CELLULAR TOXICITY OF MALACHITE GREEN AND LEUCOMALACHITE GREEN EVALUATED ON TWO RAT CELL LINES BY MTT, NRU, LDH, AND PROTEIN ASSAYS

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Abstract

The cytotoxicity of malachite green (MG) and its major metabolite, leucomalachite green (LMG) was investigated using rat FaO and L6 cell lines. Eight concentrations ranging from 0.1 to 100 μM (MG) and 10 to 1,000 μM (LMG) were tested in three independent experiments. Four cytotoxicity assays were applied in which various biochemical endpoints were assessed: mitochondrial activity (MTT assay), proliferation (total protein content assay), lysosomal activity (neutral red uptake assay), and membrane integrity (LDH leakage assay). The mean effective concentrations (EC₅₀) were calculated from the dose-response curves after 24, 48, and 72 h of exposure. The concentration- and time- dependent effects were observed. MG was very toxic and FaO cells were more sensitive than L6 cells. EC₅₀ values were <10 μM in MTT, NRU, LDH tests (in FaO cells), and in NRU, MTT (in L6 cells) indicating that the primary mechanism of toxicity is inhibition of mitochondrial and lysosomal activity. The LMG gave only slight toxic effect at the hundreds times higher concentrations, with the exception of NRU assay for which EC₅₀ values after 48 and 72 h exposure were very low (<10 μM).

Key words: malachite green, leucomalachite green, cell culture, cytotoxicity.

Malachite green (MG) is a synthetic triphenylmethane dye used to colour leather, silk, wool, cotton, and paper. Primarily, MG has been used as a therapeutic agent in aquaculture as a very effective compound in the treatment of bacterial, fungal, and protozoan infections and against parasites. Nowadays, the use of MG has become a matter of concern due to the risks it poses to consumers of treated fish. Although it was never registered as a veterinary drug for use in fish feed, illegal use of MG and its metabolites resulted in residues in fish tissues (21, 28) as well as in foodstuffs (32). MG is still used in many parts of the world due to the lack of an alternative and because it is inexpensive and readily available. It is readily soluble in water, easily absorbed by fish, and extensively metabolised. The most prevalent and persistent product in MG-treated fish is leucomalachite green (LMG) (1). Previous studies showed that both compounds are eliminated slowly, therefore a long withdrawal time is necessary (1, 17). LMG has been found to retain in fish muscle for a longer period (6).

The toxicity data on malachite green are not complete because most reports do not adequately identify the purity of the dye used. Moreover, the toxicity depends on many factors as water hardness, temperature, or amount of dissolved oxygen in water. MG is suspected to exert several toxic effects including genotoxicity, carcinogenicity, reproductive toxicity, and immunotoxicity (5–8, 10, 29, 31). It has been shown that in humans, malachite green causes severe ocular irritation with epithelial defects upon exposure (33). Therefore, further studies are required using in vivo and in vitro model systems in order to obtain more toxicological data for this environmental contaminant.

The aim of this study was to gain more insight into the mode of toxic action of both compounds at the cellular level and comparison of their cytotoxic potential against two cell lines deriving from rat hepatoma (FaO) and myocytes (L6). Four cytotoxicity assays were applied in which various biochemical endpoints were assessed: mitochondrial activity, cellular protein synthesis, lysosomal activity, and membrane integrity.

Material and Methods

Reagents. Triton X-100, neutral red (NR), dimethyl sulfoxide (DMSO), foetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Coomassie Brilliant Blue R-250 dye (TPC), trypsin-EDTA, antibiotic solution (10,000 IU/mL of penicillin, 10 mg/mL of streptomycin) were purchased from Sigma–Aldrich (USA). All other chemicals were purchased from

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commercial suppliers and were of the highest available purity.

**Cell lines.** Rat hepatoma cell line FaO (ECACC 89042701, rat skeletal muscle myoblasts; myoblastoid cells L6 (ATCC CRL-1458) were used.

**Cell cultures and treatments.** FaO cells were cultured in F-12 nutrient mixture (Ham), Kaighn’s modification with L-glutamine medium (GIBCO). L6 cells were cultured in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) medium (GIBCO).

The media were supplemented with 10% FBS, and antibiotics (penicillin 100 IU/mL, streptomycin 100 µg/mL). The cells were maintained in 75 cm² cell culture flasks at 37°C, 95% air humidity, and 5% CO₂. The cells were subcultured when they reached 70–80% confluency by dissociating with 0.25% trypsin–0.02% EDTA.

In order to perform the chosen cytotoxicity assays, the cells were grown on 96-well culture plates. For the purpose of the experiments, single cell suspensions were prepared in medium containing 2.5% FBS and adjusted to 2.5x10⁴/mL (FaO) and 1.5x10⁵/mL (L6). Then 200µl (i.e. 5x10⁴ cells/well and 3x10⁵ cells/well, respectively) were added to each well and incubated for 24 h at 37°C.

For the measurement of the toxicity for four different assays, the cells were treated with different concentrations of freshly prepared test compounds. MG was dissolved in culture medium. LMG was dissolved in DMSO. Protein synthesis was less pronounced than on MG.

**Cell viability MTT assay.** The assay is based on the reduction of tetrazolium salt MTT in live cells to formazan crystals that can be quantified. Twenty microlitres of MTT (5 mg/mL PBS/well) was added and cultures were incubated for 4 h at 37°C, 95% air humidity, and 5% CO₂. The MTT containing medium was removed and the intracellular formazan crystals were solubilised and extracted with 200 µl DMSO. The plate was shaken for 10-30 min and transferred to a microplate reader (Multiscan RC Labsystems) to measure the absorbance at 570 nm.

**Total protein content (TPC assay).** The procedure is based upon staining total cellular protein with Coomassie Brilliant Blue R 250, followed by measurement of absorbance. Following exposure to the compound, 200 µl of fixing solution (glacial acetic acid:ethanol:water, 1:50:49, v/v) was added and then stained with 200 µl of the dye solution. The plates were shaken for 10 min. Then the stain was removed and the cells were rinsed twice with 200 µl of washing solution (glacial acetic acid:ethanol:water - 5:10:85, v/v). After that, the washing solution was replaced with 200 µl of the desorbing solution (1 M potassium acetate) and plates were shaken again for 10 min. The absorbance was measured at 595 nm.

**Neutral red uptake (NRU assay).** Cells were washed with PBS-Ca²⁺ and stained for 3 h with neutral red (40 mg/mL PBS). After this time, the cells were washed with PBS-Ca²⁺. The dye from viable cells was extracted with a mixture of acetic acid, ethanol and water (1:50:49, v/v). After 20-30 min of shaking, absorbance was read at 540 nm.

**LDH leakage assay.** Commercially available Cytotoxicity Detection Kit (LDH) (Roche Diagnostics, Poland) was used. The integrity of plasma membrane was determined by monitoring the activity of cytoplasmic enzyme lactate dehydrogenase (LDH) in the extracellular incubation medium.

**Data analysis.** Results (percentage of control values) are expressed as mean ± SD (standard deviation) of at least three independent experiments. Statistical evaluation was performed using one way analysis of variance (ANOVA) followed by Dunnett’s post-hoc test.

Effects of MG and LMG on mitochondrial activity (MTT assay). EC₅₀ values for FaO cells following exposure to MG for 24, 48, and 72 h were very low, i.e. 3.3, 0.9, and <0.1 µM, respectively. For L6 cells, the corresponding values were slightly higher and ranged from 9.8 to 5.1 µM. LMG was less toxic than MG. EC₅₀ values ranged from 719 to 418 µM for FaO cells and from 436 to 108 µM for L6 cells (Table 1).

**Effects of MG and LMG on lysosomal activity (NRU assay).** EC₅₀ values of MG for both cell lines were very low: 3.4, 1.2, and 0.7 µM (FaO) and 4.2, 2.6, and <0.1 (L6). LMG, up to the concentration of 1,000 µM was shown not to interfere with the NRU in FaO cells after 24 and 48 h exposure. Exposure for 72 h resulted in EC₅₀ of 123 µM. L6 cells were more sensitive and after 24 h, EC₅₀ was 406 µM and then decreased to less than 10 µM (Table 1).

**Effects of MG and LMG on cellular protein synthesis (TPC assay).** In both cell lines, the impact of MG on protein synthesis was less pronounced than on two biochemical endpoints mentioned above. The EC₅₀ values ranged from 16 to 9.1 µM (FaO) and from 25.4 to 10.0 µM (L6). LMG was shown not to interfere with the determination of protein synthesis up to the concentration of 1,000 µM after 24 and 48 h exposure. After 72 h exposure, the EC₅₀ value was 874 and 835 µM for FaO and L6 cells, respectively (Table 1).
Effects of MG and LMG on membrane integrity (LDH leakage assay). FaO cells exposed to MG were more sensitive than L6 line. The mean EC₅₀ values were 4.1, 4.4, and 4.0 µM after 24, 48, and 72 h exposure, respectively. In L6 line, the values ranged from 25 to 9.5 µM. EC₅₀ values of LMG were very high and ranged from 940 to 362 µM (FaO) and from 853 to 346 µM (L6) (Table 1).

Fig. 1. Concentration and time-dependent response curves for MG and LMG tested in FaO cells by four assays. Results are expressed as mean ± SD of three independent experiments. * P<0.05, ** P<0.01, *** P< 0.001.
Fig. 2. Concentration and time-dependent response curves for MG and LMG tested in L6 cells by four assays. Results are expressed as mean ± SD of three independent experiments. * P<0.05, ** P<0.01, *** P< 0.001.
The MTT assay is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan dye product inside the cell. The amount of formazan generated is assumed to be directly proportional to the cell number and the best way is to use the battery of tests that evaluate different endpoints. For this reason MTT, TPC, NRU, and LDH leakage assays are the most frequently used (11, 13, 16, 26, 34). The MTT assay is based on the reduction of tetrazolium salt MTT in live cells to dark formazan. The different letters A-D (FaO) and a-d (L6) denotes significant differences (P≤0.05) among assays at 24, 48, and 72 h, respectively.

### Discussion

Assuming that toxic effects seen in a whole organism are due to prior failure of basic cellular functions, cytotoxicity studies offer a good source of information about the potential mechanism of action (9, 26). However, it is necessary to remember that the results depend on many factors such as the nature of compound, nature of assay, model used, and time of exposure (34).

Malachite green was a hundred times more toxic to both cell lines used. These results are in agreement with literature data. MG (but not LMG) has been found to be very toxic to other mammalian and fish cells (12, 24, 27, 35) as well as to two cell lines of human origin (HEp-2 and Caco-2) (30). MG is also known to be highly toxic for the marine bacteria Vibrio fisheri and was classified as “very toxic to aquatic organisms” (EC50, 30min, mg l−1), while LMG was not classified up to the concentration of 39.9 mg l−1 (15). Similar results were obtained in our previous study with the use of primary cultures of rat embryo limb buds and midbrain cells (20). In that study, MG was classified as strong teratogen. The fact that the mean inhibitory concentrations (IC50) for differentiation were close to the mean inhibitory concentrations for cell viability may suggest that the primary harmful effect of MG could be general toxicity.

Because no single test is able to gain reliable information about mechanism of cytotoxicity, the best way is to use the battery of tests that evaluate different endpoints. For this reason MTT, TPC, NRU, and LDH leakage assays are the most frequently used (11, 13, 16, 26, 34). The MTT assay is based on the reduction of tetrazolium salt MTT in live cells to dark formazan product inside the cell via mitochondrial nicotinamide adenine dinucleotide phosphate (NADPH) dependent dehydrogenase. The amount of formazan generated is assumed to be directly proportional to the cell number (23). The dye-binding (Coomassie Brilliant Blue previously Kenacid blue) assay is based upon staining total cellular protein (4). The neutral red assay is based on staining living cells by neutral red dye and was classified up to the concentration of 39.9 mg l−1 (15). Similar results were obtained in our previous study with the use of primary cultures of rat embryo limb buds and midbrain cells (20). In that study, MG was classified as strong teratogen. The fact that the mean inhibitory concentrations (IC50) for differentiation were close to the mean inhibitory concentrations for cell viability may suggest that the primary harmful effect of MG could be general toxicity.

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### Table 1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Assay</th>
<th>MG (µM)</th>
<th>LMG (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>FaO</td>
<td>MTT</td>
<td>3.3 ± 0.58A</td>
<td>0.9 ± 0.3A</td>
</tr>
<tr>
<td></td>
<td>TPC</td>
<td>16 ± 3.24B</td>
<td>13 ± 0.99B</td>
</tr>
<tr>
<td></td>
<td>NRU</td>
<td>3.4 ± 0.61A</td>
<td>1.2 ± 0.26A</td>
</tr>
<tr>
<td></td>
<td>LDH</td>
<td>4.1 ± 0.51A</td>
<td>4.4 ± 0.8C</td>
</tr>
<tr>
<td>L6</td>
<td>MTT</td>
<td>9.8 ± 0.16a</td>
<td>5.3 ± 0.2a</td>
</tr>
<tr>
<td></td>
<td>TPC</td>
<td>25 ± 0.61b</td>
<td>21 ± 0.6b</td>
</tr>
<tr>
<td></td>
<td>NRU</td>
<td>4.2 ± 0.37c</td>
<td>2.6 ± 0.18c</td>
</tr>
<tr>
<td></td>
<td>LDH</td>
<td>25 ± 0.09b</td>
<td>14 ± 0.3d</td>
</tr>
</tbody>
</table>

The different letters A-D (FaO) and a-d (L6) denotes significant differences (P≤0.05) among assays at 24, 48, and 72 h, respectively.
In our study, FaO cells appeared to be very sensitive to MG as indicated by the MTT, NRU, and LDH assays, and in L6 cells the sensitivity to MG was shown in the MTT and NRU assays. In the case of LMG, differences were less pronounced when the results of MTT, TPC, and LDH assays were compared. An exception was unusually strong cytotoxic effect (EC50 < 10 µM) obtained in NRU assay after 48 and 72 h exposure. Taken together, the results obtained in these studies revealed that the primary toxic effect of both compounds is caused by their impact on mitochondrial and/or lysosomal activity (MTT and NRU assays).

To some extent this is in agreement with the results of other authors. It has been previously reported that nuclei and mitochondria act as major targets of MG toxic action (19, 22, 25, 35). It seems that such mechanism is the most common. The literature data have shown that a large number of chemicals were reported to disturb mitochondrial functions. The example is cytotoxic events following exposure to cadmium chloride described by Fotakis and Timbrell (13). According to the new insight, MTT reduction is associated not only with mitochondria, but also with the cytoplasm and with non-mitochondrial membranes including the endosome/lysosome compartment and the plasma membrane (2).

Overall, the obtained results show that MG is strongly toxic and LMG exhibited no, or low toxicity. On the other hand, data from in vivo studies on laboratory animals revealed that exposure to LMG causes a greater number and more severe changes than exposure to MG (7). This observation is of great importance because MG (easily absorbed by fish) is extensively metabolised to the LMG, which is retained much longer in muscle. Moreover, it was found that intestinal microflora of several animal species and humans, is capable to reduce MG to LMG (14) so the risk for human health is difficult to predict. Taking into account all available observations from in vivo and in vitro studies, it seems that caution should be taken when fish from uncertain resources are consumed.

References

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