Application of the margin of exposure (MOE) approach to substances in food that are genotoxic and carcinogenic. Example: Leucomalachite green

Andrew Renwicka,*, Jean-Charles Leblancb, R. Woodrow Setzercc

a University of Southampton, UK
b French Food Safety Agency, France
c US Environmental Protection Agency, USA

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ABSTRACT

Leucomalachite green (LMG) is mutagenic and produces DNA-adducts in vivo, and is carcinogenic in rodent bioassays. Dose-response modelling of the data for hepatocellular adenomas and carcinomas in female mice gave a BMDL10 of 20 mg/kg-bw/day. Limited data are available on the concentrations present in fish for human consumption. Human exposure estimates assumed that all consumed fish is contaminated with LMG. The calculated MoEs were 4,000,000 and 400,000 respectively for average and high exposure estimates.

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Introduction

Malachite green chloride is a triphenylmethane dye used illegally in the fish culture as an antifungal agent and in dye industries. Leucomalachite green (LMG) is formed in vivo by the reduction of malachite green (MG). Although MG is not approved for use in aquaculture, its low cost and high efficacy make illicit use likely, and residues of LMG have been reported in fish for human consumption.

1. Toxicological data

1.1. Genotoxicity

The following summary is based on a joint review by the United Kingdom Committee on Carcinogenicity (UK CoM) and Committee on Mutagenicity (UK CoM) in 2004 (CoC/CoM, 2004). It provides an update to the data available to the Committee on Mutagenicity in 1999 (CoM, 1999).

Negative results were obtained on the in vitro mutagenicity of LMG from limited Salmonella and CHO/HGPRT assays, and also from a Comet assay in CHO cells. 32P-post-labelling studies using a 28-day dietary exposure have indicated that LMG induces a low level of DNA-adduct(s) in the liver of F344 rats; only a marginal response was however seen in B6C3F1 mice. DNA-adducts have been reported in the livers of mice treated with LMG, with a dose-related increase in binding. LMG did not induce micronuclei in peripheral lymphocytes or HPRT mutations in splenic lymphocytes in the 28-day study. Gene mutation studies have indicated that LMG can induce mutations in vivo in liver-DNA. Studies in Big Blue F344 rats gave equivocal results. LMG produced an increase in lacI mutations only at the highest dose tested (543 ppm in diet) and at a single time point (16 weeks). No increase was seen after 32 weeks. Studies in female Big Blue B6C3F1 mice indicated that LMG produced an increase in cII mutant frequency in liver-DNA of mice treated with 408 ppm LMG in the diet. Furthermore, it was shown that the spectrum of mutation seen in the DNA was distinct from that of the control mice. These data provide evidence of in vivo mutagenicity at the target site in the carcinogenicity bioassay.

The 2004 statement (CoC/CoM, 2004) concluded “In view of the demonstration of the induction of mutations in liver-DNA of female B6C3F1 mice, LMG should be regarded as an in vivo mutagen”.

1.2. Carcinogenicity

The CoC paper included a detailed description of a National Toxicology Program (NTP) cancer bioassay which has now been published on the NTP website (NTP, 2004, 2005), and a paper has been published in Food and Chemical Toxicology (Culp et al., 2006).

1.2.1. Rats

Groups of 48 male and female F344 rats were fed diets containing 0, 91, 272 or 543 ppm LMG for 104 weeks. The dietary levels were equivalent to average daily doses of approximately 0, 5, 15 and 30 mg LMG/kg-bw/day in the males and 0, 6, 17 and 35 mg LMG/kg-bw/day in the females. Survival was comparable in all groups apart from the females given 272 ppm where increased sur-
vival was noted. A dose-related decrease in body weight gain was seen for both sexes amounting to a 25%-reduction near termination in the females and 10–15% in the males. Liver weights were significantly increased at the two higher dose levels in the males, as were relative liver weights in the females. Relative thyroid weights were increased significantly in both sexes at the highest dose level.

At necropsy, a significant increase in mammary gland adenoma and carcinoma (combined) was seen with the effects at the top dose level exceeding the historic control range in experiments conducted at the test laboratory. In addition, in the male rats a positive trend was noted in the incidence of interstitial cell adenoma of the testes, with the increase being statistically significant at the top dose level (37/48, 42/47, 43/48 and 45/47, respectively).

A minimal increase in hepatocellular adenomas was also seen in the female rats given 91 and 543 ppm in the diet which exceeded the historical control range (1/48, 3/48, 0/48 and 3/48 at 0, 91, 272 and 543 ppm in the diet, respectively). No increase in such tumours was seen in the males (2/48, 2/47, 3/48 and 2/47 at 0, 91, 272 and 543 ppm in the diet, respectively). Non-neoplastic liver lesions (eosinophilic foci, cystic degeneration and cytoplasmic vacuolisation) were increased in both males and females. Minimal increases, not statistically significant, were seen in thyroid follicular cell adenomas and carcinomas (combined) in both the females (0/46, 1/46, 2/47 and 1/48, respectively) and males (0/47, 2/47, 1/48 and 3/48, respectively). Decreases in the incidence of mononuclear cell leukemia and pituitary gland adenoma were seen in both sexes. There was a decrease with treatment in the total numbers of animals showing a malignant neoplasm.

### 2.1. Sub-populations of interest

- **i.** there was equivocal evidence of carcinogenic activity in male F344/N rats based on an increase in interstitial cell adenoma of the testes and the occurrence of thyroid gland follicular cell adenoma or carcinoma (combined) in exposed rats,
- **ii.** there was equivocal evidence of carcinogenic activity in female F344/N rats based on a marginally increased incidence of hepatocellular adenoma and the occurrence of thyroid gland follicular cell adenoma or carcinoma (combined) in exposed rats, and
- **iii.** there was some evidence of carcinogenic activity in female B6C3F1 mice based on an increase in hepatocellular adenoma or carcinoma (combined).

Based on the peer-review it would seem probable that the data for mice on hepatocellular adenoma and carcinoma combined would be the endpoint to use for BMD/BMDL estimations and calculation of the MOE.

### 1.3. Mode of action

The mode of action was considered in the unpublished 2004 CoC report, which concluded “In view of the demonstration of the induction of mutations in liver-DNA of female B6C3F1 mice, LMG should be regarded as an in vivo mutagen”.

### 1.4. Epidemiological data

None identified.

### 1.5. Dose–response relationships

The raw incidence data for both rats and mice are given in the following table.

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>Incidence at different dietary concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 ppm (0 mg/kg/day)</td>
</tr>
<tr>
<td>Tests, interstitial cell adenoma</td>
<td>37/48</td>
</tr>
<tr>
<td>Mammary adenoma or carcinoma</td>
<td>0/48</td>
</tr>
<tr>
<td></td>
<td>0 ppm (0 mg/kg/day)</td>
</tr>
<tr>
<td>Tumours in female mice</td>
<td>3/47</td>
</tr>
</tbody>
</table>

* Increase reported as statistically significant (P < 0.05) Culp et al. (2006).

The main dose–response identified by the NTP was an increase in hepatocellular adenoma or carcinoma (combined) in female B6C3F1 mice.

### 1.6. Data quality, uncertainties and limitations

The data are from a recent NTP 2 year bioassay and therefore “gold standard”.

### 2. Human dietary exposure analysis

#### 2.1. Sub-populations of interest

None.

#### 2.2. Concentration in food

Malachite green is used to treat fungal and protozoan infections in ornamental fish. Although it is not registered for use in aquaculture for food in many countries, including Australia, Canada, the European Union, Hong Kong, Indonesia, the United States and Vietnam, residues are detected in farmed fishes.

In 2005, the Food Standards Australia New Zealand (FSANZ) conducted a Survey of Chemical Residues in Domestic and Imported Aquaculture Fish. Sixty domestic and imported aquacultured fish sampled to account for country of origin and species to best represent the aquacultured fish available for sale in Australia were collected and analysed. Leucomalachite green was quantified in 10 (17%) samples, with levels between 4 and 110 mg/kg, the limit of detection (LOD) being 2 µg/kg. The lower bound mean (assuming 0 as the concentration for non-detects) was 5 µg/kg and the upper bound mean (assuming the LOD, 2 µg/kg, as the concentration for non-detects) was 7 µg/kg (FSANZ, 2005).
Malachite green and its metabolite leucomalachite green are monitored in a limited number of countries. The United Kingdom has published detailed results since 2001 (Veterinary Residues Committee, 2001, 2002, 2003, 2004, 2005). Their monitoring programmes include:

- statutory surveillance on domestic products, following a sampling design based on national production volume, in accordance with the European Council decision 96/23/EC,
- non-statutory surveillance on imported products, targeted on areas where residues of concern are more likely to occur,
- retail surveys aimed to assess the incidence of residues in products available from retail outlets.

Recent publications, primarily concerned with analytical methods for the determination of malachite and leucomalachite green, have confirmed the prevalence and levels of these illegal colours in farmed fish (Halme et al., 2007; Tittlemier et al., 2007). A study of the effects of cooking on these colours has shown that boiling in water has little effect on the level of leucomalachite green in fish muscle. However, microwave cooking of fish resulted in some decomposition of leucomalachite (Mitrowska et al., 2007).

<table>
<thead>
<tr>
<th>Country</th>
<th>Mean consumers (ng/kg-bw/day)</th>
<th>95th percentile consumers (ng/kg-bw/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia</td>
<td>8–21</td>
<td>24–65</td>
</tr>
<tr>
<td>EU</td>
<td>2–7</td>
<td>5–16</td>
</tr>
</tbody>
</table>

2.3.2. International estimates

According to the GEMS/Food Cluster Diets (WHO, 2007), the consumption level for marine and freshwater fishes, fresh and processed, ranges between 12 g/day/capita in Cluster I (South and East Africa) and 53 g/day/capita in Cluster L (Far-Eastern Asia). There is no distinction among fish species consumed, nor are wild or farmed fishes differentiated in these diets. Combining the weighted mean LMG concentration reported in the table above (3.6 μg/kg) with the fish consumptions from the GEMS/Food Cluster Diets, assuming a body weight of 60 kg for consumers in all clusters, results in intakes ranging between 0.7 ng/kg-bw/day (Cluster I, South and East Africa) and 3.2 ng/kg-bw/day (Cluster L, Far-eastern).

2.3. Dietary exposures

2.3.1. National estimates

The FSANZ provides a national assessment of the public health risk associated with low residues of malachite green chloride and leucomalachite green in aquacultured fish (FSANZ, 2005) based on:

- the results of the Survey of Chemical Residues in Domestic and Imported Aquaculture Fish,
- consumption data from the 1995 Australian National Nutrition Survey (NNS) (N = 13858 aged 2 years and above; 989 aged 2–6 years), assuming no distinction among fish species, and including fish consumed on its own or as an ingredient in a mixed food dish.

The mean dietary exposure to leucomalachite green was estimated to range between 8 and 21 ng/kg-bw/day for the whole Australian population, including young children aged 2–6 years. The 95th percentile estimate ranged between 24 and 65 ng/kg-bw/day.

Additionally, dietary exposures were estimated using food consumption data for the general European Union (EU) population-based on data from three member states reported in an European Food Safety Authority opinion (EFSA, 2005). The food consumptions were combined with the mean concentration reported in the GB monitoring program (3.7 μg/kg) to derive dietary exposure estimates for EU adult consumers of fish products in the three reported countries. The estimates include children and consumers of fish products at a high percentile of the total population as reported in the Calipso survey (Bemrah et al., 2009). The estimates of dietary exposure in the adult EU population, assuming a 60 kg average body weight for adults and 30 kg for children, ranged between 2 and 7 ng/kg-bw at the mean, and up to 16 μg/kg-bw at the 95th percentile.

2.4. Dietary exposure assessment (values to be used in the MOE calculation)

Dietary exposure to LMG will be intermittent because its presence in fish muscle is due to the illegal use of malachite green in farmed fish. Available data suggest that only about 10–15% of fish contain residual levels of LMG. Also, the data available on fish consumption generally do not distinguish between farmed and wild-caught fish. Therefore, any estimate of LMG consumption will be conservatively high. For contaminants with this exposure pattern, the use of a population-based mean exposure estimate should be used for the dietary exposure to the substance. The national population means ranged from 2 to 21 ng/kg-bw/day (down to 0.7 ng/kg-bw/day for the international estimate using the Cluster Diet with the lowest fish intake).

The use of 5 ng/kg-bw/day, approximately the average of the mean national estimates reported for adults, is appropriate for calculating MOEs for leucomalachite green for average intakes, with a high value of 50 ng/kg-bw/day used to reflect the 95th percentile reported for Australia.

2.5. Data quality, uncertainties and limitations

Initial review of the data indicates that occurrence data for LMG in fish are very limited. Estimates of international dietary exposure are based on levels found in two countries, which is not likely to be representative of LMG concentrations that occur worldwide.

All of the dietary exposure estimates are considered to be conservatively high because it has been assumed that all fish (fresh and marine) consumed are contaminated at the mean leucomalachite green concentration reported.
3. Modelling

Logistic, logprobit, gamma, Weibull, multistage, probit and logistic models were fitted to the data, and the output of the average model used for determining the critical tumour type for risk assessment and estimation of the MOE values, using the method described by Wheeler and Bailer (2007).

3.1. BMD and BMDL

The following tumour types gave a positive trend analysis in the BMD calculations undertaken for this investigation. The data are arranged in order of increasing value of the BMDL10 values calculated by the different models.

BMD(L) values for tumours in animals treated with leucomalachite green.

<table>
<thead>
<tr>
<th>Tumour Type</th>
<th>BMD10</th>
<th>BMDL10</th>
<th>BMD05</th>
<th>BMDL05</th>
<th>BMD01</th>
<th>BMDL01</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male – testes interstitial cell adenomas</td>
<td>0.91</td>
<td>0.0003</td>
<td>0.33</td>
<td>&lt;0.0001</td>
<td>0.03</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Female – hepatocellular adenomas or carcinoma (combined)</td>
<td>39.57</td>
<td>20.44</td>
<td>20.43</td>
<td>6.81</td>
<td>4.17</td>
<td>0.0047</td>
</tr>
<tr>
<td>Female – mammary adenomas</td>
<td>39.07</td>
<td>23.46</td>
<td>17.06</td>
<td>8.81</td>
<td>1.32</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The data are in mg/kg-bw/day.
FR – female rats.
FM – female mice.
MR – male rats.

For interstitial cell adenomas in the testes of rats all models fitted the data with P values >0.41. The response at the lowest dose is similar to the responses at higher doses so that fitting of the entire dataset would be consistent with a very rapid rise in response over a very low dose range followed by a shallow increase in the range of the administered doses. Thus, the BMDL is estimated to be a value that rounds close to 0, which means the data are inadequate to put a lower bound on the estimate of the BMD. If interstitial cell adenomas in the testes of rats were selected as the most sensitive endpoint of relevance to humans the available data would be inadequate for estimation of either a reliable BMDL or a usable MOE. Selection of this endpoint would lead to the need for a new cancer bioassay with more appropriate dose selection.

The data for mammary adenomas plus carcinomas in female rats (all P values >0.40) and for hepatocellular adenomas or carcinomas (combined) in female mice (all P values >0.48) were significantly fitted by all models.

Because of the doubtful relevance of tumours of the interstitial cells of the testes in F344 rats to humans (see Cook et al., 1999 and Benford et al., this issue) and the high and variable incidences in historical control groups (69–90%), the very low BMDL10 for this endpoint was not used to calculate the MOE. Because the BMDL10 for mammary tumours is higher than that for hepatocellular tumours, the BMDL values for hepatocellular adenomas and carcinomas (combined) in female mice were used as the basis for the MOE calculation.

The average model is discussed in another paper in this issue (Benford et al., this issue). It is clear from the above table of individual models that the BMDL values derived from the logprobit model have a major effect in the average model at low BMRs of 5% and especially at 1%. The reason for this is that the parameter beta in the logprobit model is not restricted (Wheeler and Bailer, 2007) so that the fitted curve, while flat with zero slope at very low doses, can rise very steeply over a short dose range. In this model, a very low (virtually 0) BMDL is consistent with the data. If beta were restricted to be >1, then the BMDL would be substantially greater than 0. Thus it is clear that decisions made about data modelling could have a major effect on the estimated BMDLs, especially for sub-optimal dose–response data (see Section 3.3 of Benford et al., this issue for discussion). The data for the BMDL10 derived by the average model were used to calculate the MOE (see below).

3.2. T25 calculation

The T25 was calculated from the lowest dose giving a significant increase compared to controls in the study of Culp et al. (2006). The extra risk was calculated by dividing the additional risk (% tumour-bearing animals in the dosed group minus % tumour-bearing animals in the control group) by the non-affected fraction in the control population. The extra risk at the lowest dose giving a statistically significant increase = \[ ((B/100 – A/100)/(1 – A/100)) \times 100 \] where A = proportion of animals with tumours in the control group and B = proportion of animals with tumours in an exposed group.

Although increased incidences of hepatocellular adenomas and carcinomas (combined) were found in female mice at each dose level, the lowest dose showing a significant increase (P = 0.004) was at the top dose. The incidence in the control group was 6% and in the 63 mg/kg-bw/day group was 23%. Therefore, the extra risk is \[ (63/100 – (6/100))/[(1–6/100)] \times 100 = 18.1\% \] and the T25 = 25/18 × 63 mg/kg-bw/day = 87 mg/kg-bw/day.

3.3. MOE calculation

The most sensitive site for tumour development is the interstitial cells of the testes in F344 rats; but there is a very high spontaneous

Modelling of data for hepatocellular adenomas and carcinomas (combined) in female mice (see Fig. 1).

<table>
<thead>
<tr>
<th>P value</th>
<th>AIC</th>
<th>BMD10</th>
<th>BMDL10</th>
<th>BMD05</th>
<th>BMDL05</th>
<th>BMD01</th>
<th>BMDL01</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamma</td>
<td>0.80</td>
<td>150</td>
<td>35.92</td>
<td>20.16</td>
<td>17.49</td>
<td>9.81</td>
<td>3.43</td>
</tr>
<tr>
<td>Weibull</td>
<td>0.80</td>
<td>150</td>
<td>35.92</td>
<td>20.16</td>
<td>17.49</td>
<td>9.81</td>
<td>3.43</td>
</tr>
<tr>
<td>Logistic</td>
<td>0.80</td>
<td>150</td>
<td>34.80</td>
<td>18.46</td>
<td>16.48</td>
<td>8.74</td>
<td>4.17</td>
</tr>
<tr>
<td>Probit</td>
<td>0.78</td>
<td>150</td>
<td>43.49</td>
<td>29.54</td>
<td>25.30</td>
<td>18.14</td>
<td>5.96</td>
</tr>
<tr>
<td>Logistic</td>
<td>0.77</td>
<td>150</td>
<td>31.16</td>
<td>18.14</td>
<td>18.14</td>
<td>9.81</td>
<td>3.99</td>
</tr>
<tr>
<td>Multistage</td>
<td>0.51</td>
<td>152</td>
<td>39.10</td>
<td>20.02</td>
<td>17.06</td>
<td>8.81</td>
<td>1.32</td>
</tr>
<tr>
<td>Logprobit</td>
<td>0.48</td>
<td>152</td>
<td>32.39</td>
<td>14.64</td>
<td>14.64</td>
<td>6.81</td>
<td>1.92</td>
</tr>
<tr>
<td>Avg. model</td>
<td>0.70</td>
<td>150</td>
<td>39.57</td>
<td>20.44</td>
<td>20.44</td>
<td>8.81</td>
<td>1.92</td>
</tr>
</tbody>
</table>
rate of these tumours in F344 rats and tumours at this site usually arise from non-genotoxic mechanisms, although the mode of action of LMG is unclear. Because of limitations in the available data the BMDL values could not be derived for this site. The MOE has therefore been calculated for hepatocellular adenomas in female mice.

The MOE values have been calculated based on intakes of 5 ng/kg-bw/day, approximately the average of the mean national estimates reported for adults, with a high value of 50 ng/kg-bw/day used to reflect the 95th percentile reported for Australia, and the BMDL values for hepatocellular adenomas and carcinomas in female mice (BMDL10, BMDL05 and BMDL01 – calculated by the average model) treated with LMG (Section 3.1).

The MOE values for the BMDL10 are similar to those reported by FSANZ at the National level taking the non-neoplastic lesions in the liver as the most sensitive endpoint (FSANZ, 2005). The MOE values for BMDL01 are heavily influenced by the output of the log-probit model and decisions about model restriction (see above).

3.4. Modelling limitations

The design of the study was inadequate to estimate either the BMD or the BMDL for interstitial cell adenoma in rat testes. The response at the lowest dose is similar enough to the responses at higher doses that the best-fitting model has a very rapid rise in re-

<table>
<thead>
<tr>
<th>(5 ng/kg-bw/day)</th>
<th>MOE for BMDL10</th>
<th>MOE for BMDL05</th>
<th>MOE for BMDL01</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOE</td>
<td>20.44/0.000005 = 4,000,000</td>
<td>6.81/0.000005 = 1,000,000</td>
<td>0.0047/0.000005 = 1000</td>
</tr>
<tr>
<td>MOE</td>
<td>20.44/0.00005 = 400,000</td>
<td>6.81/0.00005 = 100,000</td>
<td>0.0047/0.00005 = 100</td>
</tr>
</tbody>
</table>

**Fig. 1.** Model fitting to data for hepatocellular adenomas and carcinomas in female mice.
response over a very low dose range to a plateau in the range of the administered doses. Thus, the data are inadequate for placing a lower bound on the BMD, and the BMDL is estimated to be a value that rounds to 0. The dose–response data for hepatocellular adenomas and carcinomas (combined) in female mice provided good BMD and BMDL values for BMRs of 10% and 5%, but the BMDL01 estimate was three orders of magnitude below the BMD01. Apart from the logprobit model, the different models gave BMDL10 values that varied within a factor of about 3-fold.

4. Learning points

i. The critical issue is the relevance of the animal tumours to human health; calculation of an MOE does not resolve this issue.

ii. The data on the testicular interstitial cell tumours in F344 rats, were not used because they are of doubtful relevance to humans. If they had been used, the very low BMDL10, which was three orders of magnitude lower than the BMD10, would have resulted in an MOE of less than 100.

iii. The BMDL10 values for hepatocellular adenomas or carcinomas (combined) in female mice and mammary adenomas in female rats differ from the corresponding BMDs by a factor of 2-fold or less, but the BMDL01 values can be orders of magnitude below the corresponding BMD01. An exception is the logprobit model where the choice of restriction would significantly affect the derivation of BMDLs using the average model.

iv. Only limited data are available on the concentrations of LMG in fish.

v. The MOE values are heavily dependent on the assumptions necessary to get any estimate of intake.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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