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MALACHITE GREEN page 37-68

Toxicological evaluation of certain veterinary drug residues in food

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MALACHITE GREEN

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1. EXPLANATION

Malachite green (MG) is an *N*-methylated triphenylmethane dye that is used mainly industrially for leather, wool, cotton, silk, jute, paper and certain fibres. For such purposes, large quantities of extremely variable composition have been produced. About 10–15% of all dyes are lost directly to wastewater in the dyeing process.

The chemical has been used routinely in some countries in aquaculture since the early 1930s and is considered by many in the fish industry to be an effective antifungal and antiprotozoal agent in fish, fish eggs and crayfish. It is currently

registered in some countries for use as a veterinary drug in ornamental fish, to which it is applied as a topical antiseptic or to treat parasites, fungal infections and bacterial infections in fish and fish eggs. It is not permitted in aquaculture of fish destined for human consumption. Reported types of treatment of fish include dip treatment, flush treatment, sustained culture treatment and application in feed. Extremely wide ranges of concentrations and exposure times have been used.

MG 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 and distributed to all tissues. MG is metabolically reduced by fish to the persistent colourless metabolite, leucomalachite green (LMG), and possibly other, as yet unidentified, degradation products (Plakas et al., 1996). The rate of excretion of MG (as LMG) from fish is dependent on the fat content of the fish, with more LMG being retained in fatty fish than in lean fish. Therefore, when fish that have been exposed to MG reach the consumer, the amount of LMG present in the fish is expected to be higher than that of MG, because of its longer elimination half-life. As LMG is the predominant residue found in fish tissues following exposure to MG, it is the residue of primary concern from a safety point of view.

MG has not previously been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). It was placed on the agenda of the current meeting at the request of the 17th Session of the Codex Committee on Residues of Veterinary Drugs in Foods (Codex Alimentarius Commission, 2007), which requested JECFA to consider a literature review and advise if this substance could be supported for use in food-producing animals (as the available data were probably not sufficient to derive an acceptable daily intake [ADI] and maximum residue limits [MRLs]).

Two risk assessments were provided by national authorities. In addition, a comprehensive literature search was performed.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Absorption, distribution, excretion and metabolism

(a) Bacteria

Henderson et al. (1997) investigated the reduction of MG to LMG by intestinal bacteria from humans, rats, mice and rhesus monkeys and 14 pure cultures of anaerobic bacteria representative of those found in the human gastrointestinal tract. The bacterial cultures were incubated with 300 µg of MG in 5 ml of brain–heart infusion broth for 24–48 h under anaerobic conditions. Virtually all of the MG was converted to its LMG derivative by the intestinal microflora**.** The pure bacterial cultures converted 7.3–99.3% of the MG to LMG. These results indicate the importance of the gastrointestinal tract microflora in the conversion of MG to LMG.

(b) Mice and rats

Three male and three female rats weighing about 300–325 g were given a single dose (2 mg/kg body weight [bw]) of ^{14}C-labelled MG in water by gavage. Urine and faeces were collected daily after MG administration. One week after MG administration, the liver, kidney, muscle, skin and blood were removed from the rats and stored at −20 °C prior to analysis. About 96.3% ± 5.9% (mean ± standard deviation) of the orally administered dose was excreted in the urine and faeces of rats over the 7-day study period, with more than 80% appearing in the faeces. Since the rat tissues showed only low levels of ^{14}C, they were not investigated further (Law, 1994).

In short-term feeding studies, Culp et al. (1999) showed that MG is sequentially *N*-demethylated to secondary and primary aromatic amines in rats and mice both before and after reduction to LMG. Female mice (eight per dose group; B6C3F1:Nctr BR (C57BL/6N × C3H/HeN MTV−)) as well as male rats (eight per dose group; F344:N Nctr BR) were fed 0, 100 or 600 mg MG/kg diet (equivalent to 0, 15 and 90 mg MG/kg bw per day in mice and 0, 10 and 60 mg MG/kg bw per day in rats) (as the chloride salt, ≥94% purity) or 0, 96 or 580 mg LMG/kg diet (equivalent to 0, 13.7 and 72 mg LMG/kg bw per day in mice and 0, 9.6 and 58 mg LMG/kg bw per day in rats) (≥98% purity) for 28 days. Liver extracts from the mice and rats were analysed by high-performance liquid chromatography in combination with atmospheric pressure chemical ionization mass spectrometry (HPLC-APCI/MS).

Analysis of liver extracts from rats treated with MG detected the molecular ions for MG, its mono-, di-, tri- and tetradesmethyl derivatives and MG *N*-oxide. A small, but measurable, amount of LMG was also detected. Concentrations of MG and metabolites increased with increasing dose. Similarly, in liver extracts from rats treated with LMG, primarily protonated LMG, protonated demethylated derivatives and the molecular ions of MG *N*-oxide and demethylated *N*-oxide derivatives were seen. A small, but measurable, amount of MG was also found. A dose-related increase in LMG and metabolites was observed in both rat and mouse liver extracts.

The demethylated metabolites observed in the livers of rats fed MG or LMG are presented in Figure 1 (Culp et al., 1999).

Figure 1. Demethylated metabolites in livers of rats fed MG or LMG

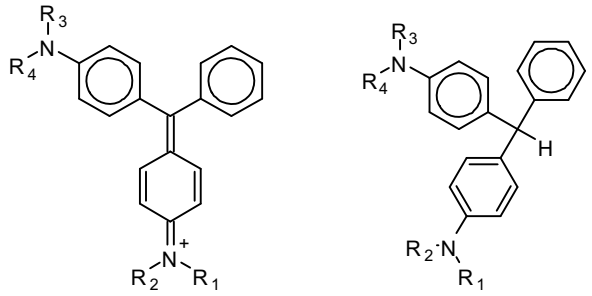


Figure 1 (contd)

Structures of MG, LMG and demethylated derivatives	Malachite green				Leucomalachite green			
	R_1	R_2	R_3	R_4	R_1	R_2	R_3	R_4
Parent molecule	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3
Desmethyl-	CH_3	CH_3	CH_3	H	CH_3	CH_3	CH_3	H
Didesmethyl- (symmetric)	CH_3	H	CH_3	H	CH_3	H	CH_3	H
Tridesmethyl-	CH_3	H	H	H	CH_3	H	H	H
Tetradesmethyl-	H	H	H	H	H	H	H	H

(c) Humans

No studies on the absorption, distribution, metabolism or excretion of MG or LMG in humans were found in a review of the literature (NTP, 2005).

2.2 Toxicological studies

2.2.1 Acute toxicity

Results of studies of the acute toxicity of MG are summarized in Table 1.

Table 1. Results of studies of the acute toxicity of MG

Species (sex)	Route	LD_{50} (mg/kg bw)	Reference
NMRI mice	Gavage	50	Clemmensen et al. (1984)
Wistar rats (M & F)	Gavage	275	Clemmensen et al. (1984)
Wistar rats (M & F)	Dermal	>2000	Clemmensen et al. (1984)
SD rats (F)	Oral	520	Meyer & Jorgenson (1983)

F, female; LD_{50}, median lethal dose; M, male.

The acute effects observed in rats were reduced motor activity, diarrhoea and piloerection, but only for the 1st day. The major findings were hyperaemia and atonia of the intestinal walls, often in conjunction with dilatation of the gastrointestinal tract as far as the substance had reached before the death of the animal. Survivors were free of symptoms after 2 days (Clemmensen et al., 1984).

2.2.2 Short-term studies of toxicity of MG

(a) Mice

In a study compliant with Good Laboratory Practice (GLP), groups of eight female and eight male mice (B6C3F1:Nctr BR (C57BL/6N × C3H/HeN MTV−))

(6–7 weeks old) were given MG (as the chloride salt, purity >94%) at concentrations of 0, 25, 100, 300, 600 or 1200 mg/kg feed (equivalent to 0, 3.75, 15, 45, 90 and 180 mg MG/kg bw per day) for 28 days. Haematology measurements included leukocyte count, erythrocyte count, haemoglobin, haematocrit, mean erythrocyte volume, mean erythrocyte haemoglobin, mean erythrocyte haemoglobin concentration, platelet count, segmented neutrophils, lymphocytes, monocytes, eosinophils and reticulocyte count. Clinical chemistry measurements included total protein, bile acids, blood urea nitrogen, creatinine, alanine aminotransferase (ALT) and alkaline phosphatase.

Female mice fed 1200 mg MG/kg diet had significantly lower body weights (91–92% of control group) at weeks 3 ($P < 0.02$) and 4 ($P < 0.03$). The body weights of male mice were not significantly affected at any of the dose levels of MG. The female mice fed 600 or 1200 mg MG/kg diet had significant decreases in the erythrocyte count and haemoglobin and haematocrit levels as compared with the control group; in male mice, significant decreases in these parameters were observed only in the 1200 mg MG/kg diet dose group. The mean erythrocyte volume was increased ($P < 0.05$) in female mice fed 300, 600 or 1200 mg MG/kg diet as compared with the control group. There was also a 1.4- to 1.9-fold increase in reticulocytes in these groups ($P < 0.05$). Male mice fed 1200 mg MG/kg diet showed a 1.6-fold increase in reticulocytes ($P < 0.05$). There were no significant histopathological changes observed in the mice fed MG (Culp et al., 1999). The no-observed-adverse-effect level (NOAEL) was 100 mg/kg feed, equivalent to 15 mg MG/kg bw per day.

(b) Rats

In a non-GLP-compliant study, groups of eight male and eight female Wistar rats were given <0, 10, 100 or 1000 mg MG/kg feed as MG oxalate (purity >90%) (equivalent to 0, 1, 10 and 100 mg MG/kg bw per day) for 28 days. Blood samples were taken after 3 weeks and analysed for alkaline phosphatase, aspartate aminotransferase (AST), urea, creatinine, glucose and methaemoglobin. The numbers of red blood cells, white blood cells and reticulocytes were counted. Differential counts of white blood cells were performed on smears and packed cell volume. All animals were autopsied, and tissues from liver, kidney, adrenals and testis were weighted and prepared for microscopy. No clinical effect of MG was observed in the treated animals, apart from apparent hyperactive behaviour in the rats in the highest dose group. These animals also had a significant reduction in weight gain and a reduced food intake. In females in the 1000 mg/kg diet group, an increase in lymphocytes and a concomitant decrease in neutrophils and a slight but significant decrease in packed cell volume were observed. The males in the 1000 mg/kg diet group showed a significant increase in plasma urea (Clemmensen et al., 1984). The NOAEL was 100 mg MG/kg feed, equivalent to 10 mg MG/kg bw per day.

In a GLP-compliant study, groups of eight female and eight male rats (F344/N Nctr BR) (6–7 weeks of age) were given MG (as the chloride salt, purity >94%) at dietary concentrations of 0, 25, 100, 300, 600 or 1200 mg/kg feed (equivalent to 0, 2.5, 10, 30, 60 or 120 mg MG/kg bw per day) for 28 days. Haematology

measurements included leukocyte count, erythrocyte count, haemoglobin, haematocrit, mean erythrocyte volume, mean erythrocyte haemoglobin, mean erythrocyte haemoglobin concentration, platelet count, segmented neutrophils, lymphocytes, monocytes, eosinophils and reticulocyte count. Clinical chemistry measurements included total protein and bile acids, blood urea nitrogen, creatinine, ALT, alkaline phosphatase, AST, glucose, cholesterol, triglycerides, gamma-glutamyl transferase (GGT), albumin, sorbitol dehydrogenase, creatine kinase, sodium, potassium, chloride, calcium and phosphorus.

In female rats, there were significant decreases in the mean body weights in the 1200 mg MG/kg diet dose group for weeks 1–4, with the animals weighing 80–83% of the control rats. Although the male rats fed 1200 mg MG/kg diet tended to have lower body weights (82–87%), compared with the control group, the differences were not significant. The female rats of the 300, 600 and 1200 mg MG/kg diet groups had significantly increased ratios of liver weights to body weights. The ratio of liver weight to body weight was significantly increased in the male rats fed 600 and 1200 mg MG/kg diet. In both sexes, there was a significant linear increasing trend in the levels of GGT, with the value in females in the 1200 mg MG/kg diet dose group being 4.2-fold greater ($P < 0.0005$) than that in the controls. Blood haematology measurements in female rats showed slight (<7%), but significant, decreases in the 1200 mg MG/kg diet dose group in erythrocyte count, haemoglobin, haematocrit, mean erythrocyte haemoglobin and mean erythrocyte haemoglobin concentration. Male rats had slight (<3%), but significant, decreases in mean erythrocyte haemoglobin in the 300, 600 and 1200 mg MG/kg diet dose groups. Seven out of eight female rats fed 1200 mg MG/kg diet had minimal to mild hepatocyte vacuolization ($P < 0.01$). The same lesion, primarily midzonal in location, was observed in one and four male rats fed 600 and 1200 mg MG/kg diet, respectively (Culp et al., 1999). The NOAEL was 100 mg MG/kg feed, equivalent to 10 mg MG/kg bw per day.

2.2.3 Short-term studies of toxicity of LMG

(a) Mice

In a GLP-compliant study, groups of eight female mice (B6C3F1/Nctr BR (C57BL/6N × C3H/HeN MTV−)) were given LMG (>98% pure) at 0, 290, 580 or 1160 mg/kg diet (equivalent to 0, 43.5, 87 and 174 mg/kg bw per day) for 28 days. Haematology measurements included leukocyte count, erythrocyte count, haemoglobin, haematocrit, mean erythrocyte volume, mean erythrocyte haemoglobin, mean erythrocyte haemoglobin concentration, platelet count, segmented neutrophils, lymphocytes, monocytes, eosinophils and reticulocyte count. Clinical chemistry measurements included total protein and bile acids, blood urea nitrogen, creatinine, ALT and alkaline phosphatase.

The female mice fed 1160 mg LMG/kg diet had significantly lower body weights (93% of the control group) at week 4. A marginally significant decrease in body weight ($P < 0.01$) from the control group was also observed in the female mice fed 580 mg LMG/kg diet at week 4. In addition, there were statistically significant linear dose trends for week 3 ($P < 0.02$) and week 4 ($P < 0.002$). All female mice

fed 1160 mg LMG/kg diet had scattered dead or degenerate cells in the transitional epithelium of the urinary bladder ($P < 0.001$). Many of the cells lacked nuclei; when visible, the nuclei were condensed or fragmented, which the authors thought suggested apoptosis. Examination of thin sections revealed that many apparent apoptotic cells were contained within phagocytic vacuoles inside viable epithelial cells. The in situ end labelling technique for detecting deoxyribonucleic acid (DNA) fragmentation showed that the cytoplasm of apparently apoptotic cells was moderately positive for the presence of DNA fragments, and condensed nuclei stained intensely for DNA fragmentation. Individual cell necrosis was not accompanied by inflammatory changes. Similar apoptosis was not seen in transitional epithelium of the bladders of female mice fed 0, 290 or 580 mg LMG/kg diet (Culp et al., 1999). The NOAEL was 290 mg LMG/kg diet, equivalent to 43.5 mg LMG/kg bw per day.

(b) Rats

In a GLP-compliant study, groups of eight male rats (F344/N Nctr BR) (6–7 weeks of age) were given 0, 290, 580 or 1160 mg LMG/kg diet (equivalent to 0, 29, 58 and 116 mg LMG/kg bw per day) for 28 days. Haematology measurements included leukocyte count, erythrocyte count, haemoglobin, haematocrit, mean erythrocyte volume, mean erythrocyte haemoglobin, mean erythrocyte haemoglobin concentration, platelet count, segmented neutrophils, lymphocytes, monocytes, eosinophils and reticulocyte count. Clinical chemistry measurements included total protein and bile acids, blood urea nitrogen, creatinine, ALT, alkaline phosphatase, AST, glucose, cholesterol, triglycerides, GGT, albumin, sorbitol dehydrogenase, creatine kinase, sodium, potassium, chloride, calcium and phosphorus.

Male rats fed 1160 mg LMG/kg diet had significantly lower body weights (91–92% of the control group) at weeks 2, 3 and 4. There were also significant decreases in body weights in the 580 mg LMG/kg diet group at weeks 3 and 4 (94% of the control group). The ratio of liver weights to body weights was significantly increased for all three dose groups as compared with the control group. GGT levels were 2.2-fold higher ($P < 0.05$) and phosphorus levels were slightly increased (10%; $P < 0.05$) in rats fed 1160 mg LMG/kg diet. In addition, erythrocyte count, haemoglobin and haematocrit levels showed slight (<6%), but significant, decreases from the controls in the 1160 mg/kg diet dose group. Hepatocyte vacuolization, primarily midzonal and centrilobular in location, was seen in seven rats fed 1160 mg LMG/kg diet ($P < 0.005$), five rats fed 580 mg LMG/kg diet ($P < 0.04$) and two rats fed 290 mg LMG/kg diet, a significant dose trend ($P < 0.0004$). Two rats fed 1160 mg LMG/kg diet and two rats fed 580 mg LMG/kg diet had apoptotic follicular epithelial cells in the thyroid gland. Morphological changes consisted of sloughed follicular cells with condensed nuclei located within the follicles. An inflammatory reaction was not present. There was evidence of follicular epithelium regeneration, since even the most severely affected follicles were still lined by viable epithelium (Culp et al., 1999). A NOAEL was not established in this study.

2.2.4 Carcinogenicity of MG

(a) Mice

In a GLP-compliant study, groups of 48 female B6C3F1/Nctr Br (C57BL/6N × C3H/HeN MTV) mice (approximately 6 weeks old) were fed 0, 100, 225 or 450 mg MG/kg diet (as the chloride, 87% pure) (equal to 0, 15, 33 and 67 mg MG/kg bw per day) for 104 weeks. NIH-31 meal and Millipore-filtered tap water were available ad libitum throughout the study, and the animals were maintained on a 12-h light–dark cycle. Homogeneity and stability tests were conducted on the diets to ensure the integrity of the test materials. Food consumption and individual body weights were recorded weekly for the first 12 weeks and approximately every 4 weeks thereafter. Complete necropsies were performed on all mice, including those that died or became moribund. All major tissues were fixed and preserved in 10% neutral buffered formalin. Tissues were processed, trimmed, embedded and stained with haematoxylin and eosin for microscopic examination. There were no treatment-related effects upon food consumption, body weights, survival or incidence of neoplasms in the female mice (Culp et al., 2006).

(b) Rats

In a GLP-compliant study, groups of 48 female F344/N Nctr Br rats were fed 0, 100, 300 or 600 mg MG/kg diet (as the chloride, 87% pure) (equal to 0, 7, 21 and 43 mg MG/kg bw per day) for 104 weeks. NIH-31 meal and Millipore-filtered tap water were available ad libitum throughout the study, and the animals were maintained on a 12-h light–dark cycle. Homogeneity and stability tests were conducted on the diets to ensure the integrity of the test materials. Food consumption and individual body weights were recorded weekly for the first 12 weeks and approximately every 4 weeks thereafter. Complete necropsies were performed on all rats, including those that died or became moribund. All major tissues were fixed and preserved in 10% neutral buffered formalin. Tissues were processed and trimmed, sectioned, embedded and stained with haematoxylin and eosin for microscopic examination.

Food consumption was not affected by treatment. The mean body weights were statistically decreased compared with controls beginning at 16, 44 and 76 weeks in rats fed 600, 300 and 100 mg MG/kg diet. These final body weights were approximately 88%, 90% and 98%, respectively, of control group weight. The survival of female rats was not affected by feeding MG. Female rats fed MG had an increasing trend (0/46, 0/48, 3/47 and 2/46) in the incidence of thyroid gland follicular cell adenoma or carcinoma, with the increase being significant only at 300 mg MG/kg diet. Hepatocellular adenomas were minimally (1/48, 1/48, 3/48 and 4/48), but not statistically significantly, increased at the two highest dose levels. In addition, a non-significant trend (2/48, 2/48, 1/48 and 5/48) in mammary gland carcinoma was observed. There was also a dose-related decreasing trend in the incidence of mononuclear cell leukaemia, with the decrease being significant in all dose groups except for the 100 mg/kg diet group rats (Culp et al., 2006). The NOAEL for non-cancer effects was 100 mg MG/kg diet, equal to 7 mg MG/kg bw per day.

2.2.5 Carcinogenicity of LMG

(a) Mice

In a GLP-compliant study, groups of 48 female B6C3F1/Nctr Br (C57BL/6N × C3H/HeN MTV) mice (approximately 6 weeks old) were fed 0, 91, 204 or 408 mg LMG/kg diet (99% pure) (equal to 0, 15, 31 or 63 mg LMG/kg bw per day) for 104 weeks. NIH-31 meal and Millipore-filtered tap water were available ad libitum throughout the study, and the animals were maintained on a 12-h light–dark cycle. Homogeneity and stability tests were conducted on the diets to ensure the integrity of the test materials. Food consumption and individual body weights were recorded weekly for the first 12 weeks and approximately every 4 weeks thereafter. Complete necropsies were performed on all mice, including those that died or became moribund. All major tissues were fixed and preserved in 10% neutral buffered formalin. Tissues were processed and trimmed, embedded, sectioned and stained with haematoxylin and eosin for microscopic examination.

There were no treatment-related effects upon food consumption, body weights or survival. A dose-related increasing trend (3/47, 6/48, 6/47 and 11/47) in the incidence of hepatocellular adenoma or carcinoma was noted, with the incidence being significant in the highest dose group (Culp et al., 2006).

(b) Rats

In a GLP-compliant study, groups of 48 female and 48 male F344/N Nctr Br rats were fed LMG (99% pure) at 0, 91, 272 or 543 mg/kg diet (equal to 0, 6, 17 and 35 mg LMG/kg bw per day for females and 0, 5, 15 and 30 mg LMG/kg bw per day for males) for 104 weeks. NIH-31 meal and Millipore-filtered tap water were available ad libitum throughout the study, and the animals were maintained on a 12-h light–dark cycle. Homogeneity and stability tests were conducted on the diets to ensure the integrity of the test materials. Food consumption and individual body weights were recorded weekly for the first 12 weeks and approximately every 4 weeks thereafter. Complete necropsies were performed on all rats, including those that died or became moribund. All major tissues were fixed and preserved in 10% neutral buffered formalin. Tissues were processed and trimmed, embedded, sectioned and stained with haematoxylin and eosin for microscopic examination.

Female and male rats fed 543 mg LMG/kg diet consumed less food, intermittently, than control rats; the same observation was noted with female rats fed 272 mg LMG/kg diet. Statistically significant body weight decreases were observed beginning at 8, 16 and 52 weeks in female rats fed 543, 272 or 91 mg LMG/kg diet, with the final body weights being approximately 77%, 90% and 95% of the control group weight, respectively. LMG had a less severe effect on the male rats. Statistically significant decreases were observed beginning at 16, 20 and 88 weeks in male rats fed 543, 272 and 91 mg LMG/kg diet, respectively, with the final body weights being approximately 89%, 93% and 99% of the control group weight, respectively. The survival of female rats was not affected by feeding LMG. The survival of male rats was also not affected by LMG, except for the 272 mg LMG/kg diet group, which had an increased survival. Female and male rats exposed to

0, 91, 272 and 543 mg LMG/kg diet also had a low incidence (0/46, 1/46, 2/47 and 1/48 and 0/47, 2/47, 1/48 and 3/46, respectively) (2–7%) of thyroid gland follicular cell adenoma or carcinoma. Although the increase was not statistically significant, this neoplasm was not detected in control rats. The mammary gland adenoma or carcinoma tumour incidence in the 0, 91, 272 and 543 mg LMG/kg diet group female rats was 0/48, 2/48, 3/48 and 4/48, respectively. Female and male rats had a dose-related decreasing trend in the incidence of mononuclear cell leukaemia, with the decrease being significant in all dose groups. With male rats only, there was a decreasing trend in pituitary gland adenoma, with the decrease being significant at all doses (Culp et al., 2006). The NOAEL for non-cancer end-points was 91 mg LMG/kg diet, equal to 5 mg LMG/kg bw per day.

2.2.6 Genotoxicity

In assays performed in compliance with GLP, the genotoxic and mutagenic effects of MG (Table 2) and LMG (Table 3) were investigated. In vitro tests for genotoxicity of MG are complicated by its strong cytotoxicity towards bacterial and mammalian cells in culture. In one of two experiments, MG (as the oxalate salt) caused mutations in *Salmonella typhimurium* strain TA98 with metabolic activation (S9 mix), but not in any other strains tested or in TA98 without metabolic activation (Clemmensen et al., 1984). MG did not induce mutations in Chinese hamster ovary cells (CHO-K1) and produced DNA damage in the comet assay in CHO-K1 cells only at cytotoxic concentrations (Fessard et al., 1999). MG (as the oxalate salt) did not produce clastogenic effects in the in vivo mouse bone marrow micronucleus test at the maximum tolerated dose of 37.5 mg/kg bw (Clemmensen et al., 1984). However, it was not known whether the test compound had reached the bone marrow. MG did not increase the occurrence of recessive spots when tested in the mammalian spot test (in mice) at doses up to 40 mg/kg bw (Jensen, 1984). MG did not induce micronuclei in erythrocytes, *Hprt* mutations in lymphocytes or (in contrast to LMG) *cII* mutations in liver cells of female Big Blue B6C3F1 transgenic mice administered 450 mg/kg diet (equal to 67.5 mg MG/kg bw per day) for up to 16 weeks (Mittelstaedt et al., 2004). MG gave rise to a single DNA adduct species, the level of which increased linearly with dose in the mouse and rat (Culp et al., 1999).

LMG is much less cytotoxic than MG to bacterial and mammalian cells in vitro and could therefore be tested for mutagenicity at higher concentrations. LMG did not induce mutations in any of the tested *S. typhimurium* strains or in Chinese hamster ovary cells (CHO-K1) and was negative in the comet assay in CHO-K1 cells (Fessard et al., 1999).

LMG did not produce any significant increase in the *lacI* mutation frequencies and changes in the mutation spectrum of *lacI* mutants in female Big Blue rats administered dietary doses of 0, 0.9, 2.7, 9.1, 27.2 or 54.3 mg LMG/kg bw per day for 4, 16 or 32 weeks (Culp et al., 2002). In addition, no effect was observed in the liver *cII* mutation frequency (Mittelstaedt et al., 2004), the *Hprt* lymphocyte mutant assay and the bone marrow micronucleus assay in these rats (Manjanatha et al., 2004). LMG gave rise to a single DNA adduct species, the level of which increased linearly with the dose (Culp et al., 2002).

Table 2. Results of tests for genotoxicity and mutagenicity with MG

Test system	Test object	Concentration	Results	References
In vitro				
Ames test[a]	*S. typhimurium* TA98, TA100, TA1535 and TA1537	0.05–160[b] µg/plate	Negative[c] except for TA98 with activation	Clemmensen et al. (1984)
Ames test[d]	*S. typhimurium* TA97, TA98, TA100 and TA102	0.01–10[b] µg/plate	Negative[e]	Fessard et al. (1999)
Hprt assay[f]	CHO-K1 cells	0.001–1 µg/ml and 1–20 µg/ml[g]	Negative[h]	Fessard et al. (1999)
SCGE (comet) assay	CHO-K1 cells	1–20[i] µg/ml	DNA damage at cytotoxic doses of >3 µg (no activation)[j] and 15 µg (activation)[k]	Fessard et al. (1999)
In vivo				
Micronucleus assay	Mice bone marrow	37.5 mg/kg bw	Negative	Clemmensen et al. (1984)
Mammalian spot test	Mice[l]	10, 20 or 40 mg/kg bw	Negative	Jensen (1984)
Micronucleus	Big Blue B6C3F1 (F) mice[m] peripheral blood	450 mg/kg diet for 4 or 16 weeks	Negative	Mittelstaedt et al. (2004)
Lymphocyte *Hprt* mutant frequency	Big Blue B6C3F1 (F) mice[m] spleen	450 mg/kg diet for 4 or 16 weeks	Negative	Mittelstaedt et al. (2004)
cII mutant frequency	Big Blue B6C3F1 (F) mice[m] liver	450 mg/kg diet for 16 weeks	Negative	Mittelstaedt et al. (2004)
DNA adduct formation	Mice[n]	0, 100 or 600 mg/kg diet for 28 days	Positive, a single DNA adduct formed[o]	Culp et al.(1999)
DNA adduct formation	Rat[p]	0, 100 or 600 mg/kg diet for 28 days	Positive[q]	Culp et al. (1999)

F, female; SCGE, single-cell gel electrophoresis.

[a] Organisation for Economic Co-operation and Development (OECD) Test Guideline, with and without metabolic activation.

Table 2 (contd)

[b] Each dose tested in triplicate, and all assays in duplicate.
[c] Cell toxicity was usually encountered at 1.28 µg/plate unless S9 was added.
[d] OECD Test Guideline 471, with and without metabolic activation.
[e] Cell toxicity above 0.5 µg/plate.
[f] OECD Test Guideline 476, with and without activation.
[g] 1–10 µg/plate without activation and 1–20 µg/plate with activation.
[h] MG was very cytotoxic and could be evaluated only for concentrations up to 0.05 µg/ml.
[i] Each dose was tested in duplicate, and at least two independent assays were performed.
[j] There was a fair relationship between cytotoxicity and DNA lesions.
[k] Cell viability decreased by <20%.
[l] Pregnant C57B1/6J Han mice were treated by gavage with 10, 20 or 40 mg MG/kg bw on days 8, 9 and 10.
[m] Twelve per group.
[n] B6C3F1:Nctr BR (C57BL/6N × C3H/HeN MTV−) female mice, eight per group.
[o] A single adduct or co-eluting adducts with a dose-related response. The mice fed the 600 mg/kg diet had a significantly higher adduct level than the corresponding (580 mg/kg diet) group fed LMG.
[p] F344:N Nctr BR male rats, eight per group.
[q] A single adduct or co-eluting adducts with a dose-related response.

Table 3. Results of tests for genotoxicity and mutagenicity with LMG

Test system	Test object	Concentration	Results	References
In vitro				
Ames test[a]	*S. typhimurium* TA97, TA98, TA100 and TA102	10–2000[b] µg/plate	Negative	Fessard et al. (1999)
Hprt assay[c]	CHO cells	5–100 µg/ml	Negative	Fessard et al. (1999)
SCGE (comet) assay	CHO cells	5–500,[d] 25–300[e] µg/ml	Negative	Fessard et al. (1999)
In vivo				
DNA adduct formation	Mice (F)[f]	0, 96 or 580 mg/kg diet for 28 days	Positive[g]	Culp et al. (1999)
Micronucleus	Big Blue B6C3F1 mice (F)[h] peripheral blood	0, 204 or 408 mg/kg diet for 4 or 16 weeks	Negative	Mittelstaedt et al. (2004)
Lymphocyte *Hprt* mutant frequency	Big Blue B6C3F1 mice (F)[h] spleen	0, 204 or 408 mg/kg diet for 4 or 16 weeks	Negative	Mittelstaedt et al. (2004)
cII mutant frequency	Big Blue B6C3F1 mice (F)[h] liver	0, 204 or 408 mg/kg diet for 16 weeks	Positive[i]	Mittelstaedt et al. (2004)
DNA adduct formation	Rat (M)[f]	0, 96 or 580 mg/kg diet for 28 days	Positive[g]	Culp et al. (1999)

Table 3 (contd)

Test system	Test object	Concentration	Results	References
lacI mutant assay[j]	Big Blue rats (F)[k]	0, 9, 27, 91, 272 or 543 mg/kg diet[l]	Increased mutant frequency only at 16 weeks for 543 mg/kg diet group[m]	Culp et al. (2002)
DNA adduct formation	Big Blue rats (F)[n]	0, 9, 27, 91, 272 or 543 mg/kg diet for 28 days	Positive at ≥91 mg/kg diet[g]	Culp et al. (2002)
Hprt lymphocyte mutant assay[o]	Big Blue rats (F)[k]	0, 9, 27, 91, 272 or 543 mg/kg diet[l]	Negative[p]	Manjanatha et al. (2004)
Bone marrow micronucleus assay	Big Blue rats (F)[k]	0, 9, 27, 91, 272 or 543 mg/kg diet[l]	Negative[q]	Manjanatha et al. (2004)
cII mutant frequency	Big Blue rats (F)[r]	0 or 543 mg/kg diet for 16 weeks	Negative[s]	Mittelstaedt et al. (2004)

F, female; M, male; SCGE, single-cell gel electrophoresis.

[a] OECD Test Guideline 471, with and without metabolic activation.

[b] Precipitated at concentrations of or higher than 500 µg/plate.

[c] OECD Test Guideline 476, with and without activation.

[d] No activation. Each dose was tested in duplicate, and at least two independent assays were performed.

[e] Activation.

[f] Eight per group.

[g] A single adduct or co-eluting adducts with a dose-related increase. The mice fed the 580 mg LMG/kg diet had a significantly lower adduct level than the corresponding group fed the 600 mg MG/kg diet.

[h] Twelve mice per group.

[l] The mutation spectrum in LMG-treated mice revealed an increase of G → T and A → T transversions, the types of mutations typical of those produced by bulky arylamine carcinogens.

[j] This assay measures mutations from base pair substitutions, frameshifts and small deletions.

[k] Six rats per group.

[l] Six-week-old rats were fed the diets for 4, 16 or 32 weeks.

[m] When corrected for clonality, the 16-week *lacI* mutation frequency was not significantly different from the clonally corrected control (Manjanatha et al., 2004).

[n] Four rats per group.

[o] The lymphocytes were obtained from the spleens of the treated animals.

[p] The mutant frequency in the lymphocytes of rats fed the control diet ranged from 3×10^{-6} to 12×10^{-6}, whereas the mutant frequency in rats fed LMG ranged from 2×10^{-6} to 11×10^{-6}.

[q] None of the LMG doses or time points showed a significant increase in Hprt over the appropriate control.

[r] Six per group.

[s] There was no increase in either cell mutant or mutation frequency in Big Blue female rats treated with 543 mg LMG/kg diet.

LMG did not induce micronuclei in erythrocytes or mutations in lymphocytes of female Big Blue B6C3F1 transgenic mice administered 30.6 or 61.2 mg LMG/kg bw per day for 16 weeks. However, LMG induced *cII* mutations with an increased frequency of guanine to thymine (G → T) and adenine to thymine (A → T) transversions in the liver cells of the female mice at the highest dose level (Mittelstaedt et al., 2004).

2.2.7 Reproductive toxicity

In a non-GLP-compliant study, Meyer & Jorgenson (1983) gavaged pregnant New Zealand White rabbits (20 per dose group) with MG (as the oxalate salt, technical grade) at doses of 0, 5, 10 or 20 mg/kg bw per day or with 150 mg thalidomide/kg bw per day from days 6 through 18 of gestation. Improper gavaging resulted in the loss of two rabbits in the 5 and 20 mg MG/kg bw per day groups. All rabbits were observed daily, and body weights were recorded on days 0, 6, 9, 12, 15, 18 and 29, when they were killed and progeny were delivered by caesarean section. Resorption sites were recorded, and all young were examined, weighed and incubated for 24 h. During incubation, pups were examined hourly for viability during the first 4 h and then after 24 h. After 24 h, all progeny were killed, sexed and examined for gross developmental anomalies. Approximately one third were dissected to check for visceral anomalies, and the remainder were examined for skeletal anomalies after staining with alizarin red S.

The rabbits treated with MG consumed less food and had lower body weights than the controls. In the three MG-treated groups, there were significant increases in pre-implantation losses and in the ratio of dead implants and decreases in the number of living fetuses. The mean body weights of the fetuses of the MG-treated groups were less than those of the control group. Viability after 24 h was not consistently affected by the MG treatment. A variety of developmental anomalies were observed in the MG-treated groups; although a dose–response was not evident, they were approximately twice those of the negative control. A NOAEL was not established. The thalidomide positive control group had decreased body weight gain for the does, number of live fetuses, body weight of fetuses and viability, along with an increase in developmental anomalies.

2.2.8 Special studies

(a) Thyroid status parameters

(i) MG

In a GLP-compliant study, groups of eight male and eight female rats (F344/N Nctr BR) (6–7 weeks of age) were fed 0 or 1200 mg MG/kg diet (as the chloride salt; equivalent to 0 or 120 mg MG/kg bw per day) for 4 or 21 days. Blood was collected for triiodothyronine (T_3), thyroxine (T_4) and thyroid stimulating hormone (TSH) analyses. The T_3 levels were significantly higher in female rats fed 1200 mg MG/kg diet as compared with the control group on day 21. The T_4 levels were significantly lower on both days 4 and 21 in the female rats in the 1200 mg MG/kg diet group as compared with the respective control groups. There were no significant changes in T_3 or T_4 levels in males or in the TSH levels in either sex (Culp et al., 1999).

(ii) LMG

Doerge et al. (1998) conducted in vitro studies on the ability of LMG to inhibit thyroid peroxidase (TPO), the enzyme that catalyses the iodination and coupling reactions required for thyroid hormone synthesis. These studies consisted of LMG inhibition of TPO-catalysed tyrosine iodination; LMG inhibition of iodination and coupling in goitre thyroglobulin; and LMG inhibition of TPO-catalysed coupling in preiodinated goitre thyroglobulin. The authors concluded that on chronic exposure, this inhibitory effect could cause thyroid follicular cell tumours through a hormonal mechanism. The authors further speculated that the TPO-catalysed oxidative demethylation of LMG to a primary arylamine could be the basis for a genotoxic mechanism for tumour formation.

In a GLP-compliant study, groups of eight male rats (F344/N Nctr BR) (6–7 weeks of age) were fed 0 or 1160 mg LMG/kg diet (equivalent to 0 or 116 mg LMG/kg bw per day) for 4 or 21 days, and blood was collected for T_3, T_4 and TSH analyses. There was no effect on T_3, but a significant decrease in T_4 and an increase in TSH levels on days 4 and 21 as compared with the respective control groups were noted (Culp et al., 1999).

(b) Cytotoxicity

(i) MG

Stammati et al. (2005) studied the cytotoxicity of MG to two tumour cell lines (Caco-2 and Hep-2). Total protein content and neutral red uptake (NRU) assays were used to assess Hep-2 viability. Proliferation capability was measured by the colony-forming ability test. Dose-dependent results were obtained with both viability assays, with 50% inhibitory concentration (IC_{50}) values of 2.03 µmol/l and 2.19 µmol/l for NRU and total protein content, respectively. From the colony-forming ability assay results, an IC_{50} value of 2.06 µmol/l was calculated. The conversion of yellow tetrazolium salt to the coloured formazan (MTT assay) and the release of cytoplasmic lactate dehydrogenase (LDH leakage) and NRU were used with the Caco-2 cells to assess cytotoxicity. The relative IC_{50} values obtained were 16.2 µmol/l, 18.4 µmol/l and 13.8 µmol/l for MTT, LDH and NRU, respectively.

(ii) LMG

Stammati et al. (2005) studied the cytotoxicity of LMG to two tumour cell lines (Caco-2 and Hep-2). Total protein content and NRU assays were used to assess Hep-2 viability. Proliferation capability was measured by the colony-forming ability test. Only a slight viability inhibition at the highest concentration tested (610 µmol/l) was observed. The results for the colony-forming ability assay were negative at all concentrations tested. The conversion of yellow tetrazolium salt to the coloured formazan (MTT assay) and the release of cytoplasmic LDH (LDH leakage) and NRU were used with the Caco-2 cells to assess cytotoxicity. No signs of cytotoxicity were observed with any of the tests.

(c) Cell transformation

Panandiker et al. (1992) assessed the cytotoxicity and morphological alterations of MG on Syrian hamster embryo (SHE) cells. Cytotoxicity was studied by determining the relative colony-forming efficiency of the MG-treated groups and the controls using logarithmically growing SHE cells. To assess enzyme activities, SHE cells were cultured and treated with MG (0.025–0.1 µg/ml) for 24 h. The cells were then collected, resuspended, sonicated and centrifuged, and the supernatant was used to determine enzyme activities. MG treatment resulted in induction of the mono-oxygenase system (aryl hydrocarbon hydroxylase and aminopyrene-*N*-demethylase activities), lipid peroxidation (superoxide dismutase activity) and catalase activity in a dose-dependent manner. These results were seen by the authors as an indication of the involvement of free radicals in the cytotoxic action of MG.

In continuing their research, Panandiker et al. (1993) used electron spin resonance analysis to show that reactive free radicals were formed during the in vitro (SHE cells) metabolism of MG.

Panandiker et al. (1994) used SHE cells to investigate the effect of MG on free radical formation, lipid peroxidation and DNA damage. SHE cells were cultured with "appropriate concentrations of MG", and procedures were applied to assess these parameters. A dose–response increase was reported for all three parameters. In addition, through the use of antioxidant enzymes (catalase and glutathione peroxidase), the authors concluded that there was an involvement of reactive free radicals in the results observed and that this could be the explanation for the genotoxicity of MG to SHE cells.

The effects of MG on cell cycle phase distribution of normal and MG-transformed SHE cells in asynchronous and synchronous cell populations were investigated by Rao et al. (1998). Treatment with MG induced a dose-dependent G2/M arrest in normal cells, but no such accumulation of cells at the G2/M phase of the cell cycle was observed in the malignantly transformed cells.

Rao et al. (2000) extended this research to show that MG-transformed SHE cells had decreased sensitivity to apoptosis compared with control cells and that an overexpression of altered p53 and bcl-2 may be conferring resistance to MG-induced apoptosis.

Fernandes et al. (1991) compared the effects of MG and phenobarbitone (PB) on the development of preneoplastic lesions during *N*-nitrosodiethylamine (DEN)-induced hepatocarcinogenesis in male Wistar rats. Sixty rats, approximately 3 months old, were randomized and placed in six groups. Group 1 served as control, whereas groups 2, 4 and 6 were given 200 mg DEN/l of tap water for 4 weeks and then DEN-free water for 2 weeks. Groups 4 and 6 were then given water containing 25 mg MG/l (equivalent to 1.88 mg MG/kg bw per day) and 500 mg PB/l tap water, respectively, for 2.5 months. Groups 3 and 5 were given tap water for the first 6 weeks and then 25 mg MG/l tap water and 500 mg PB/l tap water, respectively, for 2.5 months. The effects were monitored on the basis of the morphological appearance of the liver, histological pattern, GGT-positive foci, total GGT activity

and the induction of glycogen-deficient islands. MG and PB were found to enhance liver carcinogenesis to a significant extent when compared with either the corresponding controls or animals given DEN alone. The enhancing effect of MG at 25 mg/l of tap water is comparable with that of PB at 500 mg/l water.

Rao & Fernandes (1996) conducted a second study (10 groups with 10 rats per group) on the dose-dependent tumour promoter effects of MG in Wistar male rats (2 months of age) that were pretreated with DEN. Group 1 was the untreated control, whereas groups 2, 4, 6, 8 and 10 were given tap water containing 200 mg DEN/l for 4 weeks. A 2-week recovery period in which rats were given DEN-free water followed. Groups 4, 6, 8 and 10 were then given drinking-water containing 25, 50 or 100 mg MG/l (equivalent to 1.88, 3.75 and 7.5 mg MG/kg bw per day) or 500 mg PB/l tap water, respectively, for 22 weeks. Groups 3, 5, 7 and 9 were given tap water for an initial 6 weeks and then tap water containing 25, 50 or 100 mg MG/l (equivalent to 1.88, 3.75 or 7.5 mg/kg bw per day) or 500 mg PB/l, respectively, for the remaining 22 weeks. The rats were all killed after 28 weeks, and livers were weighed, pictures were taken for morphology, and samples were prepared for histology, GGT activity measurement and hepatocyte isolation. The isolated hepatocytes were used to measure the rate of hepatocyte DNA synthesis and LDH activity.

The body and liver weights were not affected when only MG or PB was administered. Body weights were lower in the rats in the DEN and 100 mg MG/l or 500 mg PB/l water groups. Body weights of the other dose groups were not affected. Liver to body weight ratios were increased in those rats receiving DEN, DEN and MG, and DEN and PB. Livers from all treated rats exhibited varying degrees of structural and cytological change from the controls. The livers of the DEN-treated rats showed the development of basophilic foci distinguished by a compression of the surrounding parenchyma. The livers of rats treated with only MG showed a dose-dependent incidence of mixed abnormal cells with multiple nuclei and mitotic figures. Liver hyperplasia was observed in the rats treated with PB only. MG promoted DEN-induced preneoplastic lesions to hepatocellular carcinomas in a dose-related manner. Rats treated with DEN as well as PB developed hepatocellular carcinomas. GGT-positive foci were not observed in untreated controls or in those rats treated with MG only. A dose-dependent increase in the size of the GGT-positive foci was observed in the livers from rats treated with DEN plus MG. A significant increase in GGT activity was observed in those rats treated with DEN and MG or PB. MG inhibited DNA synthesis at all concentrations tested. The increase in LDH release from the MG-treated hepatocytes into the culture medium was directly proportional to the inhibitory effect of MG on DNA synthesis.

Further studies on the tumour promotional activity of MG on DEN-induced liver tumours were conducted in the rat by Gupta et al. (2003). In order to understand the mechanism of tumour promotion, hepatic levels of proliferating cell nuclear antigen (a marker of cell proliferation) and the cell cycle regulatory proteins cyclin D1 (and its associated kinase, cdk4) and cyclin B1 (and its associated kinase, cdc2) were measured. The authors concluded that the results obtained provide strong evidence for a link between dysregulation of the two critical checkpoints of the cell cycle as one of the possible mechanisms involved during tumour promotion by MG.

2.3 Observations in humans

A healthy 3-year-old girl weighing 17.3 kg ingested about 57 g of an aquarium product containing 0.075% MG (45 mg). The child was discovered by her father with blue lips and blue nail beds. On arrival at the hospital emergency department, she was awake and crying, with generalized cyanosis, including blue head, hands, arms, feet and legs. Initial vital signs were as follows: heart rate, 115 beats per minute; respirations, 30 per minute; and temperature, 36.8 °C. An initial arterial blood gas was reported as pH 7.43; carbon dioxide partial pressure, 22.9 mmHg (3.1 kPa); oxygen partial pressure, 57.7 mmHg (7.7 kPa); bicarbonate, 15 mmol/l; oxygen saturation, 47.4%; and methaemoglobin, 50.6%. An infusion of methylene blue was begun at 2 mg/kg bw, and the child responded rapidly by becoming pink. The methaemoglobin decreased to 6.5% after 2.5 h. The child was transferred to a paediatric intensive care unit and observed for 20 h, without return of symptoms. The authors suggested that the quantity (45 mg) of MG ingested may have exceeded the capacity of the intestinal flora to reduce MG to LMG, thus leaving sufficient MG to be absorbed in its more active state (Spiller et al., 2008).

3. DIETARY EXPOSURE

Two different sets of residue data were available. The first set consisted of a number of reports on monitoring and surveillance data for MG and LMG. The concentrations found may have resulted from environmental contamination or from illegal uses. Of 3277 samples selected from these reports, 222 samples were reported positive for MG in the range from 0.2 to about 600 µg/kg fish muscle. For many of the results, it is not defined what “malachite green” means (MG, LMG or the sum of both, because the method was inadequate). However, it is possible that some national authorities have more detailed data. The details of these reports, including references, are given in the residue monograph on MG published by the Food and Agriculture Organization of the United Nations (FAO) (Annex 1, reference *195*). Estimates of dietary exposure from these data are given in section 4.3.

In the open literature, well conducted residue studies suitable to predict the concentration–time course of residues of MG in fish are available for only two species, the rainbow trout and the channel catfish. Only for trout were sufficient individual animal data available to perform a statistical evaluation. The second set of data was taken from a well conducted study using MG under realistic conditions of use in trout of a size ready for human consumption (Law, 1994). The data and the results of the assessment are described in section 4.3. A discussion of the individual studies, including references, is provided in the residue monograph on MG published by FAO (Annex 1, reference *195*).

4. COMMENTS

4.1 Biochemical data

There is only limited information available on the absorption, distribution, metabolism and excretion of MG in mammalian species, including humans. In male

and female rats given an oral dose of 2 mg ^{14}C-labelled MG/kg bw, more than 95% of the radioactivity was excreted within 7 days, with more than 80% appearing in the faeces. No attempt was made to identify any metabolites. In vitro studies have shown that MG is readily converted into LMG under anaerobic conditions by a multitude of bacterial species present in the intestinal microflora from mice, rats, rhesus monkeys and humans. Therefore, MG that is ingested can be converted to LMG by the intestinal microflora.

In short-term feeding studies, it was shown that MG is sequentially *N*-demethylated to secondary and primary aromatic amines in rats and mice both before and after reduction to LMG. Analysis of liver extracts from rats treated with MG by HPLC-APCI/MS identified MG, its mono-, di-, tri- and tetradesmethyl derivatives and MG *N*-oxide. A small, but measurable, amount of LMG was also detected. Concentrations of MG and metabolites increased with increasing dose. Similarly, LMG, demethylated derivatives, MG *N*-oxide and demethylated *N*-oxide derivatives were detected in liver extracts from rats treated with LMG. A small, but measurable, amount of MG was also detected.

MG, in contrast to LMG, is highly cytotoxic to bacteria and mammalian cells in vitro. Studies using mammalian cells showed that MG treatment resulted in induction of the CYP mono-oxygenase system, lipid peroxidation and catalase activity in a concentration-dependent manner.

4.2 Toxicological data

4.2.1 Malachite green

In short-term (28 days) feeding studies in rats with doses ranging from 1 to 120 mg MG/kg bw per day, MG produced haematological changes (decreased erythrocyte count and haemoglobin and haematocrit values) and increased the relative liver weight in both sexes. This effect was accompanied by increased levels of GGT activity in the blood. Vacuolization of hepatocytes, primarily midzonal and centrilobular, was observed at the highest dose level tested. The NOAEL was 10 mg MG/kg bw per day based on haematological changes and effects on the liver, both of which were seen at 30 mg MG/kg bw per day and higher dose levels.

When rats were dosed with 120 mg MG/kg bw per day for 21 days, there was a significant increase in the T_3 levels and a significant decrease in the T_4 levels in the females. TSH was not affected, and no effects were seen in the males.

In a study using male Wistar rats pretreated for 4 weeks with DEN, it was shown that administration of MG in the drinking-water (resulting in daily doses ranging from 1.88 to 7.5 mg/kg bw per day) for 7 months dose-dependently enhanced the DEN-induced liver carcinogenicity to a significant extent. Increases in the relative liver weights and biochemical markers for hepatic preneoplastic lesions and cell cycle regulatory proteins were noted. A subsequent biochemical study confirmed that a number of markers for liver cell proliferation and cell cycle regulation were altered. The results provided evidence for dysregulation of checkpoints of the cell cycle as a possible mechanism during promotion of hepatic preneoplastic lesions by MG.

In a teratogenicity study in rabbits, MG (0, 5, 10 or 20 mg MG/kg bw per day from gestation days 6 to 18) was reported to produce a dose-related decrease in weight gain or marked weight loss in the dams and increased incidences of fetal anomalies (gross, visceral and skeletal) in all treated groups. A NOAEL could not be established. The lack of consistent dose–response relationships in most of the effects seen in the pups is noteworthy, but might be due to the very narrow dose range studied. The study was inadequately conducted and reported, and additional studies would be needed to properly address the potential of MG to produce reproductive and developmental toxicity.

Administration of MG and its major metabolite LMG at doses up to 60 and 58 mg/kg bw per day, respectively, for 28 days gave rise to a single liver DNA adduct species (or co-eluting adducts) in male F344 rats. Females were not studied. The adduct level increased significantly as a function of the dose and did not differ between groups administered equimolar doses of MG and LMG. In female B6C3F1 mice, doses up to 90 mg/kg bw per day (600 mg/kg diet) and 87 mg/kg bw per day (580 mg/kg diet) for 28 days for MG and LMG, respectively, also gave rise to a single DNA adduct species (or co-eluting adducts), the level of which increased as a function of the dose for MG, but only minimally and borderline for LMG. Thus, in the female mouse, MG produced much higher adduct levels than LMG at equimolar doses. In contrast, in the female Big Blue F344 transgenic rat, LMG at dose levels up to 54.3 mg/kg bw per day for up to 32 weeks gave rise to a single DNA adduct species, the level of which increased linearly with the dose.

MG did not produce mutations in the Ames test using *Salmonella typhimurium* strains or in Chinese hamster ovary cells (CHO-K1) and produced DNA damage in the comet assay in CHO-K1 cells only at cytotoxic concentrations. It did not produce a clastogenic effect in the in vivo mouse bone marrow micronucleus test and did not increase the occurrence of recessive mutations when tested in the mammalian spot test in mice. In addition, MG did not induce micronuclei in erythrocytes, mutations in lymphocytes or (in contrast to LMG) *cII* mutations in liver cells of female Big Blue B6C3F1 transgenic mice administered 67.5 mg MG/kg bw per day for 16 weeks.

It is concluded that although MG DNA adducts have been identified in the liver of male rats and female mice, the weight of evidence indicates that MG has no genotoxic potential in conventional in vitro and in vivo assays and did not produce mutations in the liver of transgenic female mice at the dose levels applied.

MG was tested for carcinogenicity in female F344 rats fed diets corresponding to daily intakes of 0, 7, 21 or 43 mg/kg bw per day for 2 years. A reduced body weight gain was observed at the two highest dose levels. There was a trend in increased occurrence of thyroid gland follicular cell adenoma or carcinoma, being statistically significant only at the middle dose level. Hepatocellular adenomas were minimally (but not statistically significantly) increased at the two highest dose levels, and there was a non-significant trend in the incidence of mammary gland carcinomas in the treated animals (see Table 4). The NOAEL for non-cancer effects was 7 mg MG/kg bw per day based on the reduced body weight gain at the two highest dose levels.

Table 4. Tumours in F344 female rats administered MG (0, 7, 21 or 43 mg/kg bw per day) for 2 years

Tumour type	Tissue toxicity	DNA adducts	Genotoxicity/ mutagenicity	Other effects
Liver adenoma 1/48, 1/48, 3/48, 4/48 Historical control: 1 adenoma in 6 studies	Eosinophilic foci 5/48, 10/48, 13/48, 14/48 Necrosis at highest dose Increased relative liver weight	A single liver DNA adduct species in male rats and female mice	Negative in conventional tests and transgenic female mice Positive in comet assay in CHO cells at cytotoxic concentrations	Promotor of DEN-initiated liver tumours Decreased body weight
Thyroid adenoma and carcinoma 0/46, 0/48, 3/47, 2/46 Historical control: NCTR 1.4% and NTP 0.9%	Cystic follicles 0/46, 1/48, 1/47, 3/46 Thyroid weight was not affected			120 mg/kg bw per day: $T_3 \uparrow$, $T_4 \downarrow$ Decreased body weight
Mammary carcinoma 2/48, 2/48, 1/48, 5/48 Historical control 0.7%				Decreased body weight

NCTR, National Center for Toxicological Research (USA); NTP, National Toxicology Program (USA).

MG was also tested for carcinogenicity in female mice. No increases in tumour incidences were seen in female mice fed diets corresponding to daily intakes of 15, 33 or 67 mg MG/kg bw per day for 104 weeks. No effects on mortality or body weight gain were observed.

4.2.2 Leucomalachite green

LMG was tested in short-term (28 days) feeding studies in female B6C3F1 mice at doses ranging from 43.5 to 174 mg/kg bw per day and in male F344 rats at doses ranging from 29 to 116 mg/kg bw per day. The NOAEL in female mice was 43.5 mg LMG/kg bw per day based on reduced body weights seen at higher dose levels. All females at the highest dose level had scattered dead or degenerate cells in the transitional epithelium of the urinary bladder. When LMG was tested in male rats, increased relative liver weights were reported for all dose groups, and the animals in the two highest dose groups had significantly lower body weights. At the highest dose level, GGT activity and phosphorus levels were significantly increased, whereas the erythrocyte count, haemoglobin and haematocrit levels were significantly decreased. Vacuolization of hepatocytes, primarily midzonal and

centrilobular, was observed at all dose levels. Apoptotic follicular epithelial cells in the thyroid gland were seen in some rats at the two highest dose levels. A NOAEL could not be established in this study.

In male rats given 116 mg LMG/kg bw per day for 21 days, there was a significant increase in the serum TSH levels and a significant decrease in the T_4 levels.

LMG did not induce mutations in any of the tested *S. typhimurium* strains and in Chinese hamster ovary cells (CHO-K1) and was negative for DNA damage in the comet assay in CHO-K1 cells. It did not produce any significant increase in the *lacI* mutation frequencies or changes in the mutation spectrum of *lacI* mutants in female Big Blue rats administered dietary doses up to 54.3 mg LMG/kg bw per day for up to 32 weeks. In addition, no effect was observed in the liver *cII* mutation frequency, the *Hprt* lymphocyte mutant assay or the bone marrow micronucleus assay in these rats.

LMG did not induce micronuclei in erythrocytes or mutations in lymphocytes of female Big Blue B6C3F1 transgenic mice administered 0, 30.6 or 61.2 mg LMG/kg bw per day (0, 204 and 408 mg/kg diet) for 16 weeks. However, LMG induced *cII* mutations with an increased frequency of G → T and A → T transversions in the liver cells of the female mice at the highest dose level tested (61.2 mg LMG/kg bw per day).

LMG was tested for carcinogenicity in male and female rats fed diets corresponding to dose levels of 0, 5, 15 or 30 mg/kg bw per day or 0, 6, 17 or 35 mg/kg bw per day, respectively, for 2 years. Mortality was not affected. Reduced body weight gains were observed at the two highest dose levels in both sexes. A low, not statistically significant increase in the incidence of thyroid gland follicular cell adenoma or carcinoma (2–7%) was seen in both sexes. There was no significant effect on the incidence of hepatocellular adenomas in the rats fed LMG. The mammary gland adenoma or carcinoma incidence in female rats was 0/48, 2/48, 3/48 and 4/48 at 0, 6, 17 and 35 mg/kg bw per day, respectively. The NOAEL for non-cancer effects was 5 mg/kg bw per day based on the reduced body weight gains seen at the higher dose levels.

In female mice fed diets corresponding to intakes of 0, 15, 31 and 63 mg LMG/kg bw per day for 104 weeks, the only finding was a dose-related trend in increased incidence of hepatocellular adenomas or carcinomas (3/47, 6/48, 6/47 and 11/47, respectively), with the incidence being statistically significant in the highest dose group (Table 5).

Table 5. Tumours in B6C3F1 female mice administered LMG (0, 15, 31 or 63 mg/kg bw per day) for 2 years

Tumour type	Tissue toxicity	DNA adducts	Genotoxicity/ mutagenicity
Liver adenoma and carcinoma 3/47, 6/48, 6/47, 11/47	No toxicity reported	Liver DNA adduct species only borderline in female mice	Negative in conventional in vitro and in vivo tests and in female transgenic rats

Table 5 (contd)

Tumour type	Tissue toxicity	DNA adducts	Genotoxicity/mutagenicity
		A single liver DNA adduct species seen in male and female rats	Induced *cII* mutations in transgenic female mice

4.2.3 Consideration of mode of action for MG and LMG

The incidences of tumours in female rats administered MG for 2 years and of tumours in female mice administered LMG for 2 years are shown in Table 4 and Table 5, respectively, together with findings related to the possible mode of action.

Both MG and LMG caused a weak, statistically non-significant increase in the occurrence of thyroid gland follicular cell adenoma and carcinoma in rats. Short-term (28 days) studies showed an increase in T_3 and a decrease in T_4 after MG treatment in female rats, and LMG treatment resulted in an increase in TSH and a decrease in T_4 levels. Mechanistic studies in vitro with LMG showed an inhibition in thyroid peroxidase–catalysed tyrosine iodination. This would support the view that chronic exposure to MG and LMG could result in thyroid gland follicular cell tumours through a hormonal, thresholded mechanism.

The tumour data supporting a treatment-related effect for MG and LMG in the induction of liver carcinogenicity in rats are not strong, with only the female rats treated with the two highest doses of MG having a minimally increased (not statistically significant) occurrence of hepatocellular adenomas. Although a dose-dependent formation of a DNA adduct species was demonstrated in the livers of F344 and Big Blue rats administered MG or LMG in the diet, analyses of liver *lacI* and *cII* mutants revealed that the mutant frequencies in LMG-treated rats were similar to those of control rats and that the majority of the independent mutations in treated rats were base pair substitutions, with a mutation spectrum similar to that found for control rats. In addition, MG did not induce *cII* mutations in female Big Blue mice. These data suggest that, in the rat, MG might be promoting spontaneous lesions in a manner similar to that reported in the studies on initiation/promotion of hepatic preneoplastic lesions and tumours in male Wistar rats using DEN as an initiator and MG as a promoter.

However, in female mice fed diets containing LMG for 104 weeks, a dose-related trend in increased incidence of hepatocellular adenomas or carcinomas was reported, with the incidence being statistically significant in the highest dose group. Although hepatocellular adenomas and carcinomas in mice often are not considered to originate from a DNA-reactive mechanism when they are the only induced tumour form, the induction of *cII* mutations in liver cells by a high dose of LMG in female transgenic mice indicates that a genotoxic mechanism cannot be ruled out. Importantly, the mutation spectrum of *cII* mutations was different from that of the control mice, with a notable increase in G → T and A → T transversions. A number of studies have shown that mutagenic aromatic amines produce predominantly such transversions in Big Blue rodents.

The Committee noted that LMG produced DNA adduct species, the levels of which increased significantly with dose, in the liver of male F344 and female Big Blue rats, but had no carcinogenic effect in the liver of male and female F344 rats. In contrast, in the liver of female B6C3F1 mice, where LMG induced mutations and tumours, the level of DNA adducts was borderline. This suggests that the DNA adduct species formed after treatment with MG and LMG is of low mutagenic and carcinogenic potential. In addition, the induction of *cII* mutations by LMG in the Big Blue mouse appears to be tissue specific, as tumours developed only in the liver of B6C3F1 mice.

4.3 Preliminary dietary exposure considerations

The Committee performed preliminary dietary exposure assessments on the basis of two different sets of residue data. The first set represented results of monitoring and surveillance data, and the concentrations found may have resulted from environmental contamination or from illegal uses. The second set of data was taken from a well conducted study using MG under realistic conditions of use in fish of a size ready for human consumption.

For the first approach, the Committee examined a number of reports on monitoring and surveillance data for MG and LMG. Of 3277 samples selected from these reports, 222 samples were reported positive for MG in the range from 0.2 to about 600 µg/kg fish muscle. For many of the results, it is not defined what "malachite green" means (MG, LMG or the sum of both), because the method was inadequate. However, it is possible that some national authorities have more detailed data. The details of these reports, including references, are given in the residue monograph on MG published by FAO (Annex 1, reference *195*).

Only a subset of the above data had been systematically collected. These were monitoring data (spanning from 1995 to 2006) published in the United Kingdom on the occurrence of MG and LMG in fish muscle. If both substances were found in a sample, the Committee calculated the sum. The Committee estimated the mean level in the positive samples to be 30.7 µg/kg fish muscle and the level at the 97.5th percentile to be 138 µg/kg. Assuming the daily consumption of fish to be 300 g/person, the daily exposure to the sum of MG and LMG can be calculated to be 9.2 and 41 µg/person at the mean and 97.5th percentile, respectively. For a 60-kg person, this would be equivalent to 0.15 µg/kg bw per day and 0.69 µg/kg bw per day, respectively.

In the open literature, well conducted residue studies suitable to predict the concentration–time course of residues of MG in fish are available for only two species, the rainbow trout and the channel catfish. Only for trout were sufficient individual animal data available to perform a statistical evaluation. The data and the results of the assessment are described below. A discussion of the individual studies, including references, is provided in the residue monograph on MG published by FAO (Annex 1, reference *195*).

A study investigating the metabolite profiles and residues of MG in trout tissues was conducted in trout kept in tanks under the following conditions: water temperature, 10 ± 2 °C; pH 6.0–7.0; hardness, 5–10 mg/l; and dissolved oxygen, 9 ± 2 mg/l. All experiments and analytical work were carried out under reduced-intensity room light. Concentrations in the exposure tanks were maintained at

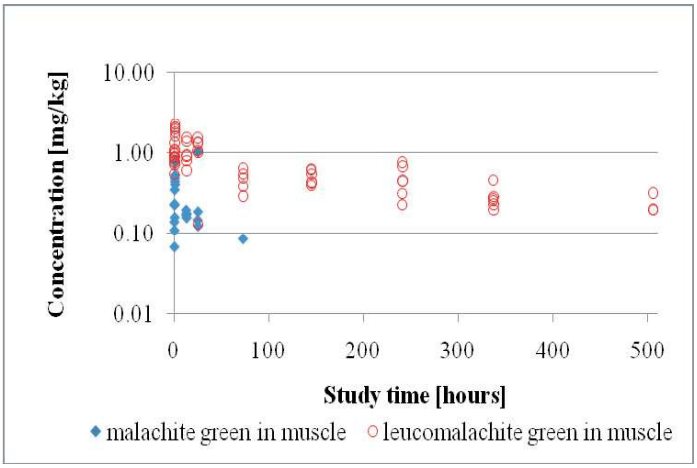
2 mg/l by a metering apparatus, using ^{14}C-labelled MG (radiochemical purity 98%) stock solution at 800 mg/l and delivering 10 ml/min of this solution to the tank.

Seventy-two randomly selected trout, each weighing about 350 g, were divided into three groups of 24 fish and put into three 200-l continuous-flow exposure tanks containing 2.0 mg ^{14}C-labelled MG/l (actual concentrations 1.84 ± 0.22 mg/l, 1.91 ± 0.29 mg/l and 1.88 ± 0.19 mg/l, respectively). A water sample (5 ml) was withdrawn from the exposure tanks every 15 min during the ^{14}C-labelled MG exposure period. After a 1-h exposure, the fish were removed to a depuration tank containing flowing, uncontaminated water. At specific time intervals during ^{14}C-labelled MG exposure and depuration, two to three trout were removed randomly from each group of fish and killed. The concentrations of total radioactive residue in tissue homogenates and the ratio of MG and LMG concentrations in an organic extract were determined. These data were used by the Committee to calculate the concentrations of MG and LMG in the tissues for the intake assessment.

The highest concentrations of residues were found in liver and kidney; however, for the intake estimates, muscle was used. In skin, there were also significant concentrations of residues; however, concentrations were not calculated for muscle and skin in natural proportions, since the concentrations in muscle were higher than those found in skin, and therefore the approach using the muscle data was slightly more conservative.

The kinetic data representing the concentrations of MG and LMG over the entire study time from the beginning of treatment until the end of the experiment at 505 h are given in Figure 2.

Figure 2. Concentration–time curves of MG and LMG in muscle of trout



The data representing the time period between the end of the treatment and 505 h were subjected to statistical analysis using one exponential term on the basis

Table 6. Parameters of the linear regression analysis of kinetic residue depletion data in trout muscle

Parameter	MG	LMG
Intercept	−0.99747	0.01994
Slope	−0.02461	−0.00352
Coefficient of correlation	−0.60012	−0.73361
Residual variance	0.58312	0.52876

of the natural logarithms of the residue contents. The parameters given in Table 6 were obtained by linear regression.

Depletion half-lives of 28 h for MG and 197 h for LMG were determined. The kinetic parameters, including the variance of the data, were used to calculate model intakes for every day of 80 years of a human lifespan, assuming daily consumption of 300 g of fish muscle. For this purpose, 29 220 approximately log-normally distributed random numbers were generated for each time point of interest, ranging from the predicted value of the regression line minus 4 times the residual variance to the same predicted value plus 4 times the residual variance. These calculations were repeated for a number of assumed slaughter times of the fish, ranging from 1 h (end of treatment) to 500 h. The results were expressed in mg MG (LMG)/kg of human body weight. The minima, maxima and several percentiles, including the median of these estimated daily intakes, were calculated. The median was used for an assessment of chronic intake. The median daily intake of LMG ranged from 7.3 µg/kg bw at 1 h to 0.87 µg/kg bw at 500 h (Table 7).

The Committee considered that the assumption of consumption of 300 g of fish contaminated with MG and LMG every day for a lifetime is a highly conservative assumption. In addition, it was assumed that the concentrations of MG and LMG would not change during cooking of the fish.

5. EVALUATION

The Committee first addressed the question of the use of MG for food-producing animals. There are no conventional studies available on the absorption, distribution, metabolism and excretion of MG in mammalian species. Although the available short- and long-term studies point to a NOAEL in the order of 10 mg/kg bw per day, the study on teratogenicity in rabbits, albeit of low quality, raises concern regarding the potential developmental toxicity of MG. Because a NOAEL could not be identified, additional studies would be needed to properly address the potential reproductive and developmental hazards of MG. In addition, following ingestion, MG is expected to be extensively reduced to LMG, primarily by the gastrointestinal microflora, before absorption, and it cannot be ruled out that LMG, the major metabolite of MG, induces hepatocellular adenomas and carcinomas in female mice via a mutagenic mode of action. Based on these considerations, the Committee considered it inappropriate to establish an ADI for MG. Therefore, the use of MG for food-producing animals cannot be supported.

Table 7. Results of an intake assessment for malachite green and leucomalachite green

	Intake (µg/kg bw per day) at various theoretical slaughter times of fish (h)														
	1.0	1.6	2.4	3.8	5.9	9.2	14.3	22.4	34.9	54.3	84.7	132.0	205.8	320.8	500.0
MG															
Lowest intake	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0
Median intake	1.8	1.8	1.7	1.7	1.6	1.5	1.3	1.1	0.8	0.5	0.2	0.1	0.0	0.0	0.0
P90	3.8	3.8	3.7	3.6	3.4	3.1	2.8	2.3	1.6	1.0	0.5	0.2	0.0	0.0	0.0
P95	4.7	4.7	4.5	4.4	4.2	3.8	3.4	2.8	2.0	1.3	0.6	0.2	0.0	0.0	0.0
P97.5	5.6	5.6	5.4	5.2	5.0	4.6	4.0	3.3	2.4	1.5	0.7	0.2	0.0	0.0	0.0
P99	6.9	6.9	6.6	6.5	6.1	5.7	5.0	4.1	2.9	1.8	0.9	0.3	0.0	0.0	0.0
Highest intake	15.6	16.2	21.1	15.2	14.2	14.1	11.8	9.8	10.4	3.8	2.0	0.6	0.1	0.0	0.0
MOE for median intake	11 128	11 225	11 540	11 940	12 609	13 664	15 426	18 921	25 713	41 351	86 780	279 316	1 715 561	29 143 431	2 364 965 091
LMG															
Lowest intake	1.5	1.4	1.5	1.3	1.5	1.1	1.3	1.2	0.9	0.8	0.8	0.5	0.3	0.3	0.1
Median intake	7.3	7.3	7.2	7.1	6.9	6.7	6.4	6.0	5.5	4.8	4.0	3.3	2.5	1.7	0.9
P90	12.6	12.6	12.3	12.2	12.0	11.7	11.3	10.7	10.0	8.9	7.8	6.4	4.8	3.3	1.7
P95	14.7	14.7	14.5	14.6	14.1	13.8	13.3	12.6	11.7	10.7	9.3	7.7	5.8	4.0	2.1
P97.5	16.9	17.0	16.6	16.8	16.4	15.8	15.3	14.5	13.5	12.5	11.0	9.1	6.9	4.6	2.4

Table 7 (contd)

	Intake (µg/kg bw per day) at various theoretical slaughter times of fish (h)														
P99	19.7	19.5	19.6	19.7	19.1	18.9	18.3	17.3	16.0	15.0	13.4	10.9	8.3	5.6	3.0
Highest intake	48.5	42.4	36.0	39.7	36.2	34.6	38.0	38.9	31.5	32.0	30.3	22.6	16.6	14.3	6.4
MOE for median intake	2 750	2 746	2 792	2 832	2 902	2 976	3 108	3 337	3 653	4 159	4 958	6 071	8 076	12 095	22 891
Sum															
Lowest intake	1.7	1.6	1.7	1.4	1.7	1.3	1.4	1.3	1.0	0.8	0.8	0.5	0.3	0.3	0.1
Median intake	9.1	9.1	8.9	8.7	8.5	8.2	7.7	7.1	6.3	5.3	4.3	3.4	2.5	1.7	0.9
P90	16.4	16.4	16.0	15.8	15.4	14.8	14.1	12.9	11.6	9.9	8.3	6.6	4.9	3.3	1.7
P95	19.4	19.4	19.0	19.0	18.3	17.6	16.7	15.4	13.8	12.0	9.9	7.9	5.9	4.0	2.1
P97.5	22.5	22.5	22.0	22.0	21.4	20.4	19.3	17.8	15.9	14.0	11.7	9.3	7.0	4.6	2.4
P99	26.7	26.4	26.2	26.3	25.2	24.6	23.3	21.4	18.9	16.8	14.3	11.2	8.4	5.6	3.0
Highest intake	64.1	58.6	57.1	54.9	50.4	48.8	49.8	48.7	42.0	35.8	32.3	23.2	16.7	14.3	6.4
MOE for median intake	2 205	2 206	2 248	2 289	2 359	2 444	2 587	2 836	3 198	3 779	4 690	5 942	8 038	12 090	22 891

MOE, margin of exposure; P, percentile.

The Committee thereafter evaluated the safety of residues of MG and LMG in fish as a result of industrial use and other sources of contamination. The Committee considered whether it could establish a margin of exposure (MOE) for non-cancer end-points. In view of the deficient database, the Committee considered it inappropriate to derive an MOE for non-cancer end-points for MG and LMG.

In the evaluation of exposure to genotoxic and carcinogenic residues, the Committee considered the induction of hepatocellular adenomas or carcinomas in female mice treated with LMG to be the pivotal effect for the risk assessment of MG and LMG as contaminants in food. Because there is no information on the conversion rate of MG to LMG in food, the Committee considered it prudent to evaluate the sum of MG and LMG in food expressed as LMG.

For substances that are genotoxic and carcinogenic, JECFA (contaminants) has suggested using an MOE approach in the risk assessment of unintentional contaminants (Annex 1, reference *176*). The present Committee agreed to this approach. The MOE is the ratio between a defined reference point on the dose–response curve for the adverse effect and the human intake of the substance. As a reference point from the dose–response curve for the pivotal adverse effect, it was suggested that the $BMDL_{10}$, which is the lower limit of a one-sided 95% confidence interval on the benchmark dose (BMDL) calculated for a benchmark response of 10% incidence above the modelled background incidence, be used.

The United States Environmental Protection Agency's benchmark dose (BMD) software (BMDS) version 1.4.1 was used for modelling the liver tumour dose–response (hepatocellular adenomas and carcinomas combined) in the LMG-treated female mice. The following dose–response models were fitted to the dose–incidence data: gamma, logistic, log-logistic, multistage, probit, log probit, quantal linear and Weibull models (Table 8). The BMD and BMDL values for an extra 10% risk compared with the modelled background incidence (BMD_{10} and $BMDL_{10}$) were estimated by performing 250 iterations.

The BMD_{10} values from the accepted models ranged from 33.5 to 43.1 mg LMG/kg bw per day, and the $BMDL_{10}$ values ranged from 18.5 to 31.2 mg LMG/kg bw per day. In order to be prudent, the Committee decided to use the more conservative lower end of this range of values for the evaluation and, to simplify the calculation, chose a $BMDL_{10}$ value of 20 mg LMG/kg bw per day as the reference point for the MOE calculation.

Assuming a daily consumption of 300 g of fish contaminated with MG and LMG, the estimated exposure to the sum of MG and LMG for a 60-kg person was 0.15 µg/kg bw per day, expressed as LMG, for the average intake and 0.69 µg/kg bw per day for the high (97.5th percentile) intake. Comparison of these mean and high-level exposures with the $BMDL_{10}$ of 20 mg/kg bw per day indicates MOEs of about 130 000 and 30 000, respectively. JECFA has previously, at its sixty-fourth meeting (Annex 1, reference *176*), considered MOEs of 10 000 or higher for unintended contaminants (polycyclic aromatic hydrocarbons and ethyl carbamate from food, excluding alcoholic beverages) to be of low concern for human health.

Table 8. BMD_{10} and $BMDL_{10}$ calculations for LMG based on the incidences of liver adenomas and carcinomas in female mice (Culp et al., 2006)

Model	Log likelihood (parameters)	*P*-value	AIC	Chi-square	*P*-value	Accept	BMD_{10} (mg/kg bw per day)	$BMDL_{10}$ (mg/kg bw per day)
Full model	−72.77 (4)							
Gamma multi-hit	−72.94 (3)	0.842	149.9	0.34	0.842	Yes	35.4	20.1
Logistic	−72.97 (2)	0.814	149.9	0.42	0.810	Yes	43.1	31.2
Log-logistic	−72.94 (3)	0.549	151.9	0.36	0.551	Yes	34.6	18.5
Multistage	−72.93 (3)	0.561	151.9	0.34	0.560	Yes	36.8	20.1
Probit	−72.96 (2)	0.882	149.9	0.40	0.818	Yes	41.9	29.5
Log-probit	−72.97 (3)	0.522	151.9	0.40	0.525	No	33.5	<0
Quantal linear	−72.94 (2)	0.842	149.9	0.34	0.842	Yes	35.4	20.1
Weibull	−72.94 (3)	0.558	151.9	0.34	0.560	No	34.8	<0
Reduced model	−75.70 (1)	0.118						

AIC, Akaike information criterion.

The Committee also performed preliminary estimates of the potential exposures in the case that MG had been used to treat fish in aquaculture and the fish had been subjected to various depletion times. For the median intake, the sum of MG and LMG was about 9 µg/kg bw per day at the end of the 1-h treatment period and about 1 µg/kg bw per day after a depletion time of 500 h. Comparison of these exposure levels with the $BMDL_{10}$ of 20 mg/kg bw per day indicates MOEs of about 2000 and 20 000, respectively. The 97.5th percentile intakes were about 23 and 2 µg/kg bw per day at the end of the 1-h treatment period and after a depletion time of 500 h, respectively, providing MOEs of about 900 and 10 000 (see Table 7).

The current Committee noted the conclusion at the sixty-fourth JECFA (Annex 1, reference *176*) and agreed that MOEs of less than 10 000 for genotoxic and carcinogenic contaminants indicate a health concern.

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