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Full Length Article

In vitro interactions of malachite green and leucomalachite green with hepatic drug-metabolizing enzyme systems in the rainbow trout (*Onchorhyncus mykiss*)

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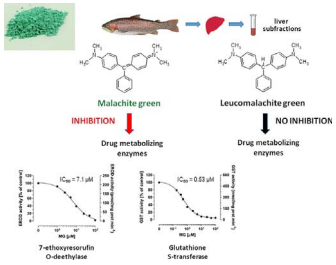
GRAPHICAL ABSTRACT



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ABSTRACT

Malachite green (MG) has been widely used in aquaculture to treat a number of microbial and parasitic diseases. It is currently banned in the EU because of the high cytotoxicity and carcinogenic activity, which is also shared by leucomalachite green (LMG), a reduced MG metabolite that can persist in fish tissues for months. There is scant information about the ability of either compound to interact with drug metabolizing enzymes in fish. Therefore we evaluated the *in vitro* effects of MG and LMG (25, 50 and 100 μM) on some DMEs and glutathione (GSH) content in rainbow trout liver subfractions. LMG did not affect any of the examined parameters. In contrast, MG proved to deplete GSH and to depress to a various extent the activities of NAD(P)H cytochrome *c* reductase, 7-ethoxycoumarin *O*-deethylase, 1-naphthol uridindiphosphoglucuronyl-transferase and maximally those of 7-ethoxyresorufin *O*-deethylase (EROD) and glutathione S-transferase (GST) accepting 1-chloro2,4-dinitrobenzene (CDNB) as substrate. The inhibition mechanisms of EROD and GST were investigated by means of non-linear Michaelis-Menten kinetics and Lineweaver-Burk plots using 0.175–8 μM MG. The calculated IC_{50} for EROD was 7.1 μM, and the inhibition appeared to be competitive (K_i 2.78 ± 0.24 μM). In the case of GST, the calculated IC_{50} was 0.53 μM. The inhibition was best described as competitive toward GSH (Ki 0.39 ± 0.02 μM) and of mixed-type toward CDNB (Ki 0.64 ± 0.06 μM). Our findings indicate that, contrary to LMG, MG behaves as a relatively strong inhibitor of certain liver DMEs and can reversibly bind GSH.

Abbreviations: BNF, β-naphthoflavone; CDNB, 1-chloro2,4-dinitrobenzene; CYP, cytochrome P450; DME, drug metabolizing enzyme; ECOD, 7-ethoxycoumarin *O*-deethylase; EROD, 7-ethoxyresorufin *O*-deethylase; GSH, glutathione; GST, glutathione S-transferase; LMG, leucomalachite green; MG, malachite green; ROS, reactive oxygen species; UGT, uridindiphosphoglucuronyl-transferase

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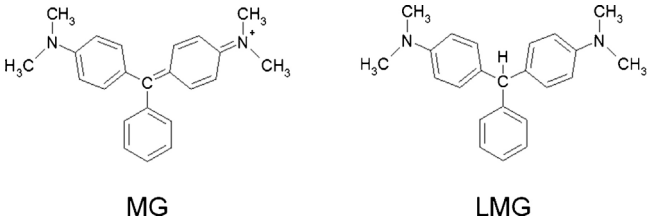

Fig. 1. Chemical structures of malachite green (MG) and its reduced metabolite leucomalachite green (LMG).

1. Introduction

Malachite green (MG), a N-methylated diaminotriphenylmethane dye (Fig. 1) has been largely employed in aquaculture as an antifungal agent and a disinfectant (Alderman, 1985). It is also used for dyeing wool, jute, cotton, leather and ceramics, and, illegally, as a food colouring agent (Hidayah et al., 2013). Although the use of MG in aquaculture is no longer permitted in the EU and in many other countries due to its cytotoxic and carcinogenic properties, it is still utilised illegally because of relatively low cost, ready availability and high efficacy against microbial and parasitic diseases (Culp and Beland, 1996). As regards EU Member States, data derived from either the National Residues Control Plans or the Rapid Alert System for Food and Feed (RASFF) indicate the presence of residues of MG or its metabolite leucomalachite green (LMG) in more than 680 fish and crustacean samples for the years 2002 to 2014 (EFSA, 2016).

Owing to its iminium structure, MG acts as an electron accepting/transferring compound, which may result in the generation of reactive oxygen species (ROS) and in glutathione (GSH) depletion ultimately leading to oxidative stress (for a review see Kovacic and Somanathan, 2014). There is also evidence that, like other cationic triarylmethane dyes, MG may form adducts with protein sulfhydrils (-SH) and/or GSH (Eldem and Özer, 2004); this property has been associated with the inhibition of a number of enzymes (*e.g.* cholinesterases) (Tacal and Özer, 2004). MG is highly cytotoxic in either mammalian (Clemmensen et al., 1984; Panandiker et al., 1992) or fish cell systems (Zhan and Braunbeck, 1995) and exhibits carcinogenic properties in rodents (NTP, 2005; Culp et al., 2006), being also able to act as a tumour promoter (Sundarrajan et al., 2001; Rao and Fernandes, 1996).

In aquatic and in mammalian species MG is extensively reduced to LMG, (Fig. 1) (Henderson et al., 1997; Culp et al., 1999), which represents the major metabolite in tissues from MG-treated fish (Machova et al., 1996; Doerge et al., 1998a) and may persist in measurable amounts in muscle and skin for months (Kietzmann et al., 1990). Besides, an oxidative biotransformation pathway entailing the generation of N-demethylated derivatives has been demonstrated in MG-exposed fish (Doerge et al., 1998a) and mammals (Culp et al., 1999), respectively, as well as in *Cunninghamella elegans*, a fungal model of mammalian xenobiotic metabolism (Cha et al., 2001). Marked differences in cytotoxicity between MG and LMG were observed in human cell lines (Stammati et al., 2005). Like MG, LMG displays carcinogenic properties; in particular, it has been implicated in the genesis of thyroid follicular cell adenomas and carcinomas in female rats (NTP, 2005) with the LMG-mediated inhibition of thyroid peroxidase (Doerge et al., 1998b) and the consequent increase in TSH output being considered the key events in tumour formation (Culp et al., 1999). Of note, in a recent opinion on MG in food (EFSA, 2016), EFSA concluded that both MG and LMG may be considered as genotoxic carcinogens.

Drug metabolizing enzymes (DMEs) play a capital role in the detoxification/bioactivation as well as in the body persistence of a vast array of both foreign and endogenous compounds, including drugs and environmental pollutants (Nebbia, 2001). In recent years, an increasing body of knowledge has allowed to characterize the biotransformation pathways as well as the expression and the catalytic activity of DMEs in most piscine species including the rainbow trout (Schlenk et al., 2008). In common with other triarylmethane dyes (*e.g.*, methylene blue, fuchsin, neutral red, safranine T), MG has been reported to inhibit some phase I (oxidative) (Beyhl, 1981) and phase II (conjugative) DMEs (Debnam et al., 1993; Glanville and Clark, 1997) in hepatic subfractions from laboratory species or humans. However, data on aquatic species are lacking nor the effects of LMG on DMEs have ever been tested.

In consideration of both the long persistence of LMG in fish tissues and the results of the aforementioned *in vitro* studies performed in mammalian species pointing to a dye-mediated overall reduction in liver DME activities, the potential exists for an MG/LMG-mediated interference on the kinetics of drugs or environmental pollutants fish may be exposed to. The aim of the present work was therefore to investigate the effects of either MG or LMG on selected oxidative and conjugative DMEs in hepatic subfractions from rainbow trout (*Oncorhynchus mykiss*), the breeding of which has gained a remarkable economic relevance in the UE as it accounts for about 50% of the overall world production (https://ec.europa.eu/fisheries/sites/fisheries/files/docs/body/pcp_en.pdf). Some experiments were conducted on liver subfractions from fish pre-treated with β-naphthoflavone (BNF). This chemical mimics the action of several pollutants such as dioxin-like compounds and polycyclic aromatic hydrocarbons in that it increases the expression and activity of cytochrome P450 (CYP) 1A-related proteins and other DMEs in mammalian and piscine species (Gooneratne et al., 1997; Novi et al., 1998).

2. Materials and methods

2.1. Chemicals

Bovine serum albumin, glucose 6-phospate and glucose-6-phosphate dehydrogenase were obtained from Boehringer (Mannheim, Germany). MG hydrochloride, LMG and all other reagents were supplied by Sigma-Aldrich (Milan, Italy).

2.2. Animals and treatments

Juvenile rainbow trouts of either sex (weight range 150–200 g) were purchased from a local fish farm (Moretto, Carmagnola, Torino, Italy) and housed in plastic tanks containing 1000 L of flowing water at 13 °C. Animals were maintained unfed during an acclimatization period of 4 weeks and thereafter randomly allotted to two groups of eight individuals. One group was treated i.p. with βNF dissolved in sterile corn oil at a dose of 100 mg/kg, while the other group received only the vehicle by the same route. After 48 h, the fish were slightly anaesthetised (25 mg/L tricaine methanesulphonate for 30 min) and sacrificed by a blow on the head. Livers were excised, rinsed in chilled 1.15% KCl and homogenized with 4 vols of ice-cold buffer (0,1 M sodium-phosphate buffer, pH 7.4, containing 0.15 M KCl and 2 mM GSH). Microsomal and cytosolic fractions were isolated by differential ultracentrifugation as reported by Bernhoft et al. (1994), pooled, frozen in liquid nitrogen and kept at −80 °C. Before freezing, microsomes were resuspended in 0.1 M phosphate buffer pH 7.4 containing 1 mM EDTA and 20% glycerol in order to obtain a protein concentration of about 30 mg/ml. Protein determination was carried out according to Lowry et al. (1951) using bovine serum albumin as the standard. Ethical permission for the experiment was granted by the Turin University Bioethical Committee and by the Italian Ministry of Health; all animal procedures were carried out in compliance with the EC Directive 86/609/EEC and with the Italian law regulating experiments on living animals (DLgs. 116/92).

2.3. Incubation procedures and biochemical assays

Malachite green or LMG was dissolved in acetonitrile (5 mM stock solutions) never exceeding 2% of the final assay volume. According to the results of preliminary experiments, this solvent was the best