

**COMMITTEE ON CARCINOGENICITY OF CHEMICALS IN FOOD,
CONSUMER PRODUCTS AND THE ENVIRONMENT**

**MALACHITE GREEN AND
LEUCOMALACHITE GREEN**

Introduction

1. Malachite green (MG) is a cationic triphenylmethane dyestuff that has been used in freshwater fisheries for the treatment of external fungal and other infections and in particular to stop fungal growth on the eggs. In June 2002 the use of MG in fish for human consumption was banned within the EU; however residues of MG, and of leucomalachite green (LMG) its lipophilic metabolite, continue to be found in UK samples of domestic and imported farmed fish. Information on the structure and properties of MG and LMG is given in Annex A. At the request of the COT, the COM provided advice on the mutagenicity of malachite green, and its lipophilic metabolite leucomalachite green (LMG), in 1999.
2. The Committee commented on the paucity of the available mutagenicity data, particularly in respect of LMG. In many instances lack of information on the purity of the MG tested was a particular problem in assessing the adequacy of the available information. Members agreed that the structure of both MG and LMG contained groupings which provide structural alerts for potential genotoxicity and thus it was important to evaluate the potential mutagenicity of both MG and its lipophilic metabolite LMG. Members also noted the results from the 1997 surveillance investigations conducted for the Veterinary Medicines Directorate which, for the first time, present information on residues of LMG and suggest that, when found, residues of this chemical in fish were higher than those of MG. On the basis of the limited data available at that time the COM felt that it would be prudent to assume that both MG and LMG were potential in-vivo mutagens.
3. The NTP have now published a report on the toxicology and carcinogenesis studies on MG chloride and LMG in rats and mice⁽⁵⁾. The peer review of this was carried out in February 2004, and few changes were made with respect to the conclusions of the carcinogenicity bioassays. The NTP report refers to a number of new mutagenicity studies, most of which have now been published. The COM was asked to consider these data, and to update their conclusions on the mutagenicity of MG and LMG at their meeting in May 2004.

Advice from COC

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4. The advice of the COC is sought on the carcinogenicity of MG and LMG based on the results of the recent NTP bioassays, and in light of the COM conclusions regarding the mutagenicity of these compounds.

Mutagenicity of MG and LMG

5. In May 2004 the COM drew the following conclusions regarding the mutagenicity of MG and LMG:

Malachite green

- (i) There is one report of a *Salmonella* assay done to an acceptable protocol; MG oxalate (>90% pure) was shown to induce mutations in TA98 in the presence of an exogenous metabolic activation system.⁽¹⁾ There is also some evidence of clastogenicity in Chinese Hamster Lung cells.⁽²⁾ MG has also been shown to produce DNA damage in CHO cells using the Comet assay.⁽⁷⁾ MG should thus be regarded as having mutagenic potential.
- (ii) Negative results were obtained in a poorly reported bone marrow micronucleus test in mice using a single oral dose of malachite green oxalate (>90% pure) at the MTD (75% of the LD50).⁽¹⁾ Members agreed that the high dose level of 37.5 mg/kg was adequate, but it was difficult to assess the value of the negative results in the absence of appropriate information on bone marrow toxicity or data to show that malachite green and/or metabolites reached the bone marrow.
- (iii) ³²P post-labelling studies using a 28 day dietary exposure have indicated that MG induces DNA adduct(s) in the liver of both F344 rats and B6C3F₁ mice.⁽⁸⁾ This has been confirmed in a separate study using 16 weeks dietary exposure.⁽⁵⁾ MG did not induce micronuclei in peripheral lymphocytes nor Hprt mutations in splenic lymphocytes in the 28 day study.
- (iv) In view of the demonstration of DNA adduct formation in samples from both rats and mice, MG should be regarded as an in-vivo mutagen.

Leucomalachite green

- (i) Data are now available on the in-vitro mutagenicity of LMG from limited *Salmonella* and CHO/HGPRT assays, and also from a Comet assay in CHO cells.⁽⁶⁾ Negative results were obtained. However in view of the limitations of these studies, and the fact that no data are available on clastogenicity, it is not possible to make an adequate assessment of the mutagenic potential of LMG from these data.

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- (ii) ³²P post-labelling studies using a 28 day dietary exposure have indicated that LMG induces a low level of DNA adduct(s) in the liver of F344 rats; only a marginal response was however seen in B6C3F₁ mice, and no conclusions can be drawn from these data.⁽⁸⁾ This has been confirmed in a separate unpublished study in mice using 16 weeks dietary exposure; there was no evidence for DNA adduct formation in the liver.⁽⁵⁾ LMG did not induce micronuclei in peripheral lymphocytes nor Hprt mutations in splenic lymphocytes in the 28 day study⁽⁷⁾; the experimental design of these studies did not optimise the chances of obtaining a positive response and no definite conclusions can be drawn.
- (iii) Gene mutation studies have indicated that LMG can induce mutations in vivo in liver DNA. Studies in Big Blue F344 rats gave equivocal results. LMG produced an increase in lac1 mutations only at the highest dose tested (543 ppm in diet) and at a single time point (16 weeks). No increase was seen after 32 weeks. Evidence was provided to suggest that this isolated positive may have been due to a disproportionate expansion of spontaneous lac1 mutations.^(9,10) However studies in female Big Blue B6C3F₁ mice indicated that LMG produced an increase in CII mutant frequency in liver DNA of mice treated with 408 ppm LMG in the diet. Furthermore it was shown that the spectrum of mutation seen in the DNA was distinct from that of the control mice. These data provide evidence of in-vivo mutagenicity at the target site in the carcinogenicity bioassay.
- (iv) In view of the demonstration of the induction of mutations in liver DNA of female B6C3F₁ mice, LMG should be regarded as an in-vivo mutagen.

Carcinogenicity

6. Prior to the results of the NTP bioassay data becoming available, there was no information available from which to assess the carcinogenicity of either MG or LMG. Data were available from a limited study to investigate the ability of MG to promote liver tumours in rats that had been initiated with DEN⁽⁶⁾. In these studies DEN was given in the drinking water for 1 month followed by MG (25 ppm) again in the drinking water for 2 ½ months. The MG treatment was reported to significantly increase the production of markers of potential hepatocarcinogenicity (gamma-glutamyltranspeptidase(GGT)-positive foci and GGT activity) by about 2-fold. This was felt to be evidence of tumour promoter activity. However it is not possible to draw any definite conclusions from this very limited study.

NTP Carcinogenicity Bioassay Results

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7. The carcinogenicity of MG was investigated in female rats and in female mice, the compound being given in the diet for 104 weeks.
8. The carcinogenicity of LMG was investigated in male and female rats and in female mice, compound again being given in the diet for 104 weeks.
9. The NTP summary of their findings (as modified by the peer review panel) is attached at Annex B and a copy of the Report itself(excluding Appendices) at Annex C..A summary of the results obtained is given below.

MG

Rat

10. Groups of 48 female rats were given diets containing 0, 100, 300 and 600 ppm MG (equivalent to average daily doses of approximately 0.1, 21 and 43mg/kg bw/day) for 104 weeks. Survival in all groups was comparable, but mean body weight gain was slightly lower at 300 and 600 ppm (approximately 10 and 12% reduction compared to controls). Relative liver weight was significantly increased at the top dose level.
11. At autopsy thyroid follicular cell adenomas and carcinomas occurred at the two higher doses [incidence of follicular cell adenomas being 0, 0, 1, and 1 and of carcinomas 0, 0, 2, 1 at 0, 100, 300 and 600 ppm in the diet respectively] The adjusted rate (for intercurrent mortality) was at 0%, 0%, 7.1% and 5.7% respectively. A low incidence of cystic follicles (0, 1, 1, 3% respectively) was reported.
12. Hepatocellular adenomas were minimally increased (not statistically significant) at the 2 higher dose levels, but exceeded the historic control range [adjusted rate 2.5%, 2.6%, 6.9% and 10.8% respectively]. The incidence of eosinophilic foci in the liver showed a dose-related increase which was statistically significant (p less than 0.05) at the top dose (5, 10, 13 and 14%).
13. Mammary gland adenomas were increase at the top dose level (5.0%, 5.2%, 2.0% and 13.0% adjusted rate respectively). The increase was not statistically significant but exceeded the historic control range.
14. There was a dose-related decrease in incidence of mononuclear cell leukaemia which was statistically significant at the two higher dose levels.

Mouse

15. Groups of 48 female mice were fed diets containing 0, 110, 225 or 450 ppm MG (equivalent to average daily doses of approximately 0, 15, 33 and 67mg/kg bw/day) for 104 weeks. Survival was comparable in all groups.

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There was a slight (5 – 10%) reduction in body weight gain at the top dose for much of the experimental period, although this was less marked at the end of the experiment. There was a dose-related decrease in absolute kidney weight, significant at the top dose; relative kidney weights were generally less than controls in all treated groups.

16. There was no increased incidence of tumours in any of the treated groups. A dose-related increase in the incidence of lymphocytic cellular infiltration of the liver was noted which was statistically significant at the top dose. Intracytoplasmic inclusions of the urinary bladder were significantly increased in exposed mice.

LMG

Rat

17. Groups of 48 male and female rats were fed diets containing 0, 91, 272 or 543 ppm LMG for 104 weeks. The dietary levels were equivalent to average daily doses of approximately 0, 5, 15 and 30mg LMG/kg bw/day in the males and 0, 6, 17 and 35mg LMG/kg bw/day in the females. Survival was comparable in all groups apart from the males given 272 ppm where increased survival was noted. A dose-related decrease in body weight gain was seen for both sexes amounting to a 25% reduction near termination in the females and 10 – 15% in the males. Liver weights were significant increase at the two higher dose levels in the males, as were relative liver weights in the females. Relative thyroid weights were increased significantly in both sexes at the highest dose level.
18. At autopsy an increase in mammary gland adenoma and carcinoma (combined) was seen with the effects at the top dose level exceeding the historic control range (adjusted rate with increasing dose levels of 0, 4.5, 6.8 and 9.3% respectively). A minimal increase in hepatocellular adenomas was also seen in the female rats given 91 and 543 ppm in the diet which exceeded the historical control range (adjusted rates 2.3, 6.7, 0.0, and 7.0% at 0, 91, 272 or 543ppm in the diet respectively). No increase in such tumours was seen in the males. Non neoplastic liver lesions (eosinophilic foci, cystic degeneration and cytoplasmic vacuolization) were generally increased in both males and females. Minimal increases, not statistically significant, were seen in thyroid follicular cell adenomas and carcinomas (combined) in both the females (adjusted rate, 0, 2.3, 4.7 and 2.4% respectively and males (adjusted rate 0, 4.8, 2.3, 7.2% respectively). Finally in the male rats a positive trend was noted in the incidence of intestinal cell adenoma of the testes, with the increase being statistically significant at the top dose level (adjusted rate 82.5, 93.3, 92.2 and 95.7% respectively). A decrease in the incidence of mononuclear cell leukemia (both sexes) and pituitary gland adenoma was seen.

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Mouse

19. Groups of female mice were fed diets containing 0, 91, 204, or 408 ppm LMG (equivalent to average daily doses of 0, 13, 31 and 63mg LMG/kg bw/day) for 104 weeks. Survival and body weight gain were comparable in all groups. Relative kidney weight was significantly reduced in all treated groups.
20. The only statistically significant ($p = 0.022$) increase in tumour incidence noted at autopsy related to hepatocellular adenoma and carcinoma combined (adjusted rate at increasing dose level 6.9, 12.8, 13.6, 24.6% respectively). These incidence of hepaticular adenoma alone was increased but this was not statistically significant (adjusted rates 6.9, 12.8, 11.4, 20.2% respectively).
21. The NTP conclusions (as agreed by the peer review panel) are given below:

MG

- (i) There was equivocal evidence of carcinogenic activity of MG in female F344/N rats based on the occurrence of thyroid gland follicular cell adenoma or carcinoma (combined) and marginal increases in hepatocellular adenoma and mammary gland carcinoma in exposed rats.
- (ii) There was no evidence of carcinogenic activity of MG in female B6C3F₁ mice.

LMG

- (i) There was evidence of carcinogenic activity of LMG in female B6C3F mice based on an increase in hepatocellular adenoma or carcinoma (combined).
- (ii) There was equivocal evidence of carcinogenic activity of LMG in male F344/N rats based on an increase in intestinal cell adenoma of the testes and the occurrence of thyroid gland follicular cell adenoma or carcinoma (combined).
- (iii) There was equivocal evidence of carcinogenic activity of LMG in female F344/N rats based on marginally increased incidences of hepatocellular adenoma or carcinoma (combined) in exposed rats.

COC Conclusions

22. The COC is asked whether it can endorse the NTP conclusions given above.

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23. Also whether, in the light of the induction of liver tumours by LMG in female mice, it should be regarded as a genotoxic carcinogen. Also is there sufficient information to regard MG as a genotoxic carcinogen?.

Secretariat
May 2004

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ANNEX A

STRUCTURE AND PHYSICO-CHEMICAL PROPERTIES OF MG AND LMG

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ANNEX B

**ABSTRACT OF NTP REPORT ON THE
TOXICOLOGY AND CARCINOGENESIS
STUDIES OF MG AND LMG PLUS
CONCLUSIONS OF
PEER REVIEW PANEL**