National Food Institute

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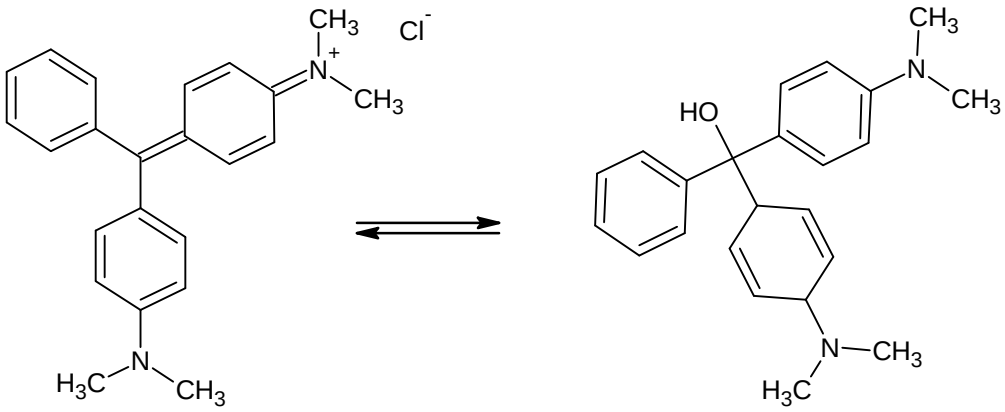
Risk Assessment of Malachite Green in Food

RISK ASSESSMENT OF MALACHITE GREEN IN FOOD



Malachite green chloride **Malachite green carbinol**

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RISK ASSESSMENT OF MALACHITE GREEN IN FOOD

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Substance name:	Malachite green
Assignment:	FVST has requested a risk assessment of malachite green. Malachite green is not authorised as a veterinary medicinal product and is therefore not allowed to be used in food producing animals.
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I. Background

Malachite green and its major metabolite leucomalachite green are occasionally found in farmed fish products on the Danish market, being imported or domestically produced. Malachite green is not authorised for use in food producing animals in the EU but nonetheless, residues of malachite green/leucomalachite green are the most frequent reason for violation of the EU rules regarding products of farmed fish. The residues found in farmed fish products may originate from environmental pollution due to past uses. If this is the case, then low concentrations of malachite green and leucomalachite green in farmed fish products may be unavoidable. Therefore the Danish Veterinary and Food Administration has requested the National Food Institute to perform a risk assessment of human exposure to malachite green and leucomalachite green in fish.

II. Introduction

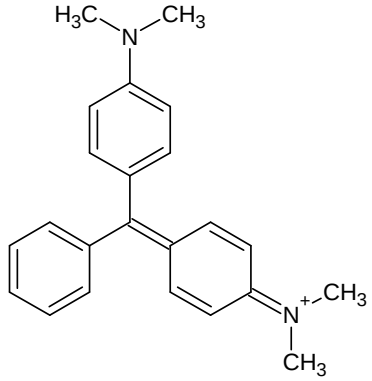
Malachite green (MG) chloride forms green crystals with a metallic luster. It is soluble in methanol and ethanol and very soluble in water (NTP 2005). In water it slowly reacts with hydroxyl ions changing into its carbinol form until equilibrium is established. Low pH favours the cation form while high pH favours the carbinol (pKa 6.9). Leucomalchite green (LMG) is the reduced form of malachite green (Plakas *et al.,* 1996).
Malachite green has been used in fish farms as an aquatic fungicide, parasiticide, antiprotozoan and bacteriocide. As an aquatic fungicide and parasiticide it is very effective and relatively cheap making it an attractive compound for fresh water fish farmers. Its use dates back to the early 1930s (NTP 2005, Srivastava *et al.* 2004). In Denmark and the EU, its use in fish farms is illegal (banned in May 1990 in Denmark). In other parts of the world outside the EU it is authorised and extensively used in fish farms. Malachite green is also used as a colorant in the paper and textile industries (Srivastava *et al.* 2004). Findings of malachite green and leucomalchite green in fish products in Denmark are listed in Table 1.

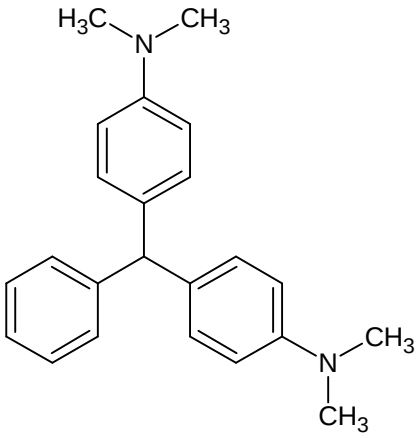
Table 1. Findings of malachite green and leucomalchite green in fish in Danmark from 1988 to 2005 (Rasmussen, 2007).

Year	Product	Origin	Nr. Samples	Positive samples[#]	Malachite green	Leucomalachite green
2005	Farmed fish	Import	5	2		5.6 and 6.1 µg/kg
2005	Farmed fish	Danish	117	1		2.7 µg/kg
2004	Farmed fish	Danish	43[*]	0[*]		
2003	Farmed fish	Danish	23	1	2 µg/kg	28 µg/kg
2002	Eel	Chinese	26	19	1** - 300 µg/kg	> 100µg/kg
2002	Farmed fish	Danish	20	0		
2001	Trout	Danish	20	0		
2000	1 Eel 5 Seatrouts 14 Trouts	Danish	20	4		< 4 µg/kg
1993	Salmon	Norwegian	54	0		
1991	Farmed fish	Danish	49	2	4 and 5 µg/kg	
1989	Farmed fish	Danish	20	6	5 – 17 µg/kg	No test
1988	Farmed fish	Danish	49	13	15 – 214 µg/kg	No test

#) In 1990 controls were extended to include leucomalchite green. Analyses were conducted by the Danish Institute for Food and Veterinary Research and the Danish Veterinary and Food Administration, Region East.
*) In addition 82 of 95 targeted samples were positive (1-60 µg/kg)
**) Limit of Detection (LOD).

- 1 Precise identification of the substance

International non-proprietary name (INN)	Malachite green
International Union of Pure and Applied Chemistry (IUPAC) name	4-[(4-dimethylaminophenyl)-phenyl-methyl]-N,N-dimethyl-aniline
Chemical Abstract Service (CAS) name	
CAS no	2437-29-8 (oxalate), 569-64-2 (chloride)
Classification *Therapeutic*	*None (used as aquatic fungicide, parasiticide, bacteriocide)*
Pharmacological	*None*
Synonyms and abbreviations	Aniline green, Basic green 4, Diamond green B, Victoria green B, Benzaldehyde green, Acryl Brilliant green, China green, Fast green and others.
Structural formula	
Molecular formula	$C_{23}H_{25}N_2^+$
Molecular weight	329.45
Degree of impurity	Variable, often of relatively low purity < 90 %.
Qualitative and quantitative composition of impurities	Leucomalachite green, degradation products, salt.
Description of physical properties: *Melting point:*	~ 159 ºC (oxalate form)
Boiling point:	Not available
Vapour pressure:	Not available
Solubility in water and organic solvents, expressed in g/l, with indication of temperature	110 g/l H_2O (24 ºC, oxalate form) Soluble in methanol and ethanol: No figures given.
Density	Not available
Refractive index, Optical rotation	Not available
pKa	*6.9*
Comments	*Malachite green is sold as various salts. Oxalate and chloride are common, but others salts are available.*

International non-proprietary name (INN)	Leucomalachite Green
International Union of Pure and Applied Chemistry (IUPAC) name	4,4′-Benzylidenebis(*N*,*N*-dimethylaniline)
Chemical Abstract Service (CAS) name	
CAS no	129-73-7
Classification *Therapeutic*	*None*
Pharmacological	*None*
Synonyms and abbreviations	
Structural formula	
Molecular formula	$C_{23}H_{26}N_2$
Molecular weight	330.47
Degree of impurity	Variable. Generally higher purity than malachite green.
Qualitative and quantitative composition of impurities	Malachite green and degradation products.
Description of physical properties: *Melting point:*	~ 100-102 ºC
Boiling point:	Not available
Vapour pressure:	Not available
Solubility in water and organic solvents, expressed in g/l, with indication of temperature	Slightly soluble in water.
Density	Not available
Refractive index, Optical rotation	Not available
Comments	*White to light coloured powder.*

- 2 Toxicodynamics and -kinetics

2.1 Toxicodynamics

No studies were reported in public literature.

2.2 Toxicokinetics (Absorption, Distribution, Metabolism and Excretion)

Henderson, A. L., Schmitt, T. C., Heinze, T. M., and Cerniglia, C. E. (1997) Reduction of malachite green to leucomalachite green by intestinal bacteria. *Appl. Environ. Microbiol.* **63**(10), 4099-4101.
Type of activity: Reduction of malachite green (MG) to leucomalachite green (LMG) by intestinal microorganisms.
Description: Reduction of malachite green to leucomalachite green was studied in several bacterial strains as well as human and other mammalian intestinal microflora. The bacterial cultures were incubated with 300 µg of MG in 5 ml of brain heart infusion broth for 24 to 48 hours under anaerobic conditions. The culture media was analyzed by HPLC using photodiode array detection.
Strains: Intestinal microflora was obtained from humans, rhesus monkeys, mice (C3H/HEN-MTV) and rats (Fischer 344). The bacterial strains tested were *Bacteroides fragilis, Bacteroides thetaiotaomicron, Bifidobacteriumadolecentis, citrobacter sp., Clostridium perfringens, Enterococcus faecali, Escherichia coli, Fusobacterium prausnitzii, Lactobacillus acidophilus, Peptostreptococcus anaerobius, Peptostreptococcus magnus, Peptostreptococcus productus, Ruminococcus albus and Ruminococcus flavofaciens.*
Effects: All intestinal microflora reduced 99-100 % of MG to LMG. The bacterial stains tested showed ability to reduce MG to LMG. Among the bacterial strains, *Clostridium perfringens, Peptostreptococcus anaerobius,* and *Escherichia coli* reduced 99 % of MG to LMG whereas *Lactobacillus acidophilus* (7.5 %), *citrobacter* sp. (12.3 %), and *Bacteroides thetaiotaomicron* (16.3 %) had the lowest activities. The remainder strains reduced from 24 – 63 % of MG to LMG. There was no reduction of MG in the negative control (without bacteria).

Conclusions

The study shows that MG can be converted into LMG by a multitude of bacterial species and mammalian intestinal microflora under anaerobic conditions, such as in the intestines. Therefore, MG that is not absorbed in the stomach is likely to be converted to LMG in the intestines.

Bauer, Von K., Danschat, H., Knöppler H.-O. und Neudegger (1988) Aufnahme und ausscheidung von Malachitgrün bei regenbogenforellen. Archiv für lebensmittelhygiene 39, 97-102.			
Type of activity: Toxicokinetic study in rainbow trout (*Salmo gairdneri*).			
Test substance: Malachite green.			
Description: MG was added to a water tank with 156 rainbow trouts to a concentration of 0.2 mg/l. The fish were exposed for 24 hours and transferred to three 200 l tanks with clean water renewal. Fish were sampled at 0, 3, 5, and 10 days and twice each week from day 10-24, and thereafter once each week until day 143. Six fish were taken for sampling at each time point except at the first sampling where 10 fish were taken. MG was measured in the water at the end of the exposure period and MG+LMG were measured in the sampled fish. The fat content in the sampled fish was also determined.			
Species	Sex & no. of Animals	Vehicle	Route
Rainbow trout (*Salmo gairdneri*)	10 to 6 per group	Water	Via water

Effects: The concentration of MG in the exposure tank declined from 0.205 mg/l to 0.005 mg/l during the 24-hour exposure period. Following the exposure there was a clear correlation between the fat content of the fish and the elimination half-life. The higher fat content, the longer excretion times. The half-life for MG + LMG was calculated to be 43.3 days in the more fatty fish. After 80 days fish with a high fat content (fat >4%) had more than 10 times higher concentration of MG + LMG than lean fish (fat < 1 %).

Conclusions

The most important result from this study is the correlation between the fat content and the half-life of LMG (which is soluble in fat) in fish. The study show that LMG can persist for more than 20 weeks in exposed fish with a high fat content.

Plakas, S. M., El Said, K. R., Stehly, G. R., Gingerich, W. H., and Allen, J. L. (1996) Uptake, tissue distribution, and metabolism of malachite green in the channel catfish (*Ictalurus punctatus*). *Canadian Journal of Fisheries and Aquatic Sciences* **53**(6), 1427-1433.

Type of activity: Toxicokinetic study in catfish (*Ictalurus punctatus*).

Test substance: Malachite green chloride (C^{14}*, 675 MBq/mmol, >98 % pure), malachite green oxalate (purity unknown) and leucomalachite green (> 98 %).

Description: Catfish were dosed intravascularly with 0.8 mg labelled MG/kg bw (1.16 MBq/mg) or MG was added to a water tank (with the fish) to a concentration of 0.8 mg/l. The fish were exposed for 1 hour and then washed and transferred to living stream tanks. In the former case blood samples were taken at 2.5, 5, 7.5, 10, 15, 20, 30, 45 minutes, 1, 2, 4, 6, 8, and 10 hours after administration. In the latter case blood samples were taken each 15 minutes during the exposure phase and 10, 20, 30, 45 minutes, 1, 2, 4, 6, 8, and 10 hours thereafter.
An additional study of the tissue distribution was done by exposing fish in a water tank with radioactively labelled MG as described above and then analysing the distribution of radioactivity in the tissues. Tissues were analysed after 0, 2, 4, 24, 96, 168 and 336 (14 days) hours. Additional fish given unlabeled MG were analyzed at day 28 and day 42.
Fish were also exposed at pH 6, 7 and 8 as above (water tank) and analyzed after the 1-hour exposure to study the uptake of MG.
To study the urinary excretion of MG four fish were exposed as above (intravascularly) and urine samples were obtained at 0-2, 2-4, 4-6, 6-24 and 24-48 hours.
Analysis was conducted using solid phase extraction, HPLC and scintillation counting.

Species	Sex & no. of Animals	Vehicle	Route
Catfish (*Ictalurus punctatus*)	5 per group	0.85 % saline solution or water	Intravascularly or via water

Effects: The half-life for intravascularly dosed fish was 6.2 hours in plasma for MG. This is the terminal half-life, as the elimination is triphasic. LMG levels increased rapidly with a peak concentration at 0.75 hours at which time the LMG concentration had surpassed the MG concentration (0.875 µg/ml vs. 0.599 µg/ml).
When exposing the fish in the water tank the MG concentration (2.77 µg/ml) exceeded the LMG concentration (1.56 µg/ml) at the end of the 1-hour exposure period. However within another hour, the LMG concentration surpassed the MG concentration. After 10 hours LMG concentrations were 30 times higher than MG concentrations. The half-life of MG in plasma was 4.7 hours. This is the terminal half-life, as the elimination is triphasic.
MG and/or metabolites were widely distributed in all tissues. Concentrations were highest in the fat and excretory tissue; lowest in muscle and plasma. The concentration of MG in plasma reached the detection limit within 1 day while LMG in plasma persisted for 14 days. In muscle MG were more persistent with a terminal half-life of 67 hours and LMG with a terminal half-life of around 240 hours. Beyond 14 days, MG could not be quantified while LMG was quantifiable for up to 42 days. Though LMG is the major metabolite from MG additional unidentified metabolites were reported (2-21 % of total radioactivity depending on sampling time).
The pH strongly influenced the uptake of MG. At pH 8 the concentration were 8 and 5 five times higher in plasma and muscle, respectively, than at pH 6. This is probably due to the carbinol (prevalent at higher pH) form being more easily absorbed than the cation.
In the urine, less than 0.5 % of the dose was excreted over a 48-hour period.

Conclusions
The study appears to be very well conducted (though purity of MG is poorly reported). From the study results it is evident that the human exposure to LMG is likely to be much higher than the exposure to MG from fish, unless the fish has been exposed only to MG shortly before slaughter. In muscle the half-life of LMG is longer than for MG, 240 hours and 67 hours, respectively. It was noted that other substances related to MG, besides LMG, were detected in the fish in significant concentrations. Whether this was a result of metabolism of MG in the fish or due to contaminants in the tested MG is not known.

Bergwerff, A. A., Kuiper, R. W., and Scherpenisse, P. (2004) Persistence of residues of malachite green in juvenile eels (*Anguilla anguilla*). *Aquaculture* **233**(1/4), 55-63.

Type of activity: Toxicokinetic study in juvenile eels (*Anguilla anguilla*).

Test substance: Malachite green (purity unknown).

Description: Juvenile eels were exposed to MG in water initially containing 0.1 mg/l MG for 24 hours (no detectable MG left after 12 hours). The eels were transferred to a new tank with MG-free water and eels were taken for analysis at regular intervals for 100 days. For the analysis the entire eel was blended and 2 g taken for (HPLC) analysis of MG and LMG.

Species	Sex & no. of Animals	Vehicle	Route
Eels (*Anguilla anguilla*)	450 animals	Water	Via water

Effects: MG concentrations peaked after 6 hours of treatment (mean: 435 µg/ml) and then quickly declined. However, MG could be detected until day 80 (LOD: 1 µg/kg). LMG peaked 72 hours after the end of treatment (mean: 831 µg/kg) and then gradually declined until it reached 15 µg/kg at day 100. No reliable elimination half-lives could be calculated. It was evident that the AUC (area under concentration curve) was many times larger for LMG than for MG. It is noteworthy that the eels did not grow during the 100 days (bw 4.1 g (start) to 3.5 g (end)).

Conclusions
The most interesting finding from this study was that MG is fairly quickly taken up by the fish (within 12 hours), and transformed to LMG, which then is only slowly excreted/degraded from the fish.

Overall conclusion
MG in water is efficiently taken up by fish and distributed to all organs. The majority is readily converted into LMG and possibly other as yet unidentified degradation products. If the exposed fish reach the consumer, the amount of LMG present is generally expected to be higher than MG, because of the longer half-life of LMG. 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.

- 3 Toxicology

3.1 Single dose toxicity (acute toxicity)

Clemmensen, S., Jensen, J. C., Jensen, N. J., Meyer, O., Olsen, P., and Würtzen, G. (1984) Toxicological studies on malachite green: a triphenylmethane dye. *Archives of Toxicology.* **56**(1), 43-45.				
Test substance: Malachite green oxalate (> 90 %).				
Species/strain	Sex & no. of Animals	Vehicle	Route	LD_{50}
Wistar rats	5 males & 5 females per group	Water	Gavage	275 mg/kg bw
Wistar rats	5 males & 5 females	Water	Dermal	No effect at 2000 mg/kg bw

LD_{50} calculations were based on results after 48 hours. Animals alive after 48 hours were free of symptoms. The administered dose range was not reported.

Species/strain	Sex & no. of Animals	Vehicle	Route	LD_{50}
Rabbits Ssc:CPH	3 (sex unknown)	Water	Eye	-

100 µl of an aqueous MG solution (concentration of 76 mg/kg) was applied to the eyes of the rabbits according to OECD guidelines (1981). The treatment resulting in marked oedema, substantial discharge and slight hyperaemia of the conjunctiva. The effects disappeared after 24 hours in two out of the three rabbits.

Conclusions
The acute oral toxicity of MG oxalate in Wistar rats, determined as the oral LD_{50}, was reported to be 275 mg/kg bw.

Meyer, F. P., and Jorgenson, T. A. (1983) Teratological and Other Effects of Malachite Green on Development of Rainbow Trout and Rabbits. *Transactions of the American Fisheries Society* **112**(6), 818-824.				
Test substance: Testing was done with malachite green oxalate (purity unknown) claimed to be zink free.				
Species	Sex & no. of Animals	Vehicle	Route	LD_{50}
Sprague-Dawley rats	40 females	Water	Oral	520 mg/kg bw

LD_{50} calculations were based on results after 24 hours.

Conclusions
The acute oral toxicity of MG oxalate in Sprague-Dawley rats, determined as the oral LD_{50}, was reported to be 520 mg/kg bw. The purity of the test substance was not reported.

Overall conclusion
The acute oral LD_{50} values of MG (as the oxalate salt) in two different rat strains was reported to be 275 and 520 mg/kg bw.

3.2 Repeated dose oral toxicity

Clemmensen, S., Jensen, J. C., Jensen, N. J., Meyer, O., Olsen, P., and Würtzen, G. (1984) Toxicological studies on malachite green: a triphenylmethane dye. *Archives of Toxicology.* **56**(1), 43-45.

Test substance: Malachite green oxalate (> 90 %).

Type of study: Short-term (28-days) repeated oral dosing test.

Description: Groups of male and female rats were fed diets containing 0, 10, 100, and 1000 mg MG/kg for 28 days. The study included analyses of clinical signs, body weights, feed consumption, haematology, serum biochemistry (alkaline phosphatase, aspartate aminotransferase, urea, creatinine glucose and methemoglobin), organ weights, and macro- and microscopic evaluations of liver, kidney, adrenals, and testes.

Species	Strain	Sex & no. of Animals	Vehicle	Route
Rats	WIST (SPF)	8 males & 8 females per group	Diet	Oral

Effects: Effects were only seen in the high-dose group (1000 mg/kg diet). In this group the animals were hyperactive, had a significant reduction in feed intake and weight gain. Among the females an increase in lymphocytes and a concomitant decrease in neutrophils was seen. There was also a significant decrease in packed cell volume. Among the males there was a significant increase in urea levels in blood.

Conclusions

Assuming that the rats consumed 100 g feed/kg bw/day (IPCS 1987), the dose levels would be 1.0, 10.0 and 100 mg MG/kg bw/day (the initial weights of the rats in the study were in the range of 0.17-0.25 kg). From this study a NOAEL of 10 mg MG/kg bw/day can be derived based on clinical signs of hyperactivity, reduced feed intake and weight gain, haematological effects (females only) and increased blood urea (males only) at the dose level of 100 mg MG/kg bw/day.

Rao, K. V., and Fernandes, C. L. (1996) Progressive effects of malachite green at varying concentrations on the development of N-nitrosodiethylamine induced hepatic preneoplastic lesions in rats. *Tumori.* **82**(3), 280-286.

Test substance: Malachite green from commercial supplier.

Type of study: Test for enhancement of N-nitrosodiethylamine induced hepatic preneoplasia using repeated oral dosing.

Description: The animals were divided into 10 groups. Five groups were treated with the liver carcinogen N-nitrosodiethylamine (DEN) in the drinking water (200 mg/l) for 4 weeks followed by 2 weeks recovery. The five groups were then given drinking water containing 0, 25, 50, or 100 mg MG/l or 500 mg phenobarbitone (a well-known liver tumour promoter) per liter for 22 weeks. The five remaining groups were treated as above but without the initial 4-week initiation treatment with DEN. Surviving animals were killed and the liver weights were recorded. Gamma-glutamyl transpeptidase activity (GGT) was measured in liver homogenates.

Species	Strain	Sex & no. of Animals	Vehicle	Route
Rats	Wistar	15 males per group	Water	Oral (drinking water)

Effects: Administration of MG alone had no effect on the relative liver weights (liver weight/body weight). However, in the groups treated with DEN there was a significant dose-dependent increase in relative liver weights in all groups administered malachite green (MG). In the 100 mg MG/kg diet group the relative liver weight slightly exceeded the positive control (phenobarbitone) and was more than twice the size of the group given DEN alone. The increase in relative liver weight was associated with a decrease in bw. Administration of MG alone had no effect on the liver GGT levels but in all the groups also treated with DEN, MG produced a significant dose-dependent increase in the GGT levels.

Conclusions

Assuming that the rats consumed 75 ml drinking water/kg bw/day (Nielsen *et al.* 2004) (the rats in the study weighing around 0.2 kg) the dose levels would be 1.88, 3.75 and 7.5 mg MG/kg bw/day. The study showed a very clear enhancing effect of MG on N-nitrosodiethylamine induced hepatic preneoplastic lesions, even at the lowest dose of 1.88 mg MG/kg bw/day. However, the relevance for human risk assessment of this type of testing is unclear. No information was given on the purity of the test compound, and as commercial MG is often highly impure, the effect could have been due to other componemts than MG. This further weakens the usefulness, if any, of this study for risk assessment purposes.

Sundarrajan, M., Fernandis, A. Z., Subrahmanyam, G., Prabhudesai, S., Krishnamurthy, S. C., and Rao, K. V. (2000) Overexpression of G1/S cyclins and PCNA and their relationship to tyrosine phosphorylation and dephosphorylation during tumor promotion by metanil yellow and malachite green. *Toxicology Letters* **116**(1-2), 119-130.

Test substance: Malachite green from commercial supplier.

Type of study: Test for enhancement of N-nitrosodiethylamine induced hepatic preneoplasia using repeated oral dosing.

Description: 2-3 month old rats (~200 g) were divided into 6 groups. Three groups were treated with the liver carcinogen N-nitrosodiethylamine (DEN) in the drinking water (200 mg/l) for 4 weeks followed by 15 days of recovery. These three groups were then given drinking water containing 0 or 100 mg MG/l, or 500 mg phenobarbitone/l for 16 weeks, respectively. The three remaining groups were treated as above but without the initial 4-week initiation treatment with DEN. At the end of the experiment the animals were killed and tyrosine specific protein kinase activity, tyrosine phosphatase acivity and western blot analysis was conducted on liver samples.

Species	Strain	Sex & no. of Animals	Vehicle	Route
Rats	Wistar	8 males per group	Water	Oral (drinking water)

Effects: As in a previous study (Rao *et al.* 1996) the relative liver weight of rats pretreated with DEN was significantly increased (2.5 times) following 16 weeks treatment with 100 mg MG/l in the drinking water compared to the controls treated only with DEN. Tyrosine phophorylation was also significantly elevated compared to the controls while there was no effect on tyrosine phosphatase activity. Western blot results showed significantly elevated levels of PCNA (proliferating cell nuclear antigen), cyclin D1 and cyclin E after DEN plus MG treatment compared to the control group treated with DEN only.

Gupta, S., Sundarrajan, M., and Rao, K. V. (2003) Tumor promotion by metanil yellow and malachite green during rat hepatocarcinogenesis is associated with dysregulated expression of cell cycle regulatory proteins. *Teratogenesis, Carcinogenesis and Mutagenesis* **Suppl 1:301-12.**, 301-312.

Description: Treatment as described above (same study). Additional western blotting was conducted along with Northern blotting

Effects: Western blot results showed elevated levels of cdk4 (significant), cyclin B1 and cdc2. Northern blot hybridisation showed that there were elevated levels of steady-state cyclin D1, cdk4, and cyclin B1 mRNAs. A transcription assay showed that the increased mRNA levels were due to an increased rate of transcription. The authors concluded that the effect of MG is linked to disruption of the cell cycle machinery at the transcriptional level.

Conclusions

Assuming that the rats consumed 75 ml drinking water/kg bw/day (Nielsen *et al.* 2004) (the rats in study weighing around 0.2 kg) the dose level would be 7.5 mg MG/kg bw/day. The authors' conclusion that MG causes cell cycle disruption at the transcriptional level in liver cells pretreated with N-nitrosodiethylamine seems plausible.

Culp, S. J., Blankenship, L. R., Kusewitt, D. F., Doerge, D. R., Mulligan, L. T., and Beland, F. A. (1999) Toxicity and metabolism of malachite green and leucomalachite green during short-term feeding to Fischer 344 rats and B6C3F1 mice. *Chemico-Biological Interactions* **122**(3), 153-170.

Test substance: Malachite green chloride (> 94 %). Impurities: LMG (1 %) and demethylated derivatives of MG (3.5 %). Leucomalachite green (> 98 %). Impurities were MG and monodesmethyl leucomalachite green.

Type of study: Short-term (28 days) repeated oral dosing study.

Description: Rats (6-7 weeks old) were fed diets containing 0, 25, 100, 300, 600, or 1200 mg MG/kg or 0, 290, 580, or 1160 mg LMG/kg for 28 days. The study included analyses of clinical signs, body weights, feed consumption, haematology (leukocyte count, erythrocyte count, haemoglobin, hematocrit, mean erythrocyte volume, mean erythrocyte haemoglobin, mean erythrocyte haemoglobin concentration, platelet count, segmented neutrophils, lymphocytes, monocytes, eosinophils and reticulocyte count), serum biochemistry (total protein, bile acids, blood urea nitrogen, creatinine, alanine aminotransferase, alkaline phosphatase, aspartate amino transferase, glucose, cholesterol, triglycerides, γ-glutamyl transferase, albumin, sorbitol dehydrogenase, creatine kinase, sodium, potassium, chloride, calcium and phosphorous), organ weights, and macroscopic and histopathological evaluations of tissues were conducted.

Species	Strain	Sex & no. of Animals	Vehicle	Route
Rats	Fischer 344/N Nctr BR	8 males* & 8 females per group	Diet	Oral

Effects: There was a significant reduction in body weight among female rats in the 1200 mg MG/kg diet group. There was a similar but smaller and not statistically significant effect among male rats in the highest dosed group. Relative liver weight were significantly elevated among female rats in the 300, 600 and 1200 mg MG/kg diet groups and for male rats in the 600 and 1200 mg/kg group. In both sexes this was followed by significant linearly increasing levels of γ-glutamyl transferase activity (at 1200 mg MG/kg diet the increase was 4.2 times the control value in females). Females in the 1200 mg/kg group had significantly lower erythrocyte counts and haemoglobin, haematocrit, mean erythrocyte haemoglobin, and mean erythrocyte concentrations. In male rats slightly, but statistically significant, lower erythrocyte levels were seen in the 300, 600, and 1200 mg MG/kg diet groups. The only histopathological changes reported were vacuolization of hepatocytes, primarily midzonal and centrilobular, among the rats fed the diet with 1200 mg MG/kg (females: 7/8, males: 4/8).
In male rats, significantly lower body weights were seen at weeks 3 and 4 after 580 mg LMG/kg diet and at weeks 2, 3 and 4 after 1160 mg LMG/kg diet. The relative liver weight was significantly increased in all three LMG dose groups. In the highest dosed group γ-glutamyl transferase activity was statistically significantly increased (2.2 times) and phosphorous levels slightly increased (10%). Erythrocyte count, haemoglobin and hematocrit showed a slight (<6%) but statistically significant decrease. Vacuolization of hepatocytes, primarily midzonal and centrilobular, was observed in 5 of 8 males after 580 mg LMG/kg and in 7 of 8 males after 1160 mg LMG/kg diet.

Description: Mice (6-7 weeks old) were fed diets containing 0, 25, 100, 300, 600, or 1200 mg MG/kg or 0, 290, 580 or 1160 mg LMG/kg for 28 days. The study included analyses of clinical signs, body weights, feed consumption, haematology (leukocyte count, erythrocyte count, haemoglobin, hematocrit, mean erythrocyte volume, mean erythrocyte haemoglobin, mean erythrocyte haemoglobin concentration, platelet count, segmented neutrophils, lymphocytes, monocytes, eosinophils and reticulocyte count), serum biochemistry (total protein and bile acids, blood urea nitrogen, creatinine, alanine aminotransferase and alkaline phosphatase).

Species	Strain	Sex & no. of Animals	Vehicle	Route
Mice	$B6C3F_1$/Nctr BR	8 males & 8 females* per group	Diet	Oral

Effects: There was a significant reduction in body weight among female mice at the highest dose level of 1200 mg MG/diet at weeks 3 and 4. There was no effect on body weight among male mice. Among female mice there was a slight but statistically significant decrease in erythrocyte count, haemoglobin and hematocrit values in the 600 and 1200 mg MG/kg diet groups. Male mice experienced similar effects but only at 1200 mg MG/kg diet. Both erythrocyte volume (1-2 %) and reticulocyte level (1.4-1.9 times) increased significantly in the 300, 600 and 1200 mg MG/kg groups among female mice.

Male mice also had increased reticulocyte levels but only at 1200 mg MG/kg.
After a LMG diet, female mice had significantly reduced body weights after 580 and 1160 mg LMG/kg diet. All female mice in the 1160 mg LMG/kg group had scattered dead or degenerate cells in the transitional epithelium of the urinary bladder. Many cells lacked nuclei and when visible the nuclei were condensed or fragmented, suggesting apoptosis. Similar effects were not seen at the lower dietary concentrations.

Description: Rats (6-7 weeks) were given diets containing 0 or 1200 mg MG or 0 or 1160 mg LMG/kg. The study was conducted for both 4 and 21 days exposure. Total triiodothyronine (T3) and thyroxine (T4) were measured along with the thyroid stimulating hormone (TSH) level.

Species	Strain	Sex & no. of Animals	Vehicle	Route
Rats	Fischer 344/N Nctr BR	8 males* & 8 females per group	Diet	Oral

Effects: There were no effects on T3, T4 or TSH levels in male rats fed MG. Among female rats fed MG there was a significant increase in T3 levels (21 days) and a significant decrease in T4 levels (4 and 21 days).
Among male rats fed LMG there was a significant increase in TSH levels (4 and 21 days) and significant decrease in T4 levels (4 and 21 days).

*Only male rats and female mice were fed leucomalachite green.

Conclusions

Assuming that the young rats (6-7 weeks) consumed 100 g feed/kg bw/day (IPCS 1987) the dose levels in the rat studies would be 2.5, 10, 30, 60, and 120 mg MG/kg bw/day and 29, 58, and 116 mg LMG/kg bw/day. For the mouse studies the doses would be 3.75, 15, 45, 90, and 180 mg MG/kg bw/day and 43.5, 87, and 174 mg LMG/kg bw/day assuming that the mice consumed 150 mg feed/kg bw/day (IPCS 1987).
The NOAEL for MG in the rat study was 100 mg MG/kg diet corresponding to 10 mg MG/kg bw/day based on an increased relative liver weights in females and slightly lower erythrocyte counts in males after 300 mg MG/kg diet corresponding to 30 mg MG/kg bw/day.
No NOEL could be established for LMG in the male rat study as increased relative liver weight was reported for all dosed groups. Thus, the LOEL was 29 mg LMG/kg bw/day. However, in the absence of histopathological changes in the liver this effect may be adaptive to the dosing rather than an adverse response.
In the mouse, the NOAEL for MG was 100 mg/kg diet, corresponding to 15 mg MG/kg bw/day based on increased erythrocyte volume and reticulocyte levels after 45 mg/kg bw/day.
For LMG, the NOAEL in females was 43.5 mg LMG/kg bw/day based on reduced body weights seen at higher dose levels.
There were no effects on T3, T4 or TSH levels in male rats fed 120 mg MG/kg bw/day. Among female rats fed MG at the same dose there was a significant increase in T3 levels (21 days) and a significant decrease in T4 levels (4 and 21 days).
Among male rats fed 116 mg LMG/kg bw/day there was a significant increase in TSH levels (4 and 21 days) and significant decrease in T4 levels (4 and 21 days).

Overall conclusion

The overall NOAEL for MG in these short-term (28 days) repeated oral dosing studies was 10 mg/kg bw/day in rats and 15 mg/kg bw in the mouse. For LMG no clear NOAEL was established in the rat as an increased relative liver weight was seen in all dosed groups. However, this effect might have been an adaptation to the exposure to LMG and not an adverse effect. In the mouse the NOAEL for LMG was 43.5 mg/kg bw/day.
In the induced hepatic preneoplasia study there was a marked dose-responsive effect of MG at all levels from 1.88 mg/kg bw/day to 7.5 mg/kg bw/day. However the appropriateness of this type of testing in regard to risk assessment remains unclear.

3.4 Reproductive toxicity including developmental effects

<table>
<tr><td colspan="4">Study of the effects on reproduction, developmental toxicity including teratogenicity</td></tr>
<tr><td colspan="4">Meyer, F. P., and Jorgenson, T. A. (1983) Teratological and Other Effects of Malachite Green on Development of Rainbow Trout and Rabbits. Transactions of the American Fisheries Society 112(6), 818-824.</td></tr>
<tr><td colspan="4">Test substance: Malachite green oxalate (technical grade).</td></tr>
<tr><td colspan="4">Description: Three groups of pregnant rabbits were given 5, 10 and 20 mg/kg bw MG in an aqueous solution by gavage at day 6 through 18 of gestation. A negative control group was given water, and positive controls were given 150 mg thalidomide/kg bw (in corn oil). Due to maladministration into the lungs the number of animals in the 5 and 20 mg/kg bw/day groups was reduced to 16. The animals were observed for signs of toxicity and body weights were recorded every third day from day 0 to day 18 and on day 29. At day 29 of gestation the animals were killed and the progeny delivered by caesarean section. The pups were monitored for viability at 1, 2, 3, 4 and 24 hours. Then they were killed, sexed and examined for gross developmental anomalies. One third of the pups were dissected to check for visceral anomalies. The remaining pups were stained with Alizarin red S for skeletal examination. Visceral anomalies detected during stripping for skeletal staining and skeletal anomalies detected during dissection were recorded.</td></tr>
<tr><td>Species: Rabbits</td><td colspan="2">Strains: New Zealand White Rabbit</td><td>Route: Gavage</td></tr>
<tr><td colspan="4">No. of pregnant females: 20-21 in each group.</td></tr>
<tr><td colspan="2">Sex: Females</td><td colspan="2">Dose levels: 5, 10 and 20 mg/kg bw</td></tr>
<tr><td colspan="4">Duration of dosing</td></tr>
<tr><td></td><td>Prior to mating</td><td>During gestation</td><td></td></tr>
<tr><td>Males</td><td>No dosing</td><td>-</td><td></td></tr>
<tr><td>Females</td><td>No dosing</td><td>13 days</td><td></td></tr>
<tr><td colspan="4">Effects (dams): Average body weights were consistently lower in the treated groups than in the controls. The MG treated animals showed a reduced feed consumption of dry feed. The animals treated with MG gained 60 g in weight at 5 mg MG/kg bw/day, lost 30 g in weight at 10 mg MG/kg bw/day and lost 60 g in weight at 20 mg/kg bw/day while the negative controls gained 230 g during the study period. The variation was however so large that only the 10 mg MG/kg bw/day group were significantly different from controls ($p<0.05$). No other signs of overt toxicity were reported for the dams.
Effects (pups): There was a significant decrease in the number of living foetuses in all treated groups (no dose response) and significant pre-implantation losses in all treated groups (dose response related). Pups from the 5 mg/kg bw/day group had a significantly lower body weight at caesarean section than negative controls. Pups from the 10 and 20 mg/kg bw/day groups were also lighter than controls but the differences were not statistically significant and the weight reduction was smaller than in the 5 mg/kg group.
All MG treated groups had a significantly higher number of anomalies than controls. There was no dose related response, though the incidence of anomalies was highest in the 20 mg/kg bw/day group. In general, the incidence of anomalies in the groups treated with MG was twice the number in the negative controls and half the number in the positive controls. All MG treated groups had significantly increased skeletal anomalies. The 5 and 20 mg/kg groups also had significantly elevated numbers of gross and visceral anomalies.</td></tr>
</table>

Conclusions

The poor reporting on the purity of test compounds generates uncertainty as to whether the toxicity observed was due to MG alone. Pooling of results from litters is incorrectly reported. Despite these shortcomings the study showed that the test compounds produced developmental toxicity and teratogenic effects. The toxic effect (dose related decreased weight gain or weight loss) seen in the dams appears too small to explain the effects upon the pups. A NOAEL could not be established because higher incidences of anomalies (gross, visceral, skeletal) were observed in all treated groups compared to controls. 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 LOAEL was 5

mg/kg bw/day, the lowest dose applied in the study. Additional reproductive toxicity studies are needed to properly assess the hazards of MG and LMG in this regard.

3.5 Mutagenicity

3.5.1 *In vitro* studies

Clemmensen, S., Jensen, J. C., Jensen, N. J., Meyer, O., Olsen, P., and Würtzen, G. (1984) Toxicological studies on malachite green: a triphenylmethane dye. *Archives of Toxicology.* **56**(1), 43-45.
Test substance: Malachite green oxalate (> 90 %).
Type of study: Ames test/ Point mutation study.
Description: Selected strains were tested according to the standard quantitative plate incorporation test (OECD 1981). Testing was done with and without metabolic activation (S-9 mix). Strains were tested at concentrations of 0.05, 0.26, 1.28, 6.4, 32 and 160 µg/plate.
Test system: *Salmonella typhimurium.*
Strains: TA 98, 100, 1535, and 1537.
Effects: Cell toxicity was usually encountered at 1.28 µg/plate without S-9 mix. There were no mutagenic effects in TA 100, 1535, and 1537 both with and without S-9 mix, and in TA 98 without S-9 mix. However, a significant increase in revertants was seen in TA 98 with S-9 mix. A supplementary investigation using a narrower concentration range showed a concentration dependency in the range of 20-70 µg/plate.

Conclusions

Malachite green caused mutations in TA-98 with metabolic activation (S-9 mix), but not in any of the other strains tested or in TA-98 without metabolic activation. Mutations in TA-98 relate to frameshift mutations that are normally seen for aromatic amines.

Fessard, V., Godard, T., Huet, S., Mourot, A., and Poul, J. M. (1999) Mutagenicity of malachite green and leucomalachite green in in vitro tests. *Journal of Applied Toxicology* **19**(6), 421-430.
Test substance: Malachite green oxalate (70.8 %) and leucomalachite green.
Type of study: Mutagenicity *in vitro* study.
Strains: Chinese hamster ovary cells (CHO-K1) and *Salmonella typhimurium* strains: TA97a, TA98, TA100, and TA102.
Test system: Ames mutagenicity test (OECD guideline 471, 1983), mammalian gene mutation test (OECD guideline 476, 1984), comet assay, and trypan blue exclusion method (cell viability measurement).
Description: Ames test was conducted with and without metabolic activation using *S. typhimurium* strains with each concentration tested in triplicate. Concentrations were 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 µg MG/per plate, and 10, 50, 100, 250, 500, 1000 and 2000 µg LMG/per plate. Appropriate positive controls were included. In the mammalian gene mutation CHO-K1 cells in a monolayer culture were exposed to test substances for 5 hours (with and without metabolic activation). Concentrations were 0.001, 0.005, 0.01, 0.05, 0.1, 0.25, 0.5 and 1 µg/ml MG and 5, 10, 50, 75 and 100 µg/ml LMG. Appropriate positive control was included. In the comet assay a monolayer culture (CHO-K1) were exposed to test substances for 1 hour (with and without metabolic activation). DNA damage was measured (in duplicate and in two independent assays). Cell viability was measured immediately after treatment (trypan blue exclusion method). Concentrations were 1, 2, 3, 4, 5 and 10 µg MG/ml (without S-9 mix), 1, 2.5, 5, 7.5, 10, 15 and 20 µg MG/ml (with S-9 mix), 5, 10, 50, 75, 100, 250 and 500 µg LMG/ml (without S-9 mix) and 25, 50, 75, 100, 125, 150, 175, 200 and 300 µg LMG/ml (with S-9 mix).

Effects: Neither MG or LMG had any mutagenic activity in the Ames test. MG was cytotoxic at concentrations higher than 0.1 µg/per plate with a clear dose-response relationship above 0.5 µg/plate. LMG was only cytotoxic at the two highest concentrations (1000 and 2000 µg/ml). No significant mutant frequencies were found in the mammalian gene mutation test for MG. MG was strongly cytotoxic at concentrations higher than 0.1 µg/ml, which limited the test range to 0.001-0.05 µg/ml. LMG was cytotoxic at concentrations higher than 500 µg/ml. Without metabolic activation mutant frequencies was above control values only at one dose (75 µg/ml). With metabolic activation, LMG significantly increased the mutant frequency at 5 µg/ml in one experiment only. In the comet assay without metabolic activation, MG produced DNA damage at 3 µg/ml and above (significant effect) inducing immediate cytotoxic effect. With metabolic activation the threshold for DNA damage was increased to 15 µg/ml again followed by a decrease in cell viability. There was no significant effect on DNA damage and cell viability for LMG, with and without metabolic activation.

Conclusions

There were very little, if any, indications of a mutagenic potential of LMG and MG. However, the mutagenic testing of MG was hampered by the strong cytotoxicity of MG towards bacterial and mammalian cells. The cytotoxicity of MG was more severe in this study than previously seen by Clemmensen *et al*. 1984. This means that TA 98 was not tested in this study in the concentration range reported to be mutagenic in the Clemmensen *et al.* study. It should be noted that the MG used in this study by Fessard *et al.* was of much lower purity compared to the MG used in the Clemmensen *et al.* study.

3.5.2 *In vivo* studies

Clemmensen, S., Jensen, J. C., Jensen, N. J., Meyer, O., Olsen, P., and Würtzen, G. (1984) Toxicological studies on malachite green: a triphenylmethane dye. *Archives of Toxicology.* **56**(1), 43-45.				
Test substance: Malachite green oxalate (> 90 %).				
Type of study: Micronucleus test/clastogen effect study.				
Description: The animals were administered the maximum tolerated dose (37.5 mg/kg bw) and killed 24, 42 and 66 hours later. Bone marrow smears were prepared from each animal and stained with May-Grünwald/Giemsa. 1000 polychromatic erythrocytes were counted. The test was conducted according to OECD guidelines 1981. A positive control group received 50 mg cyclophosphamide/kg bw.				
Test system	Strain	Sex & no. of Animals	Vehicle	Route
Mice	NMRI: BOM (SPF)	5 per group	Water (presumably)	Gavage
Effects: There were no indications of any clastogenic effect. The micronuclei count was lower in the dosed animals than in the negative control.				

Conclusions

There were no indications of any clastogenic effect. The micronuclei count was lower in the dosed animals than in the negative control. The sex of the mice was not reported. It was not demonstrated that the test compound had reached the bone marrow.

Jensen, N. J. (1984) Lack of mutagenic activity of malachite green in the mammalian spot test. *Mutation Research*. **130**(3), 248.				
Test substance: Malachite green oxalate (purity unknown).				
Type of study: Mammalian spot test.				
Description: Description: The mice were breed with T-stock mice and were administered the test substance on days 8, 9 and 10 of pregnancy. The dose levels used were 10, 20, and 40 mg/kg bw/day, respectively.				
Test system	Strain	Sex & no. of Animals	Vehicle	Route
Mice	C57B1/6J Han	Females, number not reported	Water (presumably)	Gavage
Effects: No significant increase in recessive spots in any dosed group compared to the negative controls.				

Conclusions

No reporting of group sizes. MG had no effect in the mammalian spot test

Culp, S. J., Blankenship, L. R., Kusewitt, D. F., Doerge, D. R., Mulligan, L. T., and Beland, F. A. (1999) Toxicity and metabolism of malachite green and leucomalachite green during short-term feeding to Fischer 344 rats and B6C3F1 mice. *Chemico-Biological Interactions* **122**(3), 153-170.				
Test substance: Malachite Green (> 94 %). Impurities: LMG (1 %) and demethylated derivatives of MG (3.5 %). Leucomalachite green (> 98 %). Impurities were MG and monodesmethyl leucomalachite green.				
Type of study: Genotoxicity study.				
Description: Rats (6-7 weeks old) were given diets containing 0, 100, or 600 mg MG/kg or 0, 96, or 580 mg LMG/kg for 28 days. Body weights were recorded and after the dosing period liver DNA adducts were measured using ^{32}P-postlabeling and TLC (thin layer chromatography) separation.				
Species	Strain	Sex & no. of Animals	Vehicle	Route
Rats	Fischer 344/N Nctr BR	8 males per group	Diet	Oral
Effects: MG and LMG gave rise to a single DNA adduct (or co-eluting adducts). The adduct levels increased significantly as a function of the dose. Among the rats the adduct levels did not differ between groups administered an equimolar dose of MG and LMG.				
Description: Mice (6-7 weeks) were given diets containing 0, 100, or 600 mg MG/kg or 0, 96, or 580 mg LMG/kg for 28 days. Body weights were recorded and after the dosing period liver DNA adducts were measured using ^{32}P-postlabeling and TLC separation.				
Species	Strain	Sex & no. of animals	Vehicle	Route
Mice	B6C3F$_1$/Nctr BR	8 females per group	Diet	Oral
Effects: MG and LMG gave rise to a single DNA adduct (or co-eluting adducts). The adduct levels increased significantly as a function of the dose of MG treatment. There was an apparent dose related response for LMG, but the effect was not statistically significant at any of the doses. Among the mice the adduct levels differed between groups administered an equimolar dose of MG and LMG, with MG promoting a much larger adduct generation than LMG.				

Conclusions

The quality of the study is high. Assuming that the young rats (6-7 weeks old) consumed 100 g feed/kg bw/day (IPCS 1987) the corresponding dose levels would be 10 and 60 mg MG/kg bw/day and 9.6 and 58 mg LMG/kg bw/day. For the mice the doses would be 15 and 90 mg MG/kg bw/day and 14.4 and 87 mg MG/kg bw/day, assuming that the mice consumed 150 g feed/kg bw/day.
MG and LMG gave rise to a single DNA adduct (or co-eluting adducts) in male rats. The adduct levels increased significantly as a function of the dose and did not differ between groups administered equimolar doses of MG and LMG. In female mice, MG and LMG also gave rise to a single DNA adduct (or co-eluting adducts), the levels of which increased significantly as a function of the dose for

MG but not for LMG. Thus, in the female mouse MG produced much higher adduct levels than LMG at equimolar doses.

Culp, S. J., Beland, F. A., Heflich, R. H., Benson, R. W., Blankenship, L. R., Webb, P. J., Mellick, P. W., Trotter, R. W., Shelton, S. D., Greenlees, K. J., and Manjanatha, M. G. (2002) Mutagenicity and carcinogenicity in relation to DNA adduct formation in rats fed leucomalachite green. *Mutation Research* **506-507**, 55-63.

Test substance: Leucomalachite green (98 %). Impurities were monodesmethyl LMG and MG.

Type of study: Genotoxicity study.

Description: Young rats (6 weeks old) were fed a diet with 0 (control), 9, 27, 91 or 272, 543 mg LMG/kg for either 4, 16 or 32 weeks. Body weights and feed consumption was measured weekly. At the end of the feeding period, animals were killed and their livers were used for *lacI* mutational analysis. An additional 24 rats (four per dose group) were fed the same doses for 4 weeks, killed and their livers used for DNA adduct analysis. Liver DNA adducts were measured by ^{32}P-postlabeling and TLC separation.

Test system	Strain	Sex & no. of animals	Vehicle	Route
Rats	Big Blue	6 females per group	Diet	Oral

Effects: There was no significant increase in *lacI* mutation frequency at either 4 or 32 weeks at any dose. At 16 weeks there was a significant increase in the highest dose group. However, on continuous feeding with the test substance the mutation frequency should not decline and therefore also be detectable at 32 weeks. A closer examination of the data indicated that the significant effect at 16 weeks was due to a proliferative response of background mutations.
LMG gave rise to a single DNA adduct (or co-eluting adducts) among the 91, 272 and 543 mg/kg diet groups. The adduct co-eluted with the adduct detected in a previous study (Culp *et al.* 1999) using Fischer 344 rats. The adduct levels increased significantly with a linear dose-response trend.

Conclusions

The quality of the study is high. Assuming that the young rats (6 weeks old) consumed 100 g feed/kg bw/day (IPCS 1987) the corresponding dose levels would be 9, 2.7, 9.1, 27.2 and 54.3 mg LMG/kg bw/day. LMG did not produce any significant increase in the *lacI* mutation frequencies and changes in the mutation spectrum of *LacI* mutants.

Manjanatha, M. G., Shelton, S. D., Bishop, M., Shaddock, J. G., Dobrovolsky, V. N., Heflich, R. H., Webb, P. J., Blankenship, L. R., Beland, F. A., Greenlees, K. J., and Culp, S. J. (2004) Analysis of mutations and bone marrow micronuclei in Big Blue rats fed leucomalachite green. *Mutatation Research* **547**(1-2), 5-18.

Test substance: Leucomalachite green (98 %). Impurities were monodesmethyl LMG and MG.

Type of study: Genotoxicity study.

Description: This study is a follow up of the study of Culp *et al*. 2002. The tested animals are the same. In addition to the previously conducted analysis the following examinations were performed: *Hprt* lymphocyte mutant assay of the spleen, bone marrow micronucleus assay, DNA sequencing of *LacI* mutants (liver), histopathological examination of the liver, and determination of liver cell proliferation indices (proliferating cell nuclear antigen, PCNA).

Test system	Strain	Sex & no. of animals	Vehicle	Route
Rats	Big Blue	6 females per group	Diet	Oral

Effects: There was no significant effect of LMG in the *Hprt* lymphocyte mutant assay and the bone marrow micronucleus assay. DNA sequencing of the *LacI* mutants showed that there was no significant difference in the mutation spectrum between controls and LMG treated rats (543 mg/kg diet, 16 week group). When the previously significant result was corrected for clonality the significant effect disappeared. Together this underlines that there was no effect of LMG treatment on the *lacI* mutation frequency.
Among the rats fed LMG there was a significant higher frequency of hypertrophic hepatocytes. The effect was significant in the two highest dose groups (272 and 543 mg/kg diet) after 32 weeks. At 91

mg/kg diet and below there was no effect. No other liver anomalies were observed. The cell proliferation/apoptotic index demonstrated that the rats had a significantly higher frequency of S-phase cells in the highest dose group (32 weeks). There were also more apoptotic cells but the frequency was not significantly different from the controls.

Conclusions

The study further underlines the lack of a mutagenic effect of LMG in female Big Blue rats. Following 27.2 and 54.2 mg LMG/kg bw/day for 32 weeks a significant higher frequency of hypertrophic hepatocytes were seen, probably due to an increase in liver cell proliferation. The NOAEL was 9.1 mg LMG/kg bw/day. It was not demonstrated that the test compound had reached the bone marrow.

Mittelstaedt, R. A., Mei, N., Webb, P. J., Shaddock, J. G., Dobrovolsky, V. N., McGarrity, L. J., Morris, S. M., Chen, T., Beland, F. A., Greenlees, K. J., and Heflich, R. H. (2004) Genotoxicity of malachite green and leucomalachite green in female Big Blue B6C3F1 mice. *Mutation Research* **561**(1-2), 127-138.

Test substance: Malachite green chloride (88 %). Impurities: primarily LMG and desmethyl derivatives of MG and LMG. Leucomalachite green (99 %).

Type of study: Genotoxicity study.

Description: Mice (6 weeks old) were fed diets for 4 or 16 weeks with either 0 (control) or 450 mg MG/kg, or 204 or 408 mg LMG/kg. Chemical analysis of the feed indicated that the actual doses were within 3 % of the targeted doses. Six mice from each group were killed after 4 weeks and the remainder after 16 weeks of exposure. The blood was used for micronucleus analysis, the spleen for lymphocyte *Hprt* assay, and the liver used in the *cII* mutant assay. *Hprt* mutant frequency was examined after 4 and 16 weeks of exposure in the 450 mg/kg diet MG group, and the 204 and 408 mg/kg diet LMG group. Liver *cII* mutant frequency was examined after 16 weeks of exposure in the 450 mg/kg diet MG group and in the 408 mg/kg diet LMG group. Additionally the mutations in the liver *cII* mutant spectrum were characterised.

Test system	Strain	Sex & no. of animals	Vehicle	Route
Mice	Big Blue $B6C3F_1$	12 females per group	Diet	Oral

Effects: There were no significant effects on either reticulocyte or normochromatic erythrocyte peripheral blood micronucleus frequencies. Positive controls were significantly elevated.
According to ANOVA analysis there was a small statistically significant difference among the groups in the *Hprt* mutant frequency after 4 weeks, but the difference was likely due to a low mutant frequency in the group given 204 mg LMG/kg diet. After 16 weeks there was no significant effect.
An ANOVA analysis indicated an elevated *cII* mutant frequency in the mice fed LMG but not in the mice fed MG. Results from a previous study with female Big Blue rats fed a diet containing 543 mg LMG/kg was also analysed but did not however reveal any increase in *cII* mutant frequency. Analysing the spectrum of mutations, there was no difference between the spectra from controls and mice fed MG (and rats fed MG), but there was a difference between controls and mice fed LMG. Mice fed LMG had increased frequency of G → A and A → T transversions. These transversions are the ones, which are normally produced by bulky arylamine carcinogens.

Conclusions

The experiment seems to be well conducted though the animals feed intake was not recorded. Assuming that the mice consumed 150 g feed/kg bw/day (IPCS 1987) the dose levels would be 67.5 mg MG/kg bw/day and 30.6 and 61.2 mg LMG/kg bw/day. No mutagenic effect was observed for MG. However, the results could indicate a mutagenic effect of LMG in the liver of female Big Blue mice fed the highest dose level of 61.2 mg LMG/kg bw/day for 16 weeks.

Overall conclusion

MG was reported to be mutagenic in the Ames test (only TA-98 with metabolic activations) in one study but this could not be confirmed in another study. MG did not show mutagenicity in the

mammalian spot test and showed no clastogenic effects in the micronuleous test in bone marrow of mice. MG produced a single DNA adduct in the liver of male Fisher rats and female $B6C3F_1$ mice. However, MG did not induce micronuclei in erythrocytes, mutations in lymphocytes, or mutations in liver cells of female Big Blue $B6C3F_1$ transgenic mice.
It is concluded that although MG can produce DNA adducts in the liver of male rats and female mice the weight of evidence shows that it is not genotoxic in conventional *in vitro* and *in vivo* assays and it did not produce mutations in transgenic female mice.

LMG did not show mutagenicity in the Ames test. LMG produced a single DNA adduct in the liver of male Fisher rats and to a much lesser extent in female $B6C3F_1$ mice (not significant). LMG did not induce mutations in lymphocytes, micronucleous of the bone marrow, or *lacI* mutation in the liver of female Big Blue transgenic rats. Following 27.2 and 54.2 mg LMG/kg bw/day for 32 weeks a significant higher frequency of hypertrophic hepatocytes was seen in these animals, probably due to an increase in liver cell proliferation. In female Big Blue $B6C3F_1$ transgenic mice LMG did induce *cII* mutations in liver cells, but not micronuclei in erythrocytes or mutations in lymphocytes.
It is concluded that although LMG can produce DNA adducts in the liver of male and female rats and female mice (in mice only borderline) it was not genotoxic in conventional *in vitro* and *in vivo* assays and did not induce mutations in transgenic female rats. However, in transgenic female mice LMG induced mutations in liver cells, but only at the highest dose tested.

3.6 Carcinogenicity

Culp, S. J., Mellick, P. W., Trotter, R. W., Greenlees, K. J., Kodell, R. L. and Beland, F. A. (2006) Carcinogenicity of malachite green chloride and leucomalachite green in B6C3F1 mice and F344 rats. *Food Chemical Toxicology* **44**(8), 1204-1212.

Test substance: Malachite green chloride (> 87 %). Impurities: LMG (7.5 %), N-desmethyl MG (3.8 %), N-desmethyl LMG (0.5 %) and methanol (1.4 %). Leucomalachite green (> 99 %).

Type of study: Carcinogenicity study.

Description: Young rats (6 weeks old) were fed diets with 0, 100, 300 or 600 mg MG/kg (females only) or an equimolar amount of LMG (0, 91, 272 or 543 mg/kg diet) for 104 weeks. Feed consumption and body weights were recorded weekly for the first 12 weeks and every 4 weeks thereafter. At the end of the study complete necropsies and histopathological examinations of a number of tissues were performed on all animals, including those that died prematurely or became moribund.

Species	Strain	Sex & no. of animals	Vehicle	Route
Rats	Fischer 344/N Nctr Br	48 males* & 48 females per group	Diet	Oral

Effects: Rats fed MG generally had the same feed consumption as the control group. Male rats fed LMG had a lower feed consumption at the highest dose level (543 mg/kg diet) than controls and female rats had a lower feed consumption in the 272 and 543 mg LMG/kg diet groups than controls. The diet levels of malachite green of 100, 300 and 600 mg/kg were determined as an intake of 7, 21 and 43 mg/kg bw/day, respectively. The diet levels of leucomalachite green of 91, 272 and 543 mg/kg were determined as an intake for females of 6, 17 and 35 mg/kg bw/day, respectively, and for males to 5, 15 and 30 mg/kg bw/day, respectively.
Consumption of MG and LMG reduced the body weights in the rats with a clear dose-response effect. The higher the dose the sooner the weight of the treated animals dropped behind the controls. The effect of LMG was more severe than the effect of MG. Female rats was more severely affected than male rats. Compared to controls the final body weights were 98, 90 and 88 % for groups of females treated with MG, 95, 90 and 77 % for the groups of females treated with LMG and 99, 93 and 89 % for the males treated with LMG. MG and LMG did not affect the survival of the rats (except male rats dosed with 272 ppm LMG which had increased survival).
Female rats fed MG had an increasing trend in the incidence of thyroid gland follicular cell adenoma or carcinoma (only statistically significant at the 300 mg/kg dietary level). A dose related increase in hepatocellular adenomas was also observed in the female rats fed MG (statistically significant at the 600 mg/kg dietary level). The female rats administered MG also had an increasing trend in the

incidence of mammary gland adenomas or carcinomas.
Female and male rats administered LMG also had a low incidence of thyroid gland follicular cell adenoma or carcinoma (2-7 %). Though not significant this neoplasm was not seen in controls. There was no clear increase in the incidence of hepatocellular adenomas in the rats fed LMG. Among female rats fed a diet containing 272 mg LMG/kg there was a significant increase in the incidence of mammary gland adenomas or carcinomas. The male rats given LMG had a dose related increasing trend in interstitial cell adenoma of the testis. The effect was significant at all dose groups. Mononuclear cell leukaemia was significantly reduced in a dose-related trend in rats exposed to MG (females) and LMG (males/females). Male rats also had a decreasing incidence in pituitary gland adenoma when fed LMG, with the decrease being significant at all doses. No such effect was seen among females.

Description: Young female mice (6 weeks old) were fed diets with 0, 100, 225 or 450 mg MG/kg or an equimolar amount of LMG (0, 91, 204 or 408 mg/kg) for 104 weeks. Food consumption and body weights were recorded weekly for the first 12 weeks and every 4 weeks thereafter. At the end of the study complete necropsies and histopathological examinations of a number of tissues were performed on all animals, including those that died prematurely or became moribund.

Species	Strain	Sex & no. of Animals	Vehicle	Route
Mice	B6C3F$_1$/Nctr BR	48 females per group	Diet	Oral

Effects: Female mice fed MG or LMG had the same food consumption as the control group. The diet levels of malachite green of 100, 225 and 450 mg/kg were determined as an intake of 15, 33 and 67 mg/kg bw/day, respectively. The diet levels of leucomalachite green of 91, 204 and 408 mg/kg were determined as an intake for females of 15, 31 and 63 mg/kg bw/day, respectively.
There was no effect of MG or LMG upon the body weights of the mice. MG and LMG did not affect the survival of the mice. No treatment related neoplasms were found in female mice fed MG.
Among mice, hepatocellular adenomas or carcinomas in female mice fed LMG were the only neoplasm with an increasing trend, with the incidence being significant in the highest dose group.

*Only female rats were fed malachite green.

Conclusions

This study is of high quality. A number of adenomas and carcinomas were observed in rats given MG and LMG, these were thyroid gland follicular cell adenoma or carcinoma, hepatocellular adenomas and mammary gland adenomas or carcinomas. Furthermore, the male rats given LMG had a dose related increasing trend in interstitial cell adenoma of the testis. The effect was significant at all dose groups. However, the background incidence of this tumour type is very high in the rat strain used and the relevance of the changes for human risk assessment is doubtful. The different types of adenomas and carcinomas observed in rats dosed with MG and LMG are probably caused by a non-genotoxic mechanism. This is supported by the overall conclusion from the studies on mutagenicity that MG and LMG are not genotoxic in conventional *in vitro* and *in vivo* assays in rats. The NOAEL for MG in female rats was 7 mg/kg bw/day based on a significant increase in the incidence of thyroid gland follicular cell adenoma or carcinoma. The NOAEL for LMG in rats was 6 mg/kg bw/day based on a significant increase in the incidence of mammary gland adenomas or carcinomas in female rats.
No treatment related neoplasms were found in female mice fed MG.
The clearest finding in the study is the increase in hepatocellular adenomas or carcinomas in female mice fed LMG. Generally, hepatocellular carcinomas in mice are disregarded when they are the only detectable tumour form. Stimulation of the cell division by the test compound can generate liver tumours in mice. However, because of the induction of mutations in liver cells by high doses of LMG in female transgenic mice a genetoxic mechanism cannot be excluded.

- **4 Other effects**

4.1 Contact allergy

Clemmensen, S., Jensen, J. C., Jensen, N. J., Meyer, O., Olsen, P., and Würtzen, G. (1984) Toxicological studies on malachite green: a triphenylmethane dye. *Archives of Toxicology* **56**(1), 43-45.				
Test substance: Malachite green oxalate (> 90 %).				
Type of study: Guinea pig maximization test of Magnusson and Kligman (1970).				
Description: First induction by injection of 50 µl 0.2 % solution and second induction by topical application of 400 µl 20 % solution for 48 hours. Challenge was conducted after 7 days with topical application of 0.05 %, 0.1 % and 1 % solutions respectively.				
Species	Strain	Sex & no. of animals	Vehicle	Route
Guinea pig	Scc: AL	Not reported	Water	Injection/dermal
Effects: No effect was observed. NOAEC 1 % Malachite green oxalate in water.				

Conclusions

The number of animals was not reported. This test for skin sensititation is not considered relevant for risk assessment of oral exposure to MG.

4.2 Cytotoxicity, enzyme inhibition and other in vitro studies

Rao, K. V., and Fernandes, C. L. (1996) Progressive effects of malachite green at varying concentrations on the development of N-nitrosodiethylamine induced hepatic preneoplastic lesions in rats. *Tumori.* **82**(3), 280-286.
Test substance: Malachite green from commercial supplier.
Type of study: In vitro study of MG effect on hepatocytes.
Description: Rats (wistar) liver tumours were induced with N-nitrosodiethylamine and hepatocytes were isolated. The hepatocytes were subjected to MG at concentrations of 0.025, 0.05, 0.1, 0.2 and 0.4 µg/ml. DNA synthesis was determined after 39 hours. Lactate dehydrogenase (LDH) activity was measured after 24 and 39 hours.
Test system: Hepatocytes isolated from rat tumours.
Strains: NA.
Effects: There was only a marginal effect on LDH activity after 24 hours. But after 39 hours MG produced a dose dependent inhibition of DNA synthesis (at all concentrations) and an increased LDH release (parameter for cell lysis). Inhibition of DNA synthesis was proportional to the LDH release.

Conclusions

The lack of knowledge about the composition of the test substance limits the usefulness of the result from this study. As commercial malachite green is often highly impure the effect could as well be due to a contaminant.

Stammati, A., Nebbia, C., Angelis, I. D., Albo, A. G., Carletti, M., Rebecchi, C., Zampaglioni, F., and Dacasto, M. (2005) Effects of malachite green (MG) and its major metabolite, leucomalachite green (LMG), in two human cell lines. *Toxicology In Vitro.* **19**(7), 853-858.
Test substance: Malachite green and leucomalachite green was analytical grade chemicals bought from Sigma Aldrich.
Type of study: Cytotoxicity study.
Strains: Two human tumour cell lines: Caco-2 and HEp-2.

Test system: Cytotoxicity was measured by neutral red uptake (NRU), total protein content (TPC), lactate dehydrogenase leakage (LDH), tetrazolium conversion (MTT) and proliferation capability by colony-forming ability test (CFA).
Description: Cell lines were cultured and seeded into 96 well plates and exposed MG and LMG. HEp-2 cells was exposed to 0.26, 1.4, 2.6 or 4.0 µM MG or 30, 150, 300 or 610 µM LMG for 24 hours. Caco-2 was exposed to 0.1, 1, 10, 25, 50 or 100 µM MG or 25, 50, 75 or 100 µM LMG for 24 hours. MG was dissolved in water while LMG was dissolved in DMSO (less than 1 % final conc.). Controls contained similar solvent concentrations as samples.
Effects: MG produced a clear dose dependent inhibition of the viability (NRU & TPC) of HEp-2 cells. The effect was significant at concentrations of 1.4 and 4.0 µM. CFA was also inhibited with a significant inhibition at 2.6 and 4.0 µM MG. Contrary, there was almost no effect of LMG on NRU, TPC and CFA. Only a slight inhibition of TPC was observed at 610 µM LMG (not significant). There was a significant dose related cytotoxicity of MG on Caco-2 cells measured by MTT, LDH and NRU. In the MTT testing the effect was significant from 1 µM MG (25 µM in LDH and 10 µM in NRU testing). By contrast there was no effect of LMG.

Conclusions

Poor reporting on purity of test compounds. Striking difference between the effect of MG and LMG showing the strong cytotoxicity of MG.

Doerge, D. R., Chang, H. C., Divi, R. L., and Churchwell, M. I. (1998) Mechanism for inhibition of thyroid peroxidase by leucomalachite green. *Chemical Research in Toxicology* **11**(9), 1098-1104.
Test substance: Malachite green (96 %) and leucomalachite green (99 %).
Type of study: Enzyme inhibition study.
Enzymatic reactions: Tyrosine iodination, Iodination and coupling in Goiter thyroglobulin and TPO-catalyzed coupling in preiodinated Goiter Thyroglobulin.
Test system: LMG or MG were added to enzymatic reaction mixtures and the degree of enzymatic inhibition was detected by HPLC/UV measurements.
Description: Inhibition of thyroid peroxidase (TPO) catalyzed tyrosime iodination by LMG was tested at 0, 5, 15 and 30 µM LMG. Reactions were carried out at pH 6.5 (37 ºC) and measured at various times from 0-360 seconds. Inhibition of TPO catalyzed tyrosine iodination and thyroid hormone synthesis by LMG was measured in human goiter thyroglobulin at concentrations of 0, 15 and 30 µM LMG. Reactions were carried out at pH 7.0 (37 ºC) and measured at various times from 0-60 minutes. Inhibition of TPO catalyzed formation of T3 and T4 was carried out using preiodinated goiter thyroglobulin and was tested at 0 and 30 µM LMG, and 30 µM MG. Reactions were carried out at pH 7.0 (37 ºC) and measured at various times from 0-60 minutes.
Effects: All concentration of LMG clearly inhibited the initial rate of TPO catalyzed tyrosine iodination in a dose-responsive manner. After 60 seconds, the inhibition was more than 50 % for 5 µM LMG and 85 % for 30 µM LMG. LMG also inhibited the TPO-catalyzed coupling of iodotyrosyl residues on thyroglobulin to form thyroglobulin bound T4 and T3 (assay measure both iodination of tyrosyl residues and coupling). Inhibition by 30 µM LMG was more than 80 % at 30 minutes. In the final experiment it was shown that MG and LMG both inhibited (around 50 %) the rate and extent of TPO-catalyzed coupling throughout the 60 min. study. LMG was a bit more inhibitive than MG.

Conclusions

The results show that LMG and MG have potential for disturbing the thyroid hormone homeostasis. When thyroid hormone synthesis is inhibited chronically it results in prolonged growth stimulus from TSH that can lead to thyroid follicular cell tumours.

Rao, K. V., Mahudawala, D. M., and Redkar, A. A. (2001) Abrogation of cell cycle checkpoint controls during malignant transformation of syrian hamster embryo cells is associated with decreased sensitivity to apoptosis. *Journal of Environmental Pathology, Toxicology and Oncology* **20**(3), 177-188.
Test substance: Malachite green from commercial supplier.
Type of study: In vitro study of MG effect on cell cycle checkpoint control in SHE cells.
Strains: Syrian hamster embryo (SHE) cells.
Test system: SHE cells were aseptically collected from foetuses at gestation day 10-14. Cells were then grown in liquid growth media. Cells were spread on Petri dishes and were treated with 0.05 µg MG/ml dissolved in sterile saline for five days after the seeding (negative controls were treated with sterile saline). This treatment was repeated each week for 6 weeks. After 6 weeks morphologically transformed foci were classified and one of the foci was used to establish an immortal cell line that was used for further studies.
Description: Control (untransformed) cells and transformed cells were studied using chromosomal analysis, tumorigenicity, G2/M arrest, flow cytometric analysis and MG induced apoptosis.
Effects: Chromosomal analysis revealed that the chromosomes of the transformed cell line were aneuploid contrasting the untransformed diploid cell line. The transformed cells also produced tumours in nude mice 7-10 days after injection. In G2/M analysis control cells and transformed cells were exposed to 0.1 µg MG/ml. Analysis of DNA showed that control cells accumulated progressively in the G2/M phase while transformed cells were unaffected. This indicate that the G2/M checkpoint control is defective in the transformed cell line. Using flow cytometric analysis it was shown that MG (0.0065µg/ml – 0.5 µg/ml) induced increasing apoptosis in control cells. Transformed cells only showed marginal increased apoptosis.

Conclusions

Poor reporting on purity of test compounds. The study showed the ability of MG to affect the regulation of cellular growth in SHE cells. It also showed the ability of MG to generate chromosomal damages resulting in (at least one) cell lines more resistant to the effect of MG but with defect regulatory mechanism for control of cell growth.

Overall conclusion

MG in contrast to LMG is highly cytotoxic which is likely the reason it is such an effective aquatic fungicide, parasiticide and bacteriocide. MG has been shown to inhibit DNA synthesis in hepatocytes, generate G2/M arrest in SHE cells and disturb thyroid hormone homeostasis *in vitro*. This latter effect was also seen *in vivo* in the 28 days repeated dose toxicity study at high doses and might help to explain the reported increased incidence of thyroid gland follicular cell adenoma or carcinoma in female rats in the carcinogenicity study of Culp *et al.* 2006.

4.3 Observations in humans

No studies were reported in public literature

4.4 Neurotoxicity and delayed neurotoxicity

No studies were reported in public literature

- 5 Overall conclusion

5.1 Summary of NOAELs and all relevant studies

Malachite green

Species	*Type of salt and purity*	*Study type and duration*	*NOEL/NOAEL*	*Comments*
Rats, Wistar	MG oxalate >90%	Oral LD_{50}	N/A	LD_{50} : 275 mg/kg bw.
Rats, Sprague-Dawley	MG oxalate, purity unknown	Oral LD_{50}	N/A	LD_{50} : 520 mg/kg bw.
Rats, Wistar	MG oxalate >90%	28 days repeated oral dosing	NOAEL 10 mg/kg bw/day	Hyperactivity, reduced feed intake and weight gain, haematological effects (females only) and increased blood urea (males only).
Rats, Fischer 344	MG chloride >94%	28 days repeated oral dosing	NOAEL 10 mg/kg bw/day	Increased relative liver weights in females and slightly lower erythrocyte counts in males.
Mice, B6C3F1	MG chloride >94%	28 days repeated oral dosing	NOAEL 15 mg/kg bw/day	Increased erythrocyte volume and reticulocyte levels.
Rabbit, New Zealand White	Technical grade	Developmental toxicity including teratogenicity, oral dosing	LOAEL 5 mg/kg bw/day	Incidences of anomalies (gross, visceral, skeletal) were observed in all treated groups. 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.
Rats,female Fischer 344	MG chloride >87%	2-year cancer study, oral dosing	NOAEL 7 mg/kg bw/day	Increasing trend in the incidence of thyroid gland follicular cell adenoma or carcinoma. A dose related increase in hepatocellular adenomas and an increasing trend in the incidence of mammary gland adenomas or carcinomas. Non-genotoxic mechanism.
Mice, female $B6C3F_1$	MG chloride >87%	2-year cancer study, oral dosing dosing		No treatment related neoplasms found.

Leucomalachite green

Species	*Purity of test substance*	*Study type and duration*	*NOEL/NOAEL*	*Comments*
Rats, male Fischer 344	>98%	28 days repeated oral dosing	LOEL 29 mg/kg bw/day	Increased relative liver weight was reported for all dosed groups. In the absence of histopathological changes in the liver this effect may be adaptive to the dosing rather than an adverse response.
Mice, female B6C3F1	>98%	28 days repeated oral dosing	NOAEL 43.5 mg/kg bw/day	Reduced body weight.
Rat, female Big Blue	98%	32 weeks genotoxicity study including histopathological examination of the liver, and determination of liver cell proliferation indices.	NOAEL 9.1 mg/kg bw/day	Higher frequency of hypertrophic hepatocytes was seen, probably due to an increase in liver cell proliferation.
Rats, Fischer 344	>99%	2-year cancer study, oral dosing	NOAEL 6 mg/kg bw/day	Low non significant incidence of thyroid gland follicular cell adenoma or carcinoma on meales and females. Female rats had a significant increase in the incidence of mammary gland adenomas or carcinomas. The male rats had a dose related increasing trend in interstitial cell adenoma of the testis, but the relevance of this finding is questionable. Non-genotoxic mechanism.
Mice, female $B6C3F_1$	>99%	2-year cancer study, oral dosing		Increase in hepatocellular adenomas or carcinomas in female mice. A genotoxic mechanism cannot be ruled out.

5.2 Discussion

There are no conventional studies available on the absorption, distribution, metabolism, and excretion of MG and LMG in mammalian species including humans. *In vitro* studies have shown that MG can be readily converted into LMG by a multitude of bacterial species present in the intestinal microflora from mice, rats, rhesus monkeys and humans under anaerobic conditions, such as in the intestines. Therefore, MG that is not absorbed in the stomach is likely to be converted to LMG in the intestines.

Studies in fish have shown that MG dissolved in the water is efficiently taken up by various fish species and distributed to all organs. The majority of MG is readily converted into LMG and possibly other as yet unidentified degradation products. The rate of excretion from the fish is dependent on the fat content of the fish with LMG being retained longer in fatty fish than in lean fish. In muscle tissue of catfish the elimination half-life was 67 hours for MG and 240 hours for LMG.

In contrast to LMG, MG is highly cytotoxic to bacterial and mammalian cells *in vitro*.

In vitro studies have also shown that both MG and LMG have the potential for disturbing the thyroid hormone homeostasis. In rats chronical inhibition of thyroid hormone synthesis results in prolonged stimulus from TSH of thyroid growth that may lead to thyroid follicular cell tumours.

5.2.1. Malachite green

The acute oral LD_{50} values of MG (as the oxalate salt) in two different rat strains were reported to be 275 and 520 mg/kg bw.

In a short-term (28-days) repeated oral dosing test, MG (as chloride salt) was administered in the diet to male and female mice at dose levels of 0, 3.75, 15, 45, 90, and 180 mg MG/kg bw/day. A NOAEL of 15 mg MG/kg bw/day could be derived based on increased erythrocyte volume and reticulocyte levels after 45 mg/kg bw/day. Further haematological changes were observed at higher doses.

In a short-term (28-days) repeated oral dosing test, MG (as oxalate salt) was administered in the diet to male and female rats at dose levels of 0, 1.0, 10.0, and 100 mg MG/kg bw/day. A NOAEL of 10 mg MG/kg bw/day could be derived based on clinical signs of hyperactivity, reduced feed intake and weight gain, haematological effects (females only) and increased blood urea (males only) at 100 mg MG/kg bw/day.

In a second short-term (28-days) repeated oral dosing test in male and female rats, MG (as chloride salt) was tested at dietary dose levels of 0, 2.5, 10, 30, 60, and 120 mg MG/kg bw/day. The NOAEL was 10 mg MG/kg bw/day based on increased relative liver weights in females and slightly lower erythrocyte counts in males after 30 mg MG/kg bw/day and higher. Increased relative liver weights were also recorded in males after 60 and 120 mg/kg bw/day. In both sexes the increased relative liver weight was followed by increased levels of γ-glutamyl transferase activity. However, in the absence of histopathological changes in the liver this effect may be adaptive to the dosing rather than an adverse response. The only histopathological changes reported were vacuolization of hepatocytes, primarily midzonal and centrilobular, among the rats fed the highest dose level.

In rats given 120 mg MG/kg bw/day for up to 21 days there were no effects on total triiodothyronine (T3), thyroxine (T4), and thyroid stimulating hormone (TSH) level in males whereas there was a significant increase in T3 levels and a significant decrease in T4 levels in the females.

In two studies, dose-dependent increases in relative liver weights and biochemical markers for hepatic preneoplastic lesions and cell cycle disruption were seen in male rats when MG was given at dose levels of 1.88, 3.75 and 7.5 mg MG/kg bw/day for 16-22 weeks after an initial 4-week treatment with

N-nitrosodiethylamine (DEN). However, no effects were observed when MG was given alone. The relevance of this type of testing for human risk assessment is unclear.

MG (as the oxalate salt; technical grade) has been tested for teratogenic effects in pregnant rabbits given 5, 10 and 20 mg/kg bw/day by gavage at days 6 - 18 of gestation. A dose related decreased weight gain or weight loss was seen in the dams and higher incidences of fetal anomalies (gross, visceral, and skeletal) were reported for all treated groups compared to controls. A NOAEL could not be established thus the LOAEL was 5 mg/kg bw/day. 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 reported and additional reproductive toxicity studies are needed to properly assess the hazards of MG (and LMG) in this regard.

MG and LMG gave rise to a single DNA adduct (or co-eluting adducts) in male rats. The adduct levels increased significantly as a function of the dose and did not differ between groups administered equimolar doses of MG and LMG. In female mice, MG and LMG also gave rise to a single DNA adduct (or co-eluting adducts), the levels of which increased significantly as a function of the dose for MG but not for LMG. Thus, in the female mouse MG produced much higher adduct levels than LMG at equimolar doses.

In vitro tests for genotoxicity of MG is 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 TA-98 with metabolic activation (S-9 mix), but not in any other strains tested or in TA-98 without metabolic activation. MG did not induce mutations in Chinese hamster ovary cells (CHO-K1) and only produced DNA damage in the comet assay in CHO-K1 cells at cytotoxic concentrations. MG (as oxalate) did not produce clastogenic effect in the *in vivo* mouse bone-marrow micronucleus test at the maximum tolerated dose of 37.5 mg/kg bw. 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. MG did not induce micronuclei in erythrocytes, mutations in lymphocytes, or (in contrast to LMG) *cII* mutations in liver cells of female Big Blue B6C3F_1 transgenic mice administered 67.5 mg MG/kg bw/day for 16 weeks.

It is concluded that although MG can produce DNA adducts in the liver of male rats and female mice the weight of evidence shows that it is not genotoxic in conventional *in vitro* and *in vivo* assays and it did not produce mutations in transgenic female mice.

MG was tested for carcinogenicity in female rats at dietary dose levels of 0, 7, 21, or 43 mg/kg bw/day for two years. Mortality was not affected. A reduced body weight gain was observed at the two highest dose levels. There was a trend of increased occurrence of thyroid gland follicular cell adenoma or carcinoma, being statistically significant only at the mid-dose level, and hepatocellular adenomas (significant only at the highest dose level), and a non-significant trend in the incidence of mammary gland adenomas or carcinomas in the treated animals. In the absence of a clear genotoxic effect in conventional *in vitro* and *in vivo* assays and in transgenic female mice, the occurrence of these tumours is considered due to a non-genotoxic, threshold mechanism. The NOAEL was 7 mg/kg bw/day.

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/day for 104 weeks. No effects on mortality and body weight gain were observed.

Based on the available database it is considered inappropriate to establish a TDI for malachite green. A number of shortcomings have been identified. There are no conventional studies available on the absorption, distribution, metabolism, and excretion of MG and LMG in mammalian species. The study on teratogenicity raises concerns regarding this endpoint, but the study itself is of low quality. It is not possible to derive a NOAEL for malachite green from this study as effects are seen at all doses. Additional reproductive toxicity studies are needed to properly assess the hazards of MG and LMG.

In an attempt to assess whether there is a potential risk to the Danish consumers exposed to malachite green found in fish produced in Denmark the concept of Threshold of Toxicological Concern (TTC) is applied. For MG being a aromatic amine belonging to the Cramer structural class III a threshold of intake of 1.5 µg/kg bw/day corresponding to 90 µg per person can be set according to Kroes *et al.* (2004). If this threshold was compared to the LOAEL of the teratogenicity study of 5 mg MG/kg bw/day, there would be a factor of more than 3000 between the two.

According to the latest published dietary survey in Denmark from 2000 to 2002 (Lyhne *et al.* 2005) the highest 95-percentile for daily fish intake is 64 g/day for adult males. Childrens intake of food is relatively larger than adults when compared to body weight, and to account for this a conservative intake of 100 g fish per day is used. In the Danish surveillance programme up to 214 µg MG/kg is found in fish produced in Denmark (Rasmussen, 2007). This leads to a conservatively estimated exposure of 21.4 µg MG/day, which is below the threshold of 90 µg per person per day derived using the TTC approach. At the specified conditions the exposure to malachite green from fish produced in Denmark is of low concern for the consumer.

5.2.1. Leucomalachite green

LMG was tested in a short-term (28-days) repeated oral dosing test in female mice at dietary dose levels of 0, 43.5, 87, and 174 mg LMG/kg bw/day. The NOAEL in the female mice was 43.5 mg LMG/kg bw/day based on reduced body weights seen at the higher dose levels.

When LMG was tested in a short-term (28-days) repeated oral dosing test in male rats at dietary dose levels of 29, 58, and 116 mg LMG/kg bw/day no NOEL could be established as increased relative liver weights were reported for all dosed groups. Thus, the LOEL was 29 mg LMG/kg bw/day. At the highest dose level γ-glutamyl transferase activity was statistically significantly increased. However, in the absence of histopatholotical changes in the liver this effect may be adaptive to the dosing rather than an adverse response. Vacuolization of hepatocytes, primarily midzonal and centrilobular, was observed at the two highest dose levels.

In male rats given 116 mg LMG/kg bw/day for 21 days there was a significant increase in TSH levels and a significant decrease in T4 levels.

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

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/day for either 4, 16 or 32 weeks. In addition, no effect was observed in the liver *cII* mutation frequency, the *Hprt* lymphocyte mutant assay, and the bone marrow micronucleus assay in these rats. However, it was not tested whether LMG had reached the bone marrow. LMG gave rise to a single DNA adduct, the level of which increased linearly with the dose. A significant higher frequency of hypertrophic hepatocytes was seen in the two highest dose groups after 32 weeks, probably due to increased liver cell proliferation. The NOAEL was 9.1 mg LMG/kg bw/day.

LMG did not induce micronuclei in erythrocytes or mutations in lymphocytes of female Big Blue B6C3F_1 transgenic mice administered 30.6 or 61.2 mg LMG/kg bw/day for 16 weeks. However, LMG induced *cII* mutations with an increased frequency of G → A and A → T transversions in the liver cells of the female mice at the highest dose level. It should be noted that the DNA binding as reported in the 28 days study by Culp *et al.* (1999) of LMG in female mice was very low.

It is concluded that although LMG can produce DNA adducts in the liver of male and female rats it was not genotoxic in conventional *in vitro* and *in vivo* assays and did not induce mutations in transgenic female rats. However, in transgenic female mice LMG induced mutations in liver cells, but only at the highest dose tested.

LMG was tested for carcinogenicity in male and female rats at dietary dose levels of 0, 5, 15, or 30 mg/kg bw/day or 6, 17, or 35 mg/kg bw/day, respectively. Mortality was not affected. Reduced body weight gains were observed at the two highest dose levels in either sex. A low, not statistically significant increase in the incidence of thyroid gland follicular cell adenoma or carcinoma (2-7 %) was seen in both sexes. Females at the mid dose level had a significant increase in the incidence of mammary gland adenomas or carcinomas. The male rats had a dose related decreasing incidence in pituitary gland adenoma and a parallel increasing trend in interstitial cell adenoma of the testis. However, the background incidences of these tumour types are very high in the rat strain used and the relevance of the changes for human risk assessment is doubtful. There was no clear increase in the incidence of hepatocellular adenomas in the rats fed LMG. In the absence of a clear genotoxic effect in conventional *in vitro* and *in vivo* assays and in transgenic male and female rat, the occurrence of these tumours is considered due to a non-genotoxic, thresholded mechanism. The overall NOAEL was 6 mg/kg bw/day.

In female mice fed diets corresponding to daily intakes of 0, 15, 31, and 63 mg LMG/kg bw/day for 104 weeks the only finding was a 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. Generally hepatocellular adenomas and carcinomas in mice are disregarded when they are the only induced tumour form. Stimulation of the cell division by the test compound can generate liver tumours in mice. However, the induction of mutations in liver cells by a high dose of LMG in female transgenic mice may indicate that a genotoxic mechanism cannot be ruled out.

Based on the available database it is considered inappropriate to establish a TDI for leucomalachite green. Studies on the absorption, distribution, metabolism, and excretion of LMG in mammalian species are lacking, together with studies on reproductive and developmental toxicity. However, as it cannot be ruled out that LMG is genotoxic and carcinogenic, the assessment was carried out along this line. It is assumed that this approach will also cover any possible reproductive or developmental effects.

For substances that are genotoxic and carcinogenic the Scientific Committee (SC) of the European Food Safety Authority (EFSA) has suggested to use a margin of exposure (MOE) approach in the risk assessment. 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 adverse effect the EFSA SC has suggested to use the $BMDL_{10}$ (the lower limit of a one-sided 95% confidence interval on the benchmark dose (BMD) calculated for a benchmark response (BMR) of 10 % incidence above the control (EFSA 2005).

The US EPA BMD software (BMDS) was used for modelling the liver tumour dose-response in the female mice.

BMD_{10} and $BMDL_{10}$ calculations for hepatocellular adenomas or carcinomas in female mice fed LMG.

Model	Log likelihood	p-value	AIC	Chi-square	p-value	BMD_{10} (mg/kg bw/day)	$BMDL_{10}$ (mg/kg bw/day)	Accept
Full model	-72.77							
Gamma cum.	-72.94	0.842	149.9	0.34	0.842	35.4	20.1	Yes
Logistic	-72.97	0.814	149.9	0.42	0.810	43.1	31.2	Yes
Log-logistic	-72.94	0.549	151.9	0.36	0.551	34.6	18.5	Yes
Multi-stage	-72.93	0.561	151.9	0.34	0.560	36.8	20.1	Yes
Probit	-72.96	0.822	149.9	0.40	0.818	41.9	29.5	Yes
Log probit	-72.97	0.522	151.9	0.40	0.525	33.5	< 0	No
Quantal-linear	-72.94	0.842	149.9	0.34	0.842	35.4	20.1	Yes
Quantal-quadratic	-73.15	0.680	150.3	0.77	0.682	48.2	34.7	Yes
Weibull	-72.94	0.558	151.9	0.34	0.560	34.8	< 0	No
Reduced model	-75.70	0.118						

The $BMDL_{10}$ values calculated ranged between 18.5 and 34.7 mg LMG/kg bw/day. In order to be prudent a $BMDL_{10}$ value of 20 mg LMG/kg bw/day was chosen as the reference point for the MOE calculation. It should, however, be stressed that the use of dose-response modelling on these tumour data may not be appropriate. The p value for the "reduced" model is higher than the default $p < 0,05$ that would indicate a "true" dose-response relationship. Thus neither of the models can demonstrate, that the tumour incidence is significant different from the background, even though the incidence at the highest dose level is significantly different from controls when tested individually. However, as the chi-square and p values were acceptable, it was decided to proceed with the BMD-modelling assuming a dose response relationship.

In the Danish surveillance programme up to 28 µg LMG/kg is found in fish produced in Denmark (Rasmussen, 2007). With an intake of 100 g fish/day, this leads to a conservatively estimated exposure of 2.8 µg LMG per day. For a person of 60 kg this leads to a margin of exposure (MOE) of more than 400.000 up to the reference point $BMDL_{10}$ of 20 mg/kg bw/day.

In the opinion of the EFSA SC a MOE of 10,000 or higher, if it is based on the $BMDL_{10}$ from an animal study, would be of low concern from a public health point of view and might be reasonably considered as a low priority for risk management actions.

5.3 Conclusion

The available databases on the toxicity of malachite green and leucomalachite green are not adequate for conventional risk assessment purposes. Especially the findings of teratological effects of malachite green in rabbits raise concern. Additional reproductive toxicity studies are needed to properly assess the hazards of MG and LMG. Therefore, TDI's cannot be established for neither malachite green nor leucomalachite green. However, from the available studies found in public literature it is assessed that the reported findings of malachite green (up to 214 ug/kg) and leucomalachite green (up to 28 ug/kg) in fish produced in Denmark are of low concern from a public health point of view and might be reasonably considered as a low priority for risk management actions.

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