6C3F1 mouse lymphocytes in vitro, but not in vivo. However, the dose levels used in these in vivo tests were much lower than those used in the carcinogenicity studies. The authors concluded that gentian violet is a point mutagen in bacteria and may be carcinogenic in mammalian cells by its clastogenic activity.

Gentian violet was found to break chromosomes in cultures of CHO cells (Au et al., 1978; Au & Hsu, 1979), human lymphocytes, HeLa and L cells and fibroblastic cell lines (Au et al., 1978).

2.2.5 Reproductive and developmental toxicity

(a) Multigeneration reproductive toxicity

(i) Rats

In a three-generation reproductive toxicity study, gentian violet (99% gentian violet, 1% methyl violet) was administered in feed to Fischer 344 rats at a dose of 0, 100, 300 or 600 mg/kg (equivalent to 0, 5, 15 and 30 mg/kg bw per day, respectively). F_0 animals of both sexes were randomly allocated to treatment groups and fed the medicated ration for at least 80 days. Males and females of the same dose group were then caged together for 14 days for mating, after which males were returned to their own cages. Pups from this mating (F_{1a} generation) were used for a separate study. Following this, 90 rats of each sex for the control group and 45 rats of each sex for each treatment group were selected to continue in this study. F_0 animals

were mated a second time with animals of the same treatment group, as described previously. Following the births (F_{1b} generation), one male and one female from each litter were randomly selected for further study. At 100–140 days of age, F_{1b} generation females were randomly selected for mating with randomly selected males within the same dose group to produce the F_{2a} generation. A similar procedure was used to produce the F_{2b} generation. After 100–140 days, one male and one female per litter of the F_{2b} generation were randomly selected for mating to produce the F_{3a} generation. At weaning, two males and two females per litter of the F_{3a} generation were randomly selected for histopathology of a number of organs and tissues. The test substance was administered continuously in the diet of each treatment group (i.e. during mating, gestation, lactation and the interim rest periods). Brother–sister matings were avoided. Pups in each generation were examined for gross deformities.

A dose-related effect was noted on body weight in the 600 mg/kg feed group. Animals in this group had significantly lower body weights when compared with controls or the 100 and 300 mg/kg feed groups. Gentian violet had no effect on the number of pups per litter. The fertility index and number of stillborn animals compared across the generations or across doses did not exhibit a consistent trend. The number of animals not surviving to weanling age and sex ratio did not show significant dose or generation effects. No dose-related effects on the incidence of gross deformities were noted in examinations of pups of each generation. The only significant histopathological changes noted in the F_{3a} generation were a dose-related trend for focal dilatation of the renal cortex and tubules, a statistically significant dose-related trend for necrosis of the thymus ($P < 0.012$ for males and $P < 0.0001$ for females) and a statistically significant inverse dose–response relationship for red pulp haematopoietic cell proliferation of the spleen ($P < 0.001$ for males and $P < 0.000\ 01$ for females).

The no-observed-adverse-effect level (NOAEL) for parental toxicity was 15 mg/kg bw per day, based on reductions in body weight at 30 mg/kg bw per day. A NOAEL for offspring toxicity could not be determined, as effects in the F_{3a} generation were present in all dose groups. The NOAEL for reproductive toxicity was 30 mg/kg bw per day, the highest dose tested (Littlefield, 1988).

(b) Developmental toxicity

(i) Rats

In a GLP-compliant study, groups (minimum of 20 animals) of pregnant CD rats were given 97.7% pure gentian violet by oral gavage at a dose of 0, 2.5, 5 or 10 mg/kg bw per day on gestation days 6–15. The vehicle was distilled water. Dams were weighed on gestation days 0, 6–15 and 20. Feed and water were available ad libitum. At sacrifice on gestation day 20, body, liver and gravid uterine weights and numbers of implantation sites, resorptions, and dead and live fetuses were recorded. Individual fetuses were weighed, sexed and examined for gross morphological abnormalities. All live fetuses were examined for visceral malformations using the Staples fresh tissue dissection method. Half of the fetuses were decapitated prior to dissection, and the heads were fixed in Bouin's solution for free hand sectioning and examination by Wilson's technique. All fetal carcasses were cleared and stained with alizarin red S and examined for skeletal malformations.

Three of 32 dams in the 10 mg/kg bw per day group died, whereas all other dams survived to day 20. Body weight gain was also significantly reduced in the 5 and 10 mg/kg bw per day dams. Clinical signs of toxicity (i.e. wheezing, lethargy, weakness, diarrhoea, lacrimation and rough coat) were observed to increase in a dose-related manner. There were no maternal signs of toxicity at 2.5 mg/kg bw per day. The Committee concluded that gentian violet treatment of the pregnant rats at 10 mg/kg bw per day resulted in a statistically significant increase in hydroureter, hydronephrosis and short ribs in the fetuses and clinical signs of maternal toxicity along with decreased body weight gain during treatment and the remainder of the gestation period. The fetal effects were seen only in conjunction with maternal toxicity and so were not seen at 2.5 mg/kg bw per day or at 5 mg/kg bw per day, where the maternal toxicity was limited. There were no gross malformations in any dose group.

The NOAEL for maternal toxicity was 2.5 mg/kg bw per day, based on clinical signs of toxicity at 5 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 5 mg/kg bw per day, based on fetal effects seen in conjunction with maternal toxicity at 10 mg/kg bw per day (Wolkowski-Tyl et al., 1982).

(ii) Rabbits

In a GLP-compliant study, groups of 30–40 artificially inseminated New Zealand White rabbits were given 97.7% pure gentian violet by oral gavage at a dose of 0, 0.5, 1 or 2 mg/kg bw per day on gestation days 6–19. At sacrifice, the numbers of pregnant dams in the groups were 26, 22, 21 and 23, respectively. The vehicle was distilled water. The does were weighed, prior to dosing, on days 0, 6–19 and 30 of gestation and observed for clinical signs of toxicity. At sacrifice on gestation day 30, body, liver and gravid uterine weights and numbers of implantation sites, resorptions, and dead and live fetuses were recorded. Individual fetuses were weighed, sexed and examined for gross morphological abnormalities. All live fetuses were examined for visceral malformations using the Staples fresh tissue dissection method. Half of the fetuses were decapitated after dissection, and the heads were fixed in Bouin's solution for free hand sectioning and examination by Wilson's technique. All fetal carcasses were cleared and stained with alizarin red S and examined for skeletal malformations.

Maternal mortality increased with dose (i.e. 0%, 7.4%, 15.4% and 22.6%, respectively). Maternal body weight gain was lower for all gentian violet–dosed does during treatment and gestation periods. Clinical signs, including wheezing, diarrhoea, congestion, wet nose, dyspnoea, lacrimation, anorexia and cyanosis, were observed in the dams in a dose-related manner. Fetal weights were significantly reduced in all gentian violet–treated groups compared with controls. No malformations unique to or with a higher incidence in any of the gentian violet–exposed groups were noted relative to the controls.

The authors concluded that there was no evidence of teratogenicity of gentian violet in this study with New Zealand White rabbits. Owing to the presence of maternal toxicity and significantly reduced fetal weights in all dosed groups, NOAELs could not be identified for maternal or embryo/fetal toxicity (Wolkowski-Tyl et al., 1983).

2.2.6 Special studies

Studies on mitochondria prepared from the livers of male Wistar rats provided evidence that gentian violet was a potent uncoupler of oxidative phosphorylation and that a free radical metabolite of gentian violet was not implicated in its mode of action (Moreno, Gadelha & Docampo, 1988). Gentian violet also uncouples oxidative phosphorylation in the mitochondria of unicellular protozoa, *Trypanosoma cruzi* (Gadelha et al., 1989).

Nussenzweig et al. (1953) observed that although gentian violet is a potent uncoupler of oxidative phosphorylation, this effect has not been seen in vivo in humans receiving blood that had been treated with gentian violet to protect against Chagas disease. Metabolic demethylation in the mammalian liver or species differences in the affinity of gentian violet for mitochondria were suggested as possible reasons for this different sensitivity to the uncoupling of oxidative phosphorylation.

In vitro, gentian violet has been shown to depress protein and RNA synthesis and oxygen consumption of fibroblasts (Mobacken, Ahonen & Zederfeldt, 1974). It is also shown to inhibit protein synthesis in *Trypanosoma cruzi*, probably due to inhibition of amino acid uptake in the cell (Hoffmann et al., 1995). By interacting with cell lipopolysaccharides, peptidoglycan and DNA, gentian violet damages both the bacterial and mitochondrial membrane by inducing permeability. This interferes with the electron transport mechanism at the cellular level, which could be the reason for its toxicity against bacteria and fungi. Many triphenylmethane dyes, such as malachite green, are known to inhibit the human glutathione *S*-transferase enzymes, although gentian violet is only a weak inhibitor of these enzymes (Glanville & Clark, 1997).

2.3 Observations in humans

Hodge et al. (1972) reported that the recommended therapeutic dose for anthelminthic treatment in adult humans is 2.1 mg/kg bw per day and that adverse effects were usually minimal and transient. Wright & Brady (1940) stated that the effects complained of in about one third of treated patients were gastrointestinal irritation, nausea, vomiting, diarrhoea and mild abdominal pain, which ceased on discontinuation of the treatment.

Epidemiological studies have indicated that the use of hair dyes (as a group) could be carcinogenic in humans, although these reviews could not establish a causal relationship (IARC, 1993; Rollison, Helzlsouer & Pinney, 2006; Baan et al., 2008). It is noted that the above-mentioned studies did not investigate the effects of gentian violet itself on human carcinogenicity, although gentian violet and related compounds can be used as non-oxidative direct hair dye ingredients in some countries. Diamante et al. (2009) reviewed the various reported toxicities of gentian violet in humans. Gentian violet is shown to cause dermal irritation/sensitization (Bielicky & Novák, 1969; Meurer & Konz, 1977; Lawrence & Smith, 1982), ocular irritation (Dhir et al., 1982), mucosal irritation (Slotkowski, 1957; Slotkowski & Redondo, 1966; John, 1968; Horsfield, Logan & Newey, 1976; Piatt & Bergeson, 1992) and bladder irritation (Walsh & Walsh, 1986; Kim et al., 2003; Diamante et al. 2009). An epidemic of nosebleeds in apple packers who used packing trays dyed with gentian violet has also been described (Quinby, 1968).

3. COMMENTS

The Committee reviewed studies submitted by a Member State as well as additional papers available in the published literature.

Gentian violet is structurally related to malachite green. The Committee evaluated malachite green in 2009 (Annex 1, reference *193*) and concluded that the use of malachite green in food-producing animals could not be supported. This was because its major metabolite, leucomalachite green, induces hepatocellular adenomas and carcinomas in female mice, and it could not be ruled out that this was by a genotoxic mode of action.

3.1 Biochemical data

Gentian violet is metabolized to leucogentian violet by isolated gut microflora from rats, chickens and humans. Strong binding of gentian violet to isolated gut bacteria and microsomal fractions of liver was demonstrated, and this is likely to affect the bioavailability of gentian violet. In studies in mice and rats using radiolabelled gentian violet, most of the administered dose is excreted in faeces, with urinary excretion being much less important. In mice, the excretion of gentian violet and its metabolites in urine is greater than in rats, but still represents less than 10% of the dose. Demethylation is the major metabolic pathway of biotransformation in liver microsomes, with mouse microsomes in vitro being less active than those from other rodents or chickens. In both rats and mice, the parent compound (gentian violet), its major metabolite leucogentian violet and their demethylated metabolites are found in tissues, urine and faeces.

Absorption of gentian violet from the gut is higher than that for other triphenylmethane dyes. Dosing mice and rats over 7 days demonstrated its distribution to fat, particularly in females.

3.2 Toxicological data

Critical studies are summarized in Table 15.

Table 15. Studies relevant to risk assessment

Species / study type (route)	Doses (mg/kg bw per day)	Critical end-point	NOAEL (mg/kg bw per day)	LOAEL (mg/kg bw per day)
Mouse				
Two-year study of toxicity and carcinogenicity (dietary)	Females: 0, 14.3, 35.7–39.3, 71.4	Erythropoiesis in spleen, atrophy of ovaries	–	14.3[a]
		Benign and malignant liver neoplasms (females)	–	**$BMDL_{10}$: 16.8[b]**

Table 15 (continued)

Species / study type (route)	Doses (mg/kg bw per day)	Critical end-point	NOAEL (mg/kg bw per day)	LOAEL (mg/kg bw per day)
Rat				
Two-year study of toxicity and carcinogenicity (dietary)	Males: 0, 30, 80, 160 Females: 0, 40, 100, 200	Increase in liver regeneration	–	30[a]
		Thyroid follicular cell adenocarcinoma (both sexes) and hepatocellular adenoma (males)	–	–
Three-generation study of reproductive toxicity, including developmental toxicity (dietary)	0, 5, 15, 30	Reproductive toxicity: No effects seen	30[c]	–
		Parental toxicity: Decreased body weight	15	30
		Offspring toxicity: Necrosis of thymus, focal dilatation of renal cortex and tubules, lowered red pulp haematopoietic cell proliferation in spleen	–	5[a]
Developmental toxicity study (gavage)	0, 2.5, 5, 10	Maternal toxicity: Reduced body weight gain, clinical signs	2.5	5
		Embryo and fetal toxicity: Increased hydroureter, hydronephrosis and short ribs	5	10
Rabbit				
Developmental toxicity study (gavage)	0, 0.5, 1, 2	Maternal toxicity: Increased mortality, decreased body weight gain, clinical signs	–	0.5[a]
		Embryo and fetal toxicity: Reduced fetal weight	–	0.5[a]

$BMDL_{10}$: lower 95% confidence limit on the benchmark dose for a 10% response; LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level

[a] Lowest dose tested.

[b] Pivotal study value (Littlefield, 1984; Littlefield et al., 1985).

[c] Highest dose tested.

There were few data available on the acute and short-term toxicity of gentian violet, but the reported range of LD_{50}s, from 100 to 800 mg/kg bw, shows that it is of moderate acute oral toxicity. The most common sign of toxicity was lethargy, followed by anorexia and, in some animals, diarrhoea, excessive thirst, emesis and weight loss. In 90-day studies in rats and dogs, the only reported signs were slight body weight loss and increased liver weight, respectively.

In a 24-month study, gentian violet was given to mice in the feed at a concentration of 0, 100, 300 or 600 mg/kg (equal to 0, 10.7–14.3, 32.1–35.7 and 64.3 mg/kg bw per day for males and 0, 14.3, 35.7–39.3 and 71.4 mg/kg bw per day for females, respectively). Few dose-related non-neoplastic lesions were reported, but there were statistically significant dose-related increases in erythropoiesis in the spleen and atrophy of the ovaries in females at 24 months. The LOAEL for non-carcinogenic effects was 14.3 mg/kg bw per day, the lowest dose tested. Significant, dose-related increases in neoplastic lesions were observed in both sexes, with the female mice being more sensitive. Hepatocellular adenomas and carcinomas were the most common lesions, with significant, dose-related increases found at 24 months in males and at both 18 and 24 months in females. Mortality due to liver neoplasms showed positive trends in both males and females, and there was a dose-related decrease in the time for the onset of liver neoplasms. The females also showed statistically significant dose-related increases in adenoma of the Harderian gland and in type A reticulum cell sarcoma in the urinary bladder, uterus, ovaries and vagina. The data clearly indicate that gentian violet is a multisite carcinogen in the mouse.

In a long-term study of toxicity, rats were exposed to gentian violet in the feed at a concentration of 0, 100, 300 or 600 mg/kg (equal to approximately 0, 30, 80 and 160 mg/kg bw per day for males and 0, 40, 100 and 200 mg/kg bw per day for females, respectively). Gentian violet exposure of these animals was achieved by dosing the parents of the study animals prior to and during mating, with the same dose fed to the offspring from weaning up to 24 months of age. There was a statistically significant increase in liver regeneration in all dose groups and statistically significant dose-related increases in eosinophilic foci in the liver in both sexes in both the mid- and high-dose groups. For liver centrilobular necrosis, there was a dose-related increase, but statistical significance was seen only in the 300 mg/kg feed group in males and in the 600 mg/kg feed group in females. As in mice, female rats appeared to be more sensitive than males. The incidence of thyroid adenocarcinoma was increased in males, with statistical significance at the top dose only at 24 months. Females showed a statistically significant dose–response relationship for thyroid adenocarcinoma at 24 months. The incidence of hepatocellular adenomas showed a small but significant dose–response relationship in males and a significant increase in females at 300 mg/kg feed, but not at other doses. The data indicate a carcinogenic response to gentian violet in rats, although much weaker than the response in mice.

The data show that gentian violet binds to DNA, and this, together with the cellular toxicity of gentian violet, complicates both the testing of gentian violet in vitro and the interpretation of the results. The results are somewhat varied in *Salmonella typhimurium*, with positive responses in some strains but not in others. Gentian violet was clastogenic in vitro and positive in indicator tests for DNA damage. There are few in vivo tests on gentian violet. A single in vivo test for clastogenicity (mouse

bone marrow assay) showed no evidence of clastogenic activity, but the Committee noted that the gentian violet was given via the drinking-water at lower doses (4 and 8 mg/kg bw per day) than those used in the mouse cancer bioassay (ranging from 10 to 70 mg/kg bw per day). Similarly, the other in vivo test on DNA damage in mouse lymphocytes using single intravenous doses up to 6 mg/kg bw showed no effect. The Committee concluded that, overall, the data show that gentian violet is genotoxic.

In view of the carcinogenicity of gentian violet in the mouse and rat and evidence showing genotoxicity in a number of tests, the Committee concluded that gentian violet should be considered a carcinogen acting by a genotoxic mode of action.

In a multigeneration reproductive toxicity study, rats were given gentian violet in the feed at a concentration of 0, 100, 300 or 600 mg/kg (equivalent to 0, 5, 15 and 30 mg/kg bw per day, respectively) over three generations. There were significant reductions in body weight in the top dose group in all generations. The NOAEL for parental toxicity was 15 mg/kg bw per day. In the F_{3a} generation, examined for histopathological effects, a dose-related trend for focal dilatation of the renal cortex and tubules, a statistically significant dose-related trend for necrosis of the thymus and an inverse dose–response relationship for red pulp haematopoietic cell proliferation of the spleen were seen. The effects in the F_{3a} generation were present in all dose groups, and a NOAEL for offspring toxicity could not be determined. Gentian violet had no effect on the number of pups per litter, fertility index, pup survival, sex ratio or number of stillborn animals. The NOAEL for reproductive toxicity was 30 mg/kg bw per day, the highest dose tested.

Two developmental toxicity studies were conducted in rats. In the first study, CD rats were given gentian violet at 0, 2.5, 5 or 10 mg/kg bw per day by oral gavage on days 6–15 of gestation. In the second study, the three-generation study in Fischer 344 rats described above, the F_{3b} generation was examined for teratogenic effects. In that study, gentian violet was given in the feed at a concentration of 0, 100, 300 or 600 mg/kg (equivalent to 0, 5, 15 and 30 mg/kg bw per day, respectively). CD rats appeared to be more sensitive than Fischer 344 rats to the toxicity of gentian violet, with dose-related reductions in maternal weight gain at 5 and 10 mg/kg bw per day and increased clinical signs of toxicity, significant at 10 mg/kg bw per day and limited at 5 mg/kg bw per day (maternal toxicity NOAEL of 2.5 mg/kg bw per day). In Fischer 344 rats, reduction in body weight was seen only at 30 mg/kg bw per day and not at lower doses of 5 and 15 mg/kg bw per day (maternal toxicity NOAEL of 15 mg/kg bw per day). It was also noted that malformations (hydroureter, hydronephrosis and short ribs) were seen only in the CD rats. Effects on the fetus were seen only at doses that also caused maternal toxicity. The NOAEL for embryo and fetal toxicity in CD rats was 5 mg/kg bw per day.

In a developmental toxicity study, rabbits were given gentian violet at 0, 0.5, 1 or 2 mg/kg bw per day by oral gavage on days 6–19 of gestation. Maternal mortality was increased in a dose-related manner, and maternal body weight gain was decreased in all treated groups compared with controls. Fetal weights were significantly reduced in all treated groups compared with controls. There was no evidence of teratogenic effects. Owing to the presence of maternal toxicity and significantly reduced fetal weights in all dosed groups, NOAELs could not be identified for maternal or embryo/fetal toxicity.

In humans, case reports have shown that gentian violet has been associated with dermal irritation/sensitization, ocular irritation, mucosal irritation and bladder irritation following topical or employment-related exposure, but these are not relevant to the evaluation of the safety of gentian violet in food.

4. EVALUATION

The Committee concluded that it is inappropriate to set an ADI for gentian violet because it is genotoxic and carcinogenic. Gentian violet is widely used in various ways other than as an authorized veterinary drug, and there may be residues in fish from unauthorized use or from environmental exposures. Therefore, irrespective of whether it is used as a veterinary drug, the Committee agreed that some further guidance to risk managers was needed.

The Committee determined that the pivotal study for the evaluation of gentian violet is the carcinogenicity study in mice. Although it was not possible to add the adenomas and carcinomas in liver, the dose–response relationship for the two tumour types was very similar. Accordingly, a benchmark dose (BMD) evaluation was conducted using the data for the female mouse malignant liver neoplasms at the 24-month sacrifice.

The United States Environmental Protection Agency's (USEPA) BMD software (BMDS, version 2.2) was used for modelling the dose–response relationship for malignant liver neoplasms in gentian violet–treated female mice. The following dose–response models were fitted to the dose–incidence data and resulted in an acceptable fit: gamma, logistic, log-logistic, multistage, multistage cancer, probit, log-probit and Weibull. The BMD and lower 95% confidence limit on the benchmark dose (BMDL) values for an extra 10% risk compared with the modelled background incidence (BMD_{10} and $BMDL_{10}$) were estimated by performing 250 iterations.

Table 16. BMD_{10} and $BMDL_{10}$ calculations for gentian violet based on the incidences of malignant neoplasms in female mice

Model	AIC	*P* value	Scaled residual of interest	Accepted	BMD_{10} (mg/kg bw per day)	$BMDL_{10}$ (mg/kg bw per day)
Gamma multi-hit	324.3	0.842	–0.151	Yes	24.0	18.8
Logistic	324.2	0.415	–1.016	Yes	22.3	19.5
Log-logistic	324.3	0.907	–0.093	Yes	24.7	19.6
Log-probit	324.4	0.775	0.231	Yes	25.2	19.8
Multistage	323.9	0.477	–1.03	Yes	19.9	16.8
Multistage cancer	323.9	0.477	–1.03	Yes	19.9	16.8
Probit	324.3	0.398	–1.125	Yes	20.3	17.8
Weibull	324.9	0.472	–0.562	Yes	22.9	17.8

AIC: Akaike's Information Criterion; BMD_{10}: benchmark dose for a 10% response; $BMDL_{10}$: lower 95% confidence limit on the benchmark dose for a 10% response

The BMD_{10} values from the accepted models ranged from 19.9 to 25.2 mg/kg bw per day, and the $BMDL_{10}$ values ranged from 16.8 to 19.8 mg/kg bw per day (Table 16). In order to be prudent, the Committee decided to use the more conservative lower end of this range of values for the evaluation and chose a $BMDL_{10}$ value of 16.8 mg/kg bw per day as the reference point for a margin of exposure (MOE) calculation.

The Committee estimated MOEs assuming a residue level of 0.5 µg/kg, which is a typical limit of quantification for gentian violet residues in foods, and a residue level of 5 µg/kg, which is 10 times the typical limit of quantification, as a hypothetical scenario. Assuming a daily consumption of 300 g of fish contaminated with gentian violet and its metabolites, the estimated theoretical exposures to gentian violet for a 60 kg person were 0.0025 and 0.025 µg/kg bw per day for the two residue levels, respectively. Comparison of these estimated exposures with the $BMDL_{10}$ of 16.8 mg/kg bw per day indicates MOEs of about 6.7×10^6 and 6.7×10^5, respectively. Based on considerations discussed at the sixty-fourth meeting of the Committee for unintended contaminants (Annex 1, reference *176*), these MOEs would be considered to be of low concern for human health.

However, the Committee noted that there were a number of uncertainties associated with the risk assessment, some of which were substantial. The uncertainties relate to two aspects of the data available for risk assessment. Firstly, there were insufficient residue data in food-producing animals or the environment from which to estimate dietary exposure to gentian violet, and hence assumptions had to be made. Secondly, there is very little information on the proportion of gentian violet and its metabolites in the total residue and on the carcinogenicity of the metabolites. For example, there is a published report that one of the possible metabolites of gentian violet, demethylated leucopararosaniline, is carcinogenic in rats, but no information is available on its potency. In addition, there is no information on the carcinogenicity of the major metabolite, leucogentian violet. The structure of gentian violet is similar to that of malachite green, and it is known that leucomalachite green is a more potent carcinogen than malachite green; therefore, it is possible that leucogentian violet is similarly a more potent carcinogen than gentian violet. Gentian violet and leucogentian violet are readily interconvertible in the body, and so it is likely that exposure to gentian violet will also result in exposure to leucogentian violet. Thus, there is inadequate information to determine the overall carcinogenicity of the metabolites of gentian violet (demethylated gentian violet, leucogentian violet and its demethylated metabolites).

5. REFERENCES

Aidoo A, Gao N, Neft RE, Schol HM, Hass BS, Minor TY et al. (1990). Gentian violet in bacterial and mammalian cell systems. Teratogen Carcinogen Mutagen. 10:449–62.

Au W, Hsu TC (1979). Studies on the clastogenic effects of biologic stains and dyes. Environ Mutagen. 1:27–35.

Au W, Pathak S, Collie CJ, Hsu TC (1978). Cytogenetic toxicity of gentian violet and crystal violet on mammalian cells in vitro. Mutat Res. 58:269–76.

Au W, Butler MA, Bloom SE, Matney TS (1979). Further study of the genetic toxicity of gentian violet. Mutat Res. 66:103–12.

Baan R, Straif K, Grosse Y, Secretan B, Ghissassi F, Bouvard V et al. (2008). WHO International Agency for Research on Cancer Monograph Working Group: carcinogenicity of some aromatic amines, organic dyes, and related exposures. Lancet Oncol. 9:322–3.

Bielicky T, Novák MN (1969). Contact-group sensitization to triphenylmethane dyes. Gentian violet, brilliant green, and malachite green. Arch Dermatol. 100:540–3.

Bonin AM, Farquharson GB, Baker RSU (1981). Mutagenicity of arylmethane dyes in *Salmonella*. Mutat Res. 89:21–34.

Dhir SP, Sharma SK, Munjal VP, Gupa A (1982). Keratoconjunctivitis sicca following instillation of gentian violet. Indian J Ophthalmol. 30:21–2.

Diamante C, Bergfeld WF, Belsito DV, Klaassen CD, Marks JG, Shank RC Jr et al. (2009). Final report on the safety assessment of basic violet 1, basic violet 3, and basic violet 4. Int J Toxicol. 28(6 Suppl 2):193S–204S.

Docampo R, Moreno SNJ (1990). The metabolism and mode of action of gentian violet. Drug Metab Rev. 22:161–78.

FAO/WHO (2012). Report of the Twentieth Session of the Codex Committee on Residues of Veterinary Drugs in Foods, San Juan, Puerto Rico, 7–11 May 2012. Rome: Food and Agriculture Organization of the United Nations and World Health Organization, Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission (REP 12/RVDF; http://www.codexalimentarius.org/download/report/778/REP12_RVe.pdf).

Gadelha FR, Moreno SN, De Souza W, Cruz FS, Docampo R (1989). The mitochondrion of *Trypanosoma cruzi* is a target of crystal violet toxicity. Mol Biochem Parasitol. 34:117–26.

Glanville SD, Clark AG (1997). Inhibition of human glutathione *S*-transferases by basic triphenylmethane dyes. Life Sci. 60:1535–44.

Harrelson WG Jr, Mason RP (1982). Microsomal reduction of gentian violet. Evidence for cytochrome P-450 catalyzed free radical formation. Mol Pharmacol. 22:239–42.

Hass BS, Heflich RH, McDonald JJ (1986). Evaluation of the mutagenicity of crystal violet and its metabolites in *Salmonella typhimurium* and *Escherichia coli*. Abstract of the 17th Annual Meeting of the Environmental Mutagen Society, 9–13 April 1986, Baltimore, MD, USA. Abstract No. 95.

Hodge HC, Indra J, Drobeck HP, Duprey LP, Tainter NL (1972). Acute oral toxicity of methylrosaniline chloride. Toxicol Appl Pharmacol. 22:1–5.

Hoffmann ME, Jang J, Moreno SN, Docampo R (1995). Inhibition of protein synthesis and amino acid transport by crystal violet in *Trypanosoma cruzi*. J Eukaryot Microbiol. 42:293–7.

Horsfield P, Logan FA, Newey JA (1976). Oral irritation with gentian violet. Br Med J. 2:529.

Hsu TC, Cherry LM, Pathak S (1982). Induction of chromatid breakage by clastogens in cells of G2 phase. Mutat Res. 93:185–93.

IARC (1993). Occupational exposure of hairdressers and barbers and personal use of hair colorants; some hair dyes, cosmetic colorants, industrial dyestuffs and aromatic amines. IARC [Int Agency Res Cancer] Monogr Eval Carcinog Risk Hum. 57:43–118.

John RW (1968). Necrosis of oral mucosa after local application of crystal violet. Br Med J. 1:157.

Kim SJ, Koh DH, Park JS, Ahn HS, Choi JB, Kim YS (2003). Hemorrhagic cystitis due to intravesical instillation of gentian violet completely recovered with conservative therapy. Yonsei Med J. 44:163–5.

Krishnaja AP, Sharma NK (1995). Heterogeneity in chemical mutagen–induced chromosome damage after G2 phase exposure to bleomycin, ara-C and gentian violet in cultured lymphocytes of β-thalassaemia traits. Mutat Res. 331:143–8.

Lawrence CM, Smith AG (1982). Ampliative medicament allergy: concomitant sensitivity to multiple medicaments including yellow soft paraffin, white soft paraffin, gentian violet and Span 20. Contact Dermatitis. 8:240–5.

Levin DE, Lovely TJ, Klekowski E (1982). Light-enhanced genetic toxicity of crystal violet. Mutat Res. 103:283–8.

Littlefield NA (1984). Chronic toxicity and carcinogenicity studies of gentian violet in mice. Final report. Jefferson (AK): National Center for Toxicological Research, Division of Chemical Toxicology (NCTR Technical Report for Experiment No. 304).

Littlefield NA (1988). Three-generation reproduction and toxicity studies of gentian violet in Fischer 344 rats. Jefferson (AK): National Center for Toxicological Research (NCTR Technical Report for Experiment Nos 305, 354 and 355).

Littlefield NA, Blackwell B-N, Hewitt CC, Gaylor DW (1985). Chronic toxicity and carcinogenicity studies of gentian violet in mice. Fundam Appl Toxicol. 5(5):902–12.

Littlefield NA, Gaylor DW, Blackwell B-N, Allen RR (1989). Chronic toxicity/carcinogenicity studies of gentian violet in Fischer 344 rats: two-generation exposure. Food Chem Toxicol. 27:239–47.

McDonald JJ (1989). Metabolism of gentian violet in Fischer 344 rats and B6C3F1 mice. Jefferson (AK): National Center for Toxicological Research (NCTR Technical Report for Experiment Nos 302 and 303).

McDonald JJ, Cerniglia CE (1984). Biotransformation of gentian violet to leucogentian violet by human, rat, and chicken intestinal microflora. Am Soc Pharmacol Exp Ther. 12:330–6.

McDonald JJ, North CR, Breeden CR, Lai CC, Roth RW (1984a). Synthesis and disposition of ^{14}C-labelled gentian violet in F344 rats and B6C3F1 mice. Food Chem Toxicol. 22:331–6.

McDonald JJ, Breeden CR, North BM, Roth RW (1984b). Species and strain comparison of the metabolism of gentian violet by liver microsomes. J Agric Food Chem. 32:596–600.

Meurer M, Konz B (1977). [Skin necroses following the use of a 2-per-cent Pyoctanin solution.] Hautartzt. 28:94–5 (in German with an English abstract).

Mobacken H, Ahonen J, Zederfeldt B (1974). The effect of cationic triphenylmethane dye (crystal violet) on rabbit granulation tissue. Oxygen consumption and RNA and protein synthesis in tissue slices. Acta Derm Venereol. 54:343–7.

Moreno SNJ, Gadelha FR, Docampo R (1988). Crystal violet as an uncoupler of oxidative phosphorylation in rat liver mitochondria. J Biol Chem. 263:12493–9.

Muller W, Gauthier F (1975). Interactions of heteroaromatic compounds with nucleic acids, A–T-specific non-intercalating DNA ligands. Eur J Biochem. 54:385–94.

National Toxicology Program (1986). Toxicology and carcinogenesis studies of C.I. Basic Red 9 monohydrochloride (pararosaniline) (CAS No. 569-61-9) in F344/N rats and B6C3F1 mice (feed studies). Research Triangle Park (NC): United States Department of Health and Human Services, Public Health Service, National Institutes of Health (NTP Technical Report Series No. 285; http://www.ntp.niehs.nih.gov/ntp/htdocs/LT_rpts/tr285.pdf) [cited in Docampo & Moreno, 1990].

Nussenzweig V, Sonntag R, Biancalana A, Pedriera de Freitas JL, Amato Neto V, Kloetzel J (1953). Effect of triphenylmethane dyes on *Trypanosoma cruzi* in vitro; use of gentian violet in prevention of Chagas disease by blood transfusion. Hospital (Rio de Janeiro). 44:731–44 [cited in Moreno, Gadelha & Docampo, 1988].

Piatt JP, Bergeson PS (1992). Gentian violet toxicity. Clin Pediatr (Phila). 31:756–7.

Quinby GE (1968). Gentian violet as a cause of epidemic occupational nosebleeds. Arch Environ Health. 16:485–9.

Rollison DE, Helzlsouer KJ, Pinney SM (2006). Personal hair dye use and cancer: a systematic literature review and evaluation of exposure assessment in studies published since 1992. J Toxicol Environ Health. B 9:413–39.

Rosenkranz HS, Carr HS (1971). Possible hazards in use of gentian violet. Br Med J. 3:702–3.

Seppelin [initial unknown] (1949). Unpublished data [cited in Hodge et al., 1972].

Shahin MM, von Borstel RC (1978). Comparisons of mutation induction in reversion systems of *Saccharomyces cerevisiae* and *Salmonella typhimurium*. Mutat Res. 58:1–10.

Slotkowski EL (1957). Formation of mucous membrane lesions secondary to prolonged use of one percent aqueous gentian violet. J Pediatr. 51:652–4.

Slotkowski EL, Redondo D (1966). Mucosal irritation following use of gentian violet. Am J Dis Child. 112:40–2.

Thomas SM, MacPhee DG (1984). Crystal violet: a direct-acting frame shift mutagen whose mutagenicity is enhanced by mammalian metabolism. Mutat Res. 140:165–7.

USFDA (1976). Unpublished data. United States Food and Drug Administration [cited in Littlefield et al., 1989].

Wakelin LPG, Adams A, Hunter C, Waring MJ (1981). Interaction of crystal violet with nucleic acids. Biochemistry. 20:5779–87.

Walsh C, Walsh A (1986). Haemorrhagic cystitis due to gentian violet. Br Med J. 293:732.

Wolkowski-Tyl R, Jones-Price C, Marr MC, Langhoff-Paschke L, Kimmel CA (1982). Teratologic evaluation of gentian violet (CAS No. 548-62-9) in CD rats. Final report. Research Triangle Park (NC): United States Department of Health and Human Services, Public Health Service, National Toxicology Program (NTP Study TER82079).

Wolkowski-Tyl R, Jones-Price C, Reel JR, Marr MC, Langhoff-Paschke L, Kimmel CA (1983). Teratologic evaluation of gentian violet (CAS No. 548-62-9) in New Zealand White rabbits. Final report. Research Triangle Park (NC): United States Department of Health and Human Services, Public Health Service, National Toxicology Program (NTP Study TER82080).

Wright NH, Brady FJ (1940). Studies on oxyuriasis. J Am Med Assoc. 114:861–6 [cited in Hodge et al., 1972].