(Attachment)

Veterinary Drugs Evaluation Report

Health Risk Assessment of Malachite Green and Leucomalachite Green

November, 2005

Food Safety Commission

Veterinary Drugs Expert Committee

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<Progress of Evaluation>

September 13, 2005	The Ministry of Health, Labour and Welfare requests a health risk assessment and accepts relevant documents
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September 26, 2005	34th meeting of the Veterinary Drugs Expert Committee
October 13, 2005	115th meeting of the Food Safety Commission (reporting)
October 13 to November 16, 2005	Ask for public comments on Expert Committee's report
November 22, 2005	Reportings from the Chairman of the Veterinary Drugs Expert Committee to the Chairman of the Food Safety Commission
November 24, 2005	Finalized at the 121nd meeting of the Food Safety Commission
	Notification by the Chairman of the Food Safety Commission to MHLW

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<Summary>

○ **Carcinogenicity**

　　The NTP (The National Toxicology Program) conducted two-year feed studies for Malachite green (MG) in female B6C3F$_1$ mice and female F344 rats and for Leucomalachite green (LMG) in female B6C3F$_1$ mice and male and female F344 rats. At present, these are the only data available for evaluating the carcinogenicity of MG and LMG.

　　In these studies, there were hardly any cases in which the incidence of neoplastic lesions significantly increased from that of the control; however, hepatocellular adenoma (1/48, 1/48, 3/48, 4/48) and mammary gland carcinoma (2/48, 2/48, 1/48, 5/48) in female rats exposed to MG, and thyroid gland follicular cell adenoma/carcinoma (0/47, 2/47, 1/48, 3/46) in male rats, hepatocellular adenoma (1/48, 3/48, 0/48, 3/48) in female rats and hepatocellular adenoma/carcinoma (3/47, 6/48, 6/47, 11/47) in female mice exposed to LMG could not be neglected, considering the doses and the incidence of these lesions. Moreover, there was a marked increase of eosinophilic foci in rats exposed to either MG or LMG, suggesting its relation to hepatocellular adenoma. Testicular bilateral interstitial cell adenoma was observed in male F344 rats exposed to LMG at all doses tested, and its incidence was significantly increased at the highest dose compared to the control. However, the lesion could not be used to assess the effects of LMG, due to the high spontaneous incidence of this lesion in F344 rats.

　　The data obtained so far suggests that LMG potentially acts as a liver carcinogen in female mice and a weak carcinogen in the liver and the thyroid of rats, and MG potentially acts as a weak carcinogen in the liver and the mammary gland of female rats.

○ **Genotoxicity**

　　Various genotoxicity studies have been conducted, including *in vitro* tests such as Ames test, forward mutation assay and Comet assay and *in vivo* tests such as micronucleus assay, forward mutation assay, DNA adduct formation assay by the ^{32}P-post labeling method and assays for mutations in liver *lacI* and *cII* transgenes using Big Blue mice or rats; most of these tests yielded negative results.

　　Summarizing the data related to genotoxicity, MG was mutagenic in the Comet assay but not in the *in vivo* micronucleus assay, although the latter study might not have been conducted using sufficiently high doses. DNA adduct formation was distinctly observed in the liver of rats and mice, although no mutation was detected in mouse liver and no data on mutation data have been obtained in rat liver. Meanwhile, LMG weakly induced mutation in the liver *cII* gene in mice. The compound was a potential liver carcinogen in mice, but it gave negative results in the DNA adduct formation test and the *in vitro* genetic mutation test. In rats, LMG induced liver DNA adduct formation but no *cII* mutation or *in vitro* genetic mutation. Comprehensively, these results failed to provide a univocal explanation for various *in vivo* mutations including DNA adduct formation and *cII* mutation. Nevertheless, the results obtained so far could not deny the genotoxic potentials of MG and LMG.

○ **Conclusions**

　　Carcinogenicity of MG and LMG were evaluated based on the results of two-year rodent carcinogenicity studies, which seemed to be the only adequate data available for evaluating their carcinogenicity. The data suggested that LMG acts as a liver carcinogen in female mice and a weak carcinogen in rat liver and thyroid, and MG acts as a weak carcinogen in the liver and mammary gland of female rats. Most of the neoplastic lesions observed were adenoma. Regarding genotoxic effects in target organs for carcinogenicity, MG induced liver DNA adduct formation in rats and

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mice but no genetic mutation in mouse liver. Meanwhile, LMG induced *cII* mutation but no DNA adduct formation in mouse liver. Based on the currently available data including the above data and the *in vitro* studies results, the induction of genetic mutations observed *in vivo* were hardly attributable to DNA damages, but the genotoxic potentials of MG and LMG could not be denied.

As discussed above, the mechanism of the carcinogenicity is unknown, and the carcinogenic potentials of the compounds in human are unclear. Nevertheless, the test results evaluated so far suggested that the compounds are carcinogenic in rodents and their genotoxic potentials are undeniable. Thus, it is not appropriate to settle ADIs for MG and LMG.

Health Risk Assessment of Malachite Green and Leucomalachite Green

1. Introduction

Malachite green (MG) is a green-colored synthetic triphenylmethane dye industrially used for dyeing fibers etc. Due to its moderate price, high availability and high efficacy as an antifungal agent, it has been widely used to treat fungal infections in the fisheries industry. Recently, the use of the compound on food animals has been restricted, due to its structural affinity to nucleic acids and structural similarity to substances suspected as genotoxins or carcinogens. The compound has not been evaluated by international agencies such as JECFA and IARC, although the use of the compound in the aquaculture industry is prohibited in Europe and many other countries.

In Japan, the use of MG on aquatic animals for food has been completely prohibited since July 31, 2005, according to the Ordinance on the Control of Veterinary Drugs etc. that has been partially amended in line with partial amendments to the Pharmaceutical Affairs Law (issued on June 11, 2003, enforced on July 30, 2003). Moreover, distribution of food containing MG is restricted by the Food Sanitation Law, which stipulates that, unless standards are otherwise provided, meat, eggs and fishery products must not contain any synthetic antibacterial and antifungal agents. However, neither detailed toxicity assessment nor individual residual standard has been provided for MG.

Leucomalachite green (LMG), a main metabolite of MG, is formed by the reduction of MG *in vivo*. The comparison between zones of inhibition by MG and LMG against bacteria, yeasts and fungi revealed that LMG substantially has no antimicrobial activity, which was about one-hundredth of that of MG[1]. However, in catfish exposed to 0.8 ppm MG solution for 1 hour, the serum MG level reduced to below the detection limit within 1-2 days, while LMG remained in the serum until 4 weeks after the treatment. In the muscle of catfish, MG and LMG were detected until 2 and 6 weeks after the treatment, respectively. Another report showed that the half-life of MG in the muscle of rainbow trout was 1.5 days, while the half-life of LMG ranged from 10-40 days, depending on the fat content[2]. Thus, LMG is likely to remain in the tissues of MG-exposed fish, but neither detailed toxicity assessment nor residual standard has been provided for LMG.

Although the use of MG on food animals is prohibited in Japan and many other countries, there are reported cases of MG contamination detected at import inspections etc. In addition to MG, contamination of fish by its main metabolite, LMG, has been reported in EU and Canada.
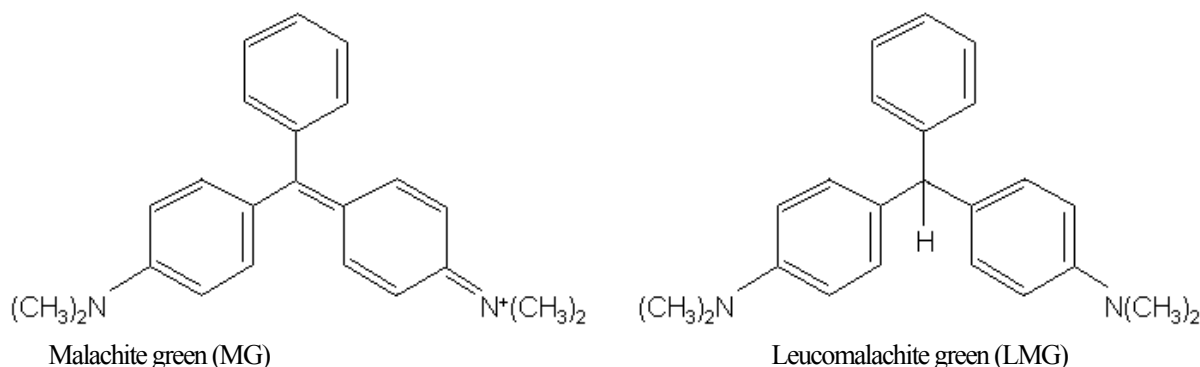
Under these circumstances, the Ministry of Health, Labour and Welfare has set out to the establishment of individual standards for MG and LMG in line with the Food Sanitation Law, and, according to the Food Safety Basic Law, has requested the Food Safety Commission to conduct a health risk assessment.

JECFA has not conducted any toxicity studies or established any international standards for MG or LMG. However, EU has settled MRPL (Minimum Required Performance Limit)[1] for the sum of MG and LMG in aquaculture products as 2 µg/kg.

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[1] Minimum required performance limit is explained as "minimum content of an analyte in a sample, which at least has to be detected or confirmed. It is intended to harmonise the analytical performance etc. of methods for substances for which no permitted limit has been established." The value is established to harmonise the detection performance of a substance in the EU community.

2. Outline of the Drugs to be Evaluated
Chemical name

Malachite green (MG)   Leucomalachite green (LMG)

Chemical formula   : $C_{23}H_{25}N_2$ (malachite green), $C_{23}H_{26}N_2$ (leucomalachite green)
Molecular weight   : 329.47 (MG), 330.48 (LMG)
Appearance at room temperature: MG is a blue-green crystal, LMG is a white crystal
Solubility   : soluble in water

3. Safety of MG and LMG

MG and LMG have not been subjected to any systematic toxicity studies generally required for veterinary drugs. However, the U.S. NTP[2] has conducted a subacute toxicity test for 28 days as a pilot test and a carcinogenicity study for two years in rodents. There are several published reports on genotoxicity studies and a joint statement released by the U.K. COM[3] and COC[4(3)]. Findings currently available on the safety of MG and LMG are summarized below.

[Subacute toxicity test][(4)]
<Malachite green>

The U.S. NTP has conducted a subacute toxicity study by feeding male and female F344/N rats (8 rats per group) and B6C3F$_1$ mice (8 mice per group) with diets containing MG chloride (0, 25, 100, 300, 600, 1200 ppm; male rats 0, 3, 12, 40, 70, 175 mg/kg bw, female rats 0, 3, 12, 40, 75, 190 mg/kg bw, male mice 0, 4, 18, 50, 100, 220 mg/kg bw, female mice 0, 5, 20, 65, 120, 250 mg/kg bw) for 28 days.

Although survival rates during the study were not affected in rats or mice, low body weight and decreased weight gain were observed in animals exposed to 1200 ppm, suggesting that the dose would be inappropriate for a two-year study. Toxicological findings included anemic conditions such as low Ht values (in male mice exposed to 300 ppm and higher, female mice exposed to 600 ppm and higher and female rats exposed to 1200 ppm), low Hb values (in male and female mice exposed to 300 ppm and higher and male and female rats exposed to 1200 ppm) and low erythrocyte counts (in female mice exposed to 100 ppm and higher, male mice exposed to 300 ppm and higher and female rats exposed to 1200 ppm). Hepatotoxicity of the compound was suggested in rats based on increased relative and absolute liver weights in females exposed to 300 ppm and higher, increased relative liver weights in males

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[2] National Toxicology Program

[3] COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

[4] COMMITTEE ON CARCINOGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

exposed to 600 ppm and higher, blood chemistry data revealing increased γ-glutamyltransferase activity in females exposed to 600 ppm and higher and histopathological examinations revealing liver cytoplasmic vacuolation in male and female rats exposed to 1200 ppm.

Females were more sensitive to toxic effects of the compound than males, both in mice and rats.

<Leucomalachite green>

The U.S. NTP has conducted a subacute toxicity study by feeding male F344/N rats (8 rats per group) and female B6C3F$_1$ mice (8 mice per group) with diets containing LMG (0, 290, 580, 1160 ppm; male rats 0, 30, 60, 115 mg/kg bw, female mice 0, 60, 110, 220 mg/kg bw) for 28 days.

Although survival rates during the study were not affected in rats or mice, decreased weight gain was observed in animals exposed to 580 ppm and higher and low body weight was observed in animals exposed to 1160 ppm, suggesting that 1160 ppm would be inappropriate for a two-year study. Toxicological findings included anemic conditions such as low Ht and Hb values and low erythrocyte counts in animals exposed to 1160 ppm. In rats, relative liver weights were increased in all the exposed groups, and the absolute liver weight was also increased at 1160 ppm. In mice, there was an increase in relative liver weight in females exposed to 1160 ppm. Like MG, hepatotoxicity of LMG was suggested in rats based on increased γ-glutamyltransferase activity at 1160 ppm and histopathological examinations revealing liver cytoplasmic vacuolation at 580 ppm and higher. Moreover, apoptosis of transitional epithelium of the urinary bladder was found in mice exposed to1160 ppm.

These findings indicated that rodents were more sensitive to toxic effects of LMG than to those of MG.

[Carcinogenicity test for two years in mice][5]
<Malachite green>

A two-year carcinogenicity test has been reported in female B6C3F$_1$ mice (48 mice per group). The mice were fed diets containing MG chloride at 0, 100, 225, 450 ppm (corresponding to approximately 0, 15, 33, 67 mg/ kg bw/day).

No significant change was observed in survival rate, general clinical conditions, mean body weight or feed consumption.

Regarding organ weights, absolute right kidney weights were decreased at 225 ppm and higher as were the relative weights at 100 and 225 ppm. The absolute weight of the left kidney was decreased at 450 ppm, and the relative weight was decreased at 225 ppm.

At autopsy and histopathological examinations, a dose-related increase in the incidence of intracytoplasmic inclusions in transitional epithelium of the urinary bladder was observed in all the exposed groups (7/47, 15/46, 34/45, 39/48). No effect was observed on general conditions and mortality. The toxicological significance of the intracytoplasmic inclusions is unknown, but the inclusions are considered to be degradation products. Otherwise, exposure to MG had no particular effect on the incidence of neoplastic and pre-neoplastic lesions.

<Leucomalachite green>

A two-year carcinogenicity test has been reported in female B6C3F$_1$ mice (48 mice per group). The mice were fed diets containing LMG at 0, 91, 204, 408 ppm (corresponding to 0, 13, 31, 63 mg/ kg bw/day).

No significant change was observed in survival rate, general clinical conditions, mean body weight or feed consumption.

Regarding organ weights, relative kidney weights were decreased in all the exposed groups.

At autopsy and histopathological examinations, a dose-related increase in the incidence of intracytoplasmic inclusions in transitional epithelium of the urinary bladder was observed in all the exposed groups. Regarding neoplastic and pre-neoplastic lesions, hepatocellular adenoma was observed in all the exposed and control groups, and hepatocellular carcinoma was observed at 204 ppm and higher. The incidence of the adenoma was not significantly increased from that of the control but exceeded the historical control range at 91 and 408 ppm. The combined incidence of adenoma and carcinoma increased in a dose-related manner. At 408 ppm, it showed a marked increase compared to the control and exceeded the historical control range. The lesions were histologically similar to those occurring spontaneously. The incidences of individual types of lesions were as follows.

	0ppm	91ppm	204ppm	408ppm
Non-neoplastic lesions				
Transitional epithelium of the urinary bladder: intracytoplasmic inclusions	14/46	33/48	44/47	44/44
Neoplastic lesions				
Hepatocellular adenoma[1]	3/47	6/48	5/47	9/47
Hepatocellular carcinoma	0/47	0/48	1/47	2/47
Hepatocellular adenoma/carcinoma[2]	3/47	6/48	6/47	11/47

1 Historical incidence 26/563 (4.6%), range 0-11%

2 Historical incidence 34/563 (6.0%), range 0-11%

[Carcinogenicity test for two years in rats][(5)]
<Malachite green>

A two-year carcinogenicity test has been reported in female F344/N rats (48 mice per group). The rats were fed diets containing MG chloride at 0, 100, 300, 600 ppm (corresponding to approximately 0, 7, 21, 43 mg/ kg bw/day).

No significant change was observed in survival rate, feed consumption or general clinical conditions.

Body weights of rats exposed to 300 ppm and higher were generally less than those of the controls.

Regarding organ weights, the relative liver weight was increased at 600 ppm.

At autopsy and histopathological examinations, formation of cysts composed of thyroid gland follicular epithelial cells was observed in all the exposed groups, and hyperplasia was observed in rats exposed to 300 ppm and higher. Eosinophilic foci in the liver were observed in all the exposed and control groups and were significantly increased at 600 ppm. Regarding neoplastic and pre-neoplastic lesions in the thyroid gland, follicular cell adenoma and adenocarcinoma were observed in rats exposed to 300 ppm and higher. Combined incidence of adenoma and adenocarcinoma exceeded the historical control range. Hepatocellular adenoma occurred in all the exposed and control groups, and its incidence exceeded the historical control range. Mammary gland carcinoma was observed in all the exposed and control groups, and its incidence exceeded (not statistically significant) the historical control range at 600 ppm. Mononuclear cell leukemia occurred in all the exposed and control groups; its incidence decreased in a dose-related manner and was significantly decreased at 300 and 600 ppm. The incidences of individual types of lesions were as follows.

	0ppm	100ppm	300ppm	600ppm
Non-neoplastic lesions				
Thyroid gland follicular cell cyst	0/46	1/48	1/47	3/46
Thyroid gland follicular cell hyperplasia	0/46	0/48	1/47	2/46

Liver eosinophilic focus	5/48	10/48	13/48	**14**/48
Neoplastic lesions				
Thyroid gland follicular cell adenoma	0/46	0/48	1/47	1/46
Thyroid gland follicular cell adenocarcinoma	0/46	0/48	2/47	1/46
Thyroid gland follicular cell adenoma/adenocarcinoma [1]	0/46	0/48	3/47	2/46
Hepatocellular adenoma[2]	1/48	1/48	3/48	4/48
Mammary gland carcinoma [3]	2/48	2/48	1/48	5/48
Pituitary gland adenoma	26/48	36/47	32/46	29/45
Pituitary gland adenoma/adenocarcinoma[4]	26/48	36/47	32/46	30/45
Mononuclear cell leukemia [5]	19/48	17/48	10/48	1/48

*Bold letters represent data that were significantly different from the control group

1 Historical incidence 7/517 (1.4%), range 0-3%

2 Historical incidence 1/541 (0.2%), range 0-0.6%

3 Historical incidence 4/534 (0.7%), range 0-4%

4 Historical incidence 306/528 (58.0%), range 51-68%

5 Historical incidence 188/542 (34.7%), range 13-45%

<Leucomalachite green>

A two-year carcinogenicity test has been conducted in F344/N rats (48 males and 48 females per group). The rats were fed diets containing LMG at 0, 91, 272, 543 ppm (corresponding to approximately 0, 5, 15, 30 mg/kg bw/day for males, approximately 0, 6, 17, 35 mg/kg bw/day for females).

The survival rate in male rats exposed to 272 ppm exceeded that of the control. Survival rates in other treatment groups were similar to that of the control.

No significant change was observed in general clinical conditions.

Body weights of females exposed to 272 ppm and higher and males exposed to 543 ppm were low throughout the study. Males exposed to 272 ppm and females exposed to 91 ppm weighed low during the second year of the study.

Feed consumption by males and females exposed to 543 ppm was intermittently decreased than that by the controls. Feed consumption by females exposed to 272 ppm was intermittently decreased than that by the controls during the second year of the study.

Regarding organ weights, relative and absolute liver weights in males and relative liver weights in females were increased at 272 ppm and higher. Relative thyroid weights in males and females exposed to 543 ppm were increased.

At autopsy and histopathological examinations, formation of cysts composed of thyroid follicular epithelial cells was observed in males exposed to 543 ppm and females exposed to 91 and 543 ppm. Hyperplasia was observed in all the exposed and control groups in males and the 543 ppm and control groups in females. In the liver, eosinophilic foci, cystic degeneration and cytoplasmic vacuolation were observed in all the exposed and control groups. Increases in eosinophilic foci in all the exposed groups in males and females, cystic degeneration in all the exposed male groups and vacuolation in males exposed to 91 ppm and females exposed to 272 ppm and higher were statistically significant. Regarding neoplastic and pre-neoplastic lesions in the thyroid gland, thyroid gland follicular cell adenoma and adenocarcinoma (combined) were observed in all the exposed groups. Combined incidence of adenoma and adenocarcinoma exceeded the historical control range in males and females exposed to 543 ppm and 272 ppm, respectively. Hepatocellular adenoma occurred in the female control, 91 and 543 ppm groups, and its incidence in these groups exceeded the historical control range. In females, mammary gland adenoma and adenocarcinoma were

observed in all groups, and their combined incidence exceeded (not statistically significant) the historical control range at 543 ppm. In males, testicular bilateral interstitial cell adenoma was observed in all the exposed and control groups, and its incidence was significantly increased at 543 ppm compared to the control. Meanwhile, mononuclear cell leukemia occurred in all the exposed and control groups, but its incidence was decreased in the exposed males and females. Incidence of pituitary gland adenoma was decreased in the exposed males but not in females. The incidences of individual types of lesions were as follows.

Males

	0ppm	91ppm	272ppm	543ppm
Non-neoplastic lesions				
Thyroid gland follicular cell cyst	0/47	0/47	0/48	3/46
Thyroid gland follicular cell hyperplasia	2/47	1/47	3/48	3/46
Liver eosinophilic focus	3/48	**14**/47	**19**/48	**33**/47
Liver cystic degeneration	4/48	**18**/47	**13**/48	**19**/47
Liver cytoplasmic vacuolation	9/48	**21**/47	10/48	13/47
Neoplastic lesions				
Thyroid gland follicular cell adenoma	0/47	2/47	0/48	1/46
Thyroid gland follicular cell adenocarcinoma	0/47	0/47	1/48	2/46
Thyroid gland follicular cell adenoma/adenocarcinoma[1]	0/47	2/47	1/48	3/46
Hepatocellular adenoma[2]	2/48	2/47	3/48	2/47
Testicular bilateral interstitial cell adenoma[3]	22/48	30/47	38/48	**39**/47
Mononuclear cell leukemia[4]	29/48	16/47	19/48	7/47
Pituitary gland adenoma	30/45	19/46	21/48	13/45

*Bold letters represent data that were significantly different from the control group

1　Historical incidence 2/511(0.4%), range 0-2%

2　Historical incidence 4/548(0.7%), range 0-2%

3　Historical incidence 469/547(85.7%), range 69-90%

4　Historical incidence 240/550(43.6%), range 31-58%

Females

	0ppm	91ppm	272ppm	543ppm
Non-neoplastic lesions				
Thyroid gland follicular cell cyst	0/46	1/46	0/47	2/48
Thyroid gland follicular cell hyperplasia	1/46	0/46	0/47	3/48
Liver eosinophilic focus	3/48	**12**/48	**20**/48	**16**/48
Liver cystic degeneration	3/48	2/48	5/48	3/48
Liver cytoplasmic vacuolation	5/48	5/48	**17**/48	**22**/48
Neoplastic lesions				
Thyroid gland follicular cell adenoma	0/46	0/46	0/47	1/48
Thyroid gland follicular cell adenocarcinoma	0/46	1/46	2/47	0/48
Thyroid gland follicular cell adenoma/adenocarcinoma[1]	0/46	1/46	2/47	1/48
Hepatocellular adenoma[2]	1/48	3/48	0/48	3/48
Mammary gland adenoma	0/48	1/48	1/48	2/48
Mammary gland adenocarcinoma	0/48	1/48	2/48	2/48
Mammary gland	0/48	2/48	3/48	4/48

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adenoma/adenocarcinoma[3]				
Mononuclear cell leukemia[4]	17/48	8/48	5/48	8/48
Pituitary gland adenoma	26/47	23/47	17/45	20/46

*Bold letters represent data that were significantly different from the control group

1 Historical incidence 7/517(1.4%), range 0-3%

2 Historical incidence 1/541(0.2%), range 0-1%

3 Historical incidence 9/534(1.7%), range 0-6%

4 Historical incidence 188/543(34.6%), range 13-45%

[Genotoxicity study]

Results from *in vitro* and *in vivo* tests are summarized below.

<Malachite green>

in vitro

Test systems	Cells/animals	Doses	Results	References
Ames test	*S. typhimurium* TA98,TA100,TA1535, TA1537	0.05, 0.26, 1.28, 6.4, 32, 160 µg/plate (-S9)	Negative[1]	(6)
		0.05, 0.26, 1.28, 6.4, 32, 160µg/plate (+S9)	Positive[2] (on TA98 at 6.4µg/plate and higher)	(6)
	S. typhimurium TA98	10～150µg/plate (+S9)	Positive[3] (30µg/mL and higher)	(6)
	S. typhimurium TA98,TA100, TA1537	Dose unknown (-S9)	Negative	(7)
	S. typhimurium TA97,TA98,TA100,TA102,TA104,TA1535	0.1～10µg/plate (±S9)[4]	Negative	(4), (5)
	S. typhimurium TA97,TA98,TA100,TA102	0.01～10µg/plate (±S9)	Negative[5]	(8)
Forward mutation assay	CHO/HPRT	0.001～0.05µg/mL (-S9) 5h+7 days	Negative[6]	(8)
		0.01～1µg/mL (+S9) 5h+7 days	Negative	(8)
Comet assay	CHO	1, 2, 3, 4, 5, 10µg/mL (-S9)	Positive[7] (3µg/mLand higher)	(8)
		1～20µg/mL (+S9)	Positive[8] (15µg/mLand higher)	(8)

1 Bacterial growth was inhibited at 1.28µg/plate

2 No description on bacterial growth inhibitory concentration was found

3 Bacterial growth was inhibited at 100µg/plate and higher. Incidence of reverse mutation increased dose-dependently at 20-70µg/plate

4 Hamster- and rat-derived S9mixes were used.

5 Bacterial growth was inhibited at10µg/plate in the absence of S9, except for TA98. In the presence of S9, the compound was tested up to 10µg/plate, based on the results from preliminary cytotoxicity studies.

6 Marked cytotoxicity was observed at 0.1µg/mL and higher. Positive results were obtained in only one trial (1/2) at 0.01µg/ml.

7 Cell survivals decreased to about 80% at 3µg/mL, about 70% at 4 and 5µg/mL and about 30% at 10µg/mL.

8 Cell survivals slightly decreased (to about 80-90%) at 15µg/mL and higher

in vivo

Test systems	Animals/cells	Doses	Results	References
Micronucleus assay	Mouse bone marrow	24, 42, 66 hours after single oral administration of 37.5 mg/kg	Negative	(6)
	Rat bone marrow	Single intraperitoneal injection of 1.094～8.750 mg/kg per day for three days	Negative[1]	(4)
	Mouse peripheral blood	25-1200 ppm in feed for 28 days	Negative	(4)
	Female Big Blue B6C3F$_1$ mouse peripheral blood	450 ppm in feed for 4 weeks[2]	Negative	(5), (9)
		450 ppm in feed for 16 weeks[2]	Negative	(5), (9)

Forward mutation assay (HPRT)	Female Big Blue B6C3F$_1$ mouse spleen lymphocytes	450 ppm in feed for 4 weeks	Negative[2]	(5), (9)
		450 ppm in feed for 16 weeks	Negative	(5), (9)
Mutation assay (mouse liver cII gene mutation assay)	Female Big Blue B6C3F$_1$ mouse liver cII gene	450 ppm in feed for 16 weeks	Negative	(5), (9)
^{32}P post-labeling analysis	Female B6C3F$_1$ mouse liver DNA	0, 100, 600 ppm in feed for 28 days	Significant increase of adducts at 600 ppm	(10)
	Male F344 rat liver DNA	0, 100, 600 ppm in feed for 28 days	Significant increase of adducts at all doses	(10)
	Female B6C3F$_1$ mouse liver DNA	450 ppm in feed for 28 days	Significant increase of adducts	(5)

1 Significant increase was observed at 4.375mg/kg bw but not at other doses.
2 Purity of 88% (major portion of the impurities (12%) was attributed to leucomalachite green and the remainder to demethylated forms of MG or LMG)

\<Leucomalachite green\>

in vitro

Test systems	Animals/cells	Doses	Results	References
Ames test	*S. typhimurium* TA97, TA98,TA100,TA102	10～2000 μg/plate (±S9)	Negative[1]	(8)
Forward mutation assay	CHO/HPRT	5～100μg/mL (-S9) 5h+7 days	Negative[2]	(8)
		5～100μg/mL (+S9) 5h+7 days	Negative[3]	(8)
Comet assay	CHO	5～500μg/mL (-S9)	Negative	(8)
		25～300μg/mL (+S9)	Negative	(8)

1 Survival rates were decreased to 35-45% of that of the control at1000μg/plate and higher. Test substance precipitated at 500μg/plate and higher.
2 The compound was tested up to 100μg/mL, based on the preliminary studies in which marked cytotoxicity was observed at 500 μg/mL and higher.
3 The compound was tested up to 100μg/mL, based on the preliminary studies in which marked cytotoxicity was observed at 500 μg/mL and higher.

in vivo

Test systems	Animals/cells	Doses	Results	References
Micronucleus assay	Female Big Blue rat bone marrow	0, 9, 27, 91, 272 ,543 ppm in feed for 4, 16, 32 weeks	Negative	(11)
	Female B6C3F$_1$/NctrBR mouse peripheral blood	0, 290, 580, 1160 ppm in feed for 28 days	Positive[1] (290,580 ppm)	(4)
	Female Big Blue B6C3F$_1$ mouse peripheral blood	204,408 ppm in feed for 4 weeks	Negative	(5), (9)
		204,408 ppm in feed for 16 weeks	Negative	(5), (9)
	Female Big Blue B6C3F$_1$ mouse bone marrow	0, 9, 27, 91, 272 ,543 ppm in feed for 4, 16, 32 weeks	Negative	(11)
Forward mutation assay (HPRT)	Female Big Blue B6C3F$_1$ mouse spleen lymphocytes	204 ppm in feed for 4 weeks	Negative	(5), (9)
		408 ppm in feed for 16 weeks	Negative	(5), (9)
	Female Big Blue rat spleen lymphocytes	0, 9, 27, 91, 272 ,543 ppm in feed for 4, 16, 32 weeks	Negative	(11)
^{32}P post-labelling analysis	Female B6C3F$_1$ mouse liver DNA	0, 204, 408 ppm in feed for 28 days	Negative	(5)
	Female B6C3F$_1$ mouse liver DNA	0, 96, 580 ppm in feed for 28 days	Negative	(10)
	Male F344 rat liver DNA	0, 96, 580 ppm in feed for 28 days	Significant increase of adducts at 580ppm	(10)
	Female Big Blue rat liver DNA	0, 9, 27, 91, 272, 543 ppm in feed for 4 weeks	Dose-related significant increase of DNA adducts at 91ppm and higher	(12)
Mutation assay (liver lacI gene mutation assay)	Female Big Blue rat liver DNA	0, 91, 272, 543 ppm in feed for 4, 16, 32 weeks	Negative[2]	(12)

Mutation assay (liver cII gene mutation assay)	Female Big Blue B6C3F$_1$ mouse liver cII gene	408 ppm in feed for 16 weeks	Positive	(5), (9)
	Female Big Blue F344 rat liver cII gene	543 ppm in feed for 16 weeks	Negative	(5), (9)

1   Slight increase observed at 1160 ppm but not statistically significant
2   Increase observed at 543 ppm after16 weeks

In vitro studies including Ames test, forward mutation assay and Comet assay were conducted.

Ames tests were conducted using *Salmonella typhimurium* strains TA97, TA98, TA100, TA102, TA104, TA1535 and TA1537. Since MG showed an antibacterial property, it was not tested at high doses except in few tests. LMG was tested up to 2000μg/plate. MG was found to be mutagenic on TA98 (10-150 μg/plate) in the presence of S9 in an Ames test[6]; however, in a later study[8], the compound could only be tested at doses up to 10μg/plate, due to its antibacterial property found in the preliminary test. The report also discussed the possible causes for the lack of reproducible results, and suggested the need for further studies to verify the reproducibility of the mutagenicity of MG on TA98 (+S9). LMG was demonstrated to have no mutagenic activity for doses up to 2000μg/plate. The forward mutation assay was conducted using the *Hprt* locus of CHO, and neither of the compounds was mutagenic. LMG was tested up to 100μg/mL, while MG was tested only up to 0.05 and 1 μg/mL in the absence and presence of S9, respectively, due to its cytotoxicity. However, in the presence of S9, the cytotoxicity of MG was attenuated and could not be detected, even at the highest dose, indicating that the doses used for the assay might have been too low. The Comet assay was conducted using CHO, and the highest doses tested were 10 and 20 μg/mL in the absence and presence of S9, respectively, for MG and 500 and 300 μg/mL in the absence and presence of S9, respectively, for LMG. MG was reported to be mutagenic, both in the presence and absence of the metabolic activation system. Regarding the cytotoxic effects observed at doses giving mutagenic effects, there was a 20-70% decrease in cell survival in the absence of S9 but only a 10-20% decrease in the presence of S9. LMG was not mutagenic at any of the doses tested. No paper has been published on studies assessing the induction of chromosomal aberrations.

In vivo studies including micronucleus assay, forward mutation assay, DNA adduct formation assay by the ^{32}P-post labeling method and assays for mutations in liver *lacI* and *cII* transgenes using Big Blue mice or rats were conducted. Micronucleus assays of MG and LMG were conducted on mouse and rat bone marrows and mouse peripheral blood. In 2004, NTP reported that micronuclei were induced in peripheral blood of mice (B6C3F$_1$/NctrBR) following 28-days exposure to LMG in feed, but in another assay reported by NTP in 2005, negative results were obtained in peripheral blood of mice (Big Blue B6C3F$_1$) after 4- or 16-weeks exposure to LMG in feed. All other assays gave negative results. Forward mutation assays were conducted on lymphocyte HPRT mutation in mouse spleens for MG and in mouse and rat spleens in LMG, all of which gave negative results. DNA adduct formation assays by the ^{32}P-post labeling method were performed on liver DNAs from female B6C3F$_1$ mice and male F344 rats for MG and female B6C3F$_1$ mice, male F344 rats and female Big Blue rats for LMG. MG induced adduct formation in both animals, while LMG induced adduct formation in rats but not in mice. In assays for mutations in liver *lacI* and *cII* transgenes using Big Blue mice or rats, MG did not induce *cII* gene mutation in mice, while LMG induced *cII* gene mutation in mice but not in rats. In the *lacI* mutation assay for LMG in rats, the mutant frequency was significantly increased at 16 weeks but not at 32 weeks after exposure to the highest dose. Moreover, when clonality was taken into account, the mutant frequency at week 16 was not significantly different from that of the control[11].

MG induced DNA adduct formation in livers of mice and rats assessed by the ^{32}P-post labeling method, but the

compound gave negative results in the micronucleus and forward mutation assays in mice. Thus, it is unlikely that the DNA adduct formation would be fixed as chromosomal aberrations or genetic mutations. However, most of the micronucleus and forward mutation assays were performed by feed treatments using doses selected based on those used in carcinogenicity tests, which might have been too low for genotoxicity assays. No relevant data was available for rats.

LMG induced DNA adduct formation in rat liver but not in mouse liver, assessed by the ^{32}P-post labeling method. The compound gave negative results in all but one micronucleus assay and all HPRT mutation assays. However, these assays were performed by feed treatments using doses selected based on those used in carcinogenicity tests, which might have been too low for genotoxicity assays. In assays using transgenic animals (Big Blue), LMG weakly induced mutation in liver *cII* gene in mice but not in *cII* or *lacI* gene in rats. The compound gave negative and positive results in mice and rats, respectively, in DNA adduct formation assays, which contradicted the results from the mutation assays.

4. Health Risk Assessment
[Carcinogenicity]

NTP has performed two-year carcinogenicity studies by feed treatment in female B6C3F$_1$ mice and female F344 rats for MG and in female B6C3F$_1$ mice and male and female F344 rats for LMG. Some studies were performed using only females, due to a finding from a 28-days pilot study that females were more sensitive to toxic effects.

The NTP has presented the following conclusions for the carcinogenicities of MG and LMG in the report in 2005. The conclusions have been supported by a joint statement by the U.K. COM and COC.

There was no evidence of carcinogenic activity of MG in female B6C3F$_1$ mice orally exposed to doses up to 450 ppm. There was equivocal evidence of carcinogenic activity[5] in female F344 rats, based on the marginal increases in incidences of thyroid gland follicular cell adenoma/adenocarcinoma (combined), hepatocellular adenoma and mammary gland carcinoma.

There was some evidence of carcinogenic activity[6] of LMG in female B6C3F$_1$ mice, based on the increased incidences of hepatocellular adenoma and adenoma/carcinoma (combined). There was equivocal evidence of carcinogenic activity of LMG in male F344 rats, based on the marginally increased incidences of thyroid gland follicular cell adenoma/adenocarcinoma (combined) and testicular bilateral interstitial cell adenoma. There was equivocal evidence of carcinogenic activity of LMG in female F344 rats, based on the marginally increased incidences of thyroid gland follicular cell adenoma/adenocarcinoma (combined) and hepatocellular adenoma.

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[5] NTP classifies the evidence of carcinogenicity into four levels, i.e. clear evidence, some evidence, equivocal evidence and no evidence. NTP further described "equivocal evidence of carcinogenic activity" as "studies that are interpreted as showing a marginal increase of neoplasmas that may be chemical related."

[6] NTP further described "some evidence of carcinogenic activity" as "studies that are interpreted as showing a chemical-related increased incidence of neoplasms (malignant, benign, or combined) in which the strength of the response is less than that required for clear evidence."

At present, these are the only data available for evaluating the carcinogenicity of MG and LMG. In these studies, there were hardly any cases in which the incidence of neoplastic lesions significantly increased from that of the control; however, hepatocellular adenoma (1/48, 1/48, 3/48, 4/48) and mammary gland carcinoma (2/48, 2/48, 1/48, 5/48) in female rats exposed to MG, and thyroid gland follicular cell adenoma/carcinoma (0/47, 2/47, 1/48, 3/46) in male rats, hepatocellular adenoma (1/48, 3/48, 0/48, 3/48) in female rats and hepatocellular adenoma/carcinoma (3/47, 6/48, 6/47, 11/47) in female mice exposed to LMG could not be neglected, considering the doses and the incidence of these lesions. Moreover, there was a marked increase of eosinophilic foci in rats exposed to either MG or LMG, suggesting its relation to hepatocellular adenoma. Testicular bilateral interstitial cell adenoma was observed in male F344 rats exposed to LMG at all doses tested, and its incidence was significantly increased at the highest dose compared to the control. However, the lesion could not be used to assess the effects of LMG, due to the high spontaneous incidence of this lesion in F344 rats.

The data obtained so far suggests that LMG potentially acts as a liver carcinogen in female mice and a weak carcinogen in the liver and the thyroid gland of rats, and MG potentially acts as a weak carcinogen in the liver and the mammary gland of female rats.

[Genotoxicity]

Various genotoxicity studies have been conducted, including *in vitro* tests such as Ames test, forward mutation assay and Comet assay and *in vivo* tests such as micronucleus assay, forward mutation assay, DNA adduct formation assay by the ^{32}P-post labeling method and assays for mutations in liver *lacI* and *cII* transgenes using Big Blue mice or rats; most of these tests yielded negative results.

MG was mutagenic on TA98 in the presence of the metabolic activation system in an *in vitro* Ames test, gave positive results in the Comet assay, both in the presence and absence of the metabolic activation system, and increased DNA adducts in the livers of female B6C3F$_1$ mice and male F344 rats in the DNA adduct formation assay by the ^{32}P-post labeling method. LMG was not mutagenic in *in vitro* tests; however, in *in vivo* tests, it was weakly mutagenic in the micronucleus test using mouse peripheral blood and in the assay for mutation in liver *cII* transgene using Big Blue mice and formed DNA adducts in the livers of male F344 rats and female Big Blue rats in the DNA adduct formation assay by the ^{32}P-post labeling method. Regarding these results, the U.K. COM and COC have released a joint statement in 2004 and claimed that MG should be considered as an *in vivo* mutagen, based on the fact that it induced DNA adduct formation in rats and mice. The joint statement also concluded that LMG should be considered as an *in vivo* mutagen, based on the fact it increased frequency of liver *cII* gene mutants in female Big Blue mice.

Summarizing the data related to genotoxicity, MG was mutagenic in the Comet assay but not in the *in vivo* micronucleus assay, although the latter study might not have been conducted using sufficiently high doses. DNA adduct formation was distinctly observed in the livers of rats and mice, although no mutation was detected in mouse liver and no data on mutation has been obtained in rat liver. Meanwhile, LMG weakly induced mutation in the liver *cII* gene in mice. The compound was a potential liver carcinogen in mice, but it gave negative results in the DNA adduct formation test and the *in vitro* genetic mutation test. In rats, LMG induced liver DNA adduct formation but no *cII* mutation or *in vitro* genetic mutation. Comprehensively, these results failed to provide a univocal explanation for various *in vivo* mutations including DNA adduct formation and *cII* mutation. Nevertheless, the results obtained so far could not deny the genotoxic potentials of MG and LMG. Further studies are required to reach a reliable conclusion.

The results of two-year rodent carcinogenicity studies, which seemed to be the only adequate data available for evaluating the carcinogenicity of MG and LMG, suggested that LMG acts as a liver carcinogen in female mice and a weak carcinogen in rat liver and thyroid gland, and MG acts as a weak carcinogen in the liver and mammary gland of female rats. Most of the neoplastic lesions observed were adenoma. Regarding genotoxic effects in target organs for carcinogenicity, MG induced liver DNA adduct formation in rats and mice but no genetic mutation in mouse liver. Meanwhile, LMG induced *cII* mutation but no DNA adduct formation in mouse liver. Based on the currently available data including the above data and the *in vitro* studies results, the induction of genetic mutations observed *in vivo* were hardly attributable to DNA damages, but the genotoxic potentials of MG and LMG could not be denied.

As discussed above, the mechanism of the carcinogenicity is unknown, and the carcinogenic potentials of the compounds in human are unclear. Nevertheless, the test results evaluated so far suggested that the compounds are carcinogenic in rodents and that their genotoxic potentials are undeniable. Thus, it is not appropriate to settle ADIs for MG and LMG.

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