

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

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

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1647120> since 2019-01-08T13:35:14Z

Published version:

DOI:10.1016/j.toxlet.2017.07.900

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

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

Carlo Nebbia^{a*}, Flavia Girolami^a, Monica Carletti^a, Laura Gasco^b, Ivo Zoccarato^b, Alessandra Giuliano Albo^{a#}

^a *Department of Veterinary Sciences, University of Torino, Grugliasco, Italy*

^b *Department of Agricultural, Forest and Food Sciences, University of Torino, Grugliasco, Italy*

[#] *Present affiliation: ABLE Biosciences, Bioindustry Park Silvano Fumero SpA, Collettero Giacosa, Italy*

* Corresponding author. Tel: +39 011 670 9015; Fax: +39 011 670 9016.

E-mail address: carlo.nebbia@unito.it (C. Nebbia)

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 μ M) 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 μ M MG. The calculated IC₅₀ for EROD was 7.1 μ M, and the inhibition appeared to be competitive (K_i 2.78 \pm 0.24 μ M). In the case of GST, the calculated IC₅₀ was 0.53 μ M. The inhibition was best described as competitive toward GSH (K_i 0.39 \pm 0.02 μ M) and of mixed-type toward CDNB (K_i 0.64 \pm 0.06 μ M). Our findings indicate that, contrary to LMG, MG behaves as a relatively strong inhibitor of certain liver DMEs and can reversibly bind GSH.

Keywords: malachite green; leucomalachite green; trout; drug-metabolizing enzymes; enzyme inhibitors; inhibition kinetics

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.

1. Introduction

Malachite green (MG), a N-methylated diaminotriphenylmethane dye (Figure 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 leuco-malachite 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 sulfhydryls (-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 & Fernandes, 1996).

In aquatic and in mammalian species MG is extensively reduced to LMG, (Figure 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, safranin 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-phosphate 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 gr) were purchased from a local fish farm (Moretto, Carmagnola, Torino, Italy) and housed in plastic tanks containing 1000 litres 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 β NF 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 hours, 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 volumes of ice-cold buffer (0,1M sodium-phosphate buffer, pH 7.4, containing 0.15M KCl and 2mM 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 0.1M phosphate buffer pH 7.4 containing 1mM 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 were 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 compromise between the solubility of the chemicals under investigation and the inhibitory effects on the tested enzyme activities. Unless otherwise

specified, final concentrations of 100, 50 or 25 μ M of either compound were used; control incubations were performed with solvent only. The effects on both CYP content and spectral properties were detected diluting 3 mg of microsomal proteins in phosphate buffer 0.1M pH 7.4 either in the presence or in the absence of NADPH 1 mM. After 10 minutes of aerobic incubation at 37 °C with MG or LMG, the reactions were quenched by placing the flasks in an ice water bath. Aliquots were then removed and the haemoprotein content was measured as the dithionite-reduced minus carbon monoxide differential spectrum (Fent and Bucheli, 1994).

To evaluate the effects on DMEs, test compounds were directly added to vessels containing microsomal or cytosolic proteins in the assay buffer and incubated for 3 minutes; the reactions were started by adding the appropriate cofactors, with the exception of glutathione *S*-transferase (GST) which was monitored after substrate addition. In particular, NAD(P)H- cytochrome *c* reductase was determined using 0.02 mg of protein in the presence of 2.5 mM NADPH or 0.17 mM NADH, respectively (Fent and Bucheli, 1994). The O-deethylation of 2 μ M 7-ethoxyresorufin (EROD) and the O-deethylation of 0.8 mM 7-ethoxycoumarin (ECOD) were measured as described by Nebbia et al. (2003) with 0.15 and 0.3 mg of protein, respectively. The 1-naphthol uridindiphosphoglucuronyl-transferase (UGT) activity was assayed spectrofluorometrically using 0.25% Triton X100-activated microsomes (0.2 mg of protein) (Bock et al., 1979). For EROD, ECOD, and UGT only preparations from β NF-induced animals were used due to low basal activities, amounting to 62 \pm 5 pmol/min/mg of protein, 47 \pm 4 pmol/min/mg protein and 1.2 \pm 0.2 nmol/min/mg protein, respectively. The activity of GST accepting 1-chloro-2,4-chlorodinitrobenzene (CDNB) as a substrate was measured by a modification of the method of Habig et al., (1974): briefly, 0.025 mg of cytosolic protein were added to 0.1M potassium-phosphate buffer (pH 7.4) containing GSH 1 mM and CDNB 2.5 mM and the formation of the GSH-CDNB adduct was monitored at 340 nm. All assays were carried out at 30°C except for GST and EROD, which were respectively measured at 25°C and at 37°C. Finally, to study the effects on GSH, cytosolic fractions were diluted in a phosphate buffer 0.1 M (pH 8)-EDTA 5 mM, incubated for 10 minutes at 37°C and GSH content was determined in appropriate aliquots with Ellmann's reagent (Gergel' and Cederbaum, 1997).

2.4. Kinetic studies and data analysis

Enzyme activities exhibiting more than 50% inhibition at the lowest tested MG concentration (25 μ M) (i.e. EROD and GST) were subjected to both concentration-response studies and kinetic analysis.

The dye concentration required to cause 50% inhibition, the IC₅₀ value, was determined by plotting a sigmoid dose response curve of the percent of the remaining enzyme activity vs. the logarithm of graded inhibitor concentrations (1-100 μ M) using GraphPad Prism version 7.1 for Windows (GraphPad Software Inc., La Jolla, CA, USA).

For the inhibition kinetic studies, apparent K_m and V_{max} values were determined by the non linear regression curve fit using the Michaelis Menten equation (GraphPad Prism version 7.1 for Windows). The K_i values and the type of inhibition were determined from the best fit using the same scientific software. Kinetic analysis was also performed by means of Lineweaver-Burk plot. EROD kinetic studies were conducted with substrate concentrations in the range 0.125 - 4 μ M and inhibitor (MG) concentrations of 0, 2, 4, or 8 μ M. The kinetic analysis of GST activity in the presence of MG (0, 0.175, 0.35, 0.7 and 1.4 μ M) was accomplished with respect to both the G site (binding the co-substrate GSH) and the H site (binding the substrate) (Eaton and Bammler, 1999), i.e. alternatively changing either GSH (0.5, 1, 2 or 4 mM) or CDNB concentrations (0.125, 0.166, 0.25, 0.5, 1 or 2 mM) while holding constant the other one at 1mM..

2.5. Statistics

Unless otherwise indicated, data represent mean \pm S.E.M. of three experiments performed at least in triplicate. Where appropriate, data were subjected to statistical evaluation by means of one-way ANOVA followed by Bonferroni's Test and the level of significance was set at $P < 0.05$.

3. Results

Malachite green

3.1. Effects on drug-metabolizing enzymes

3.1.1. Cytochrome P450 content, spectral characteristics and phase I enzymes

The effects of MG on the haemoprotein content and on phase I enzymes are shown in Table 1. In either control- or BNF-microsomes the incubation of MG at 50 or 100 μ M caused a slight loss of CYP content; no measurable levels of cytochrome P420 (P420) could be detected (data not shown). Similar results were obtained after the addition of NADPH 1mM. The incubation of microsomal fractions in the presence of MG led to a concentration-related inhibition of NADPH- and NADH- cytochrome *c* reductases: both were statistically significant depressed already at the lowest MG concentration (25 μ M) in either control- (-20% and -10 %, respectively) or BNF-fractions (-37% and -16%, respectively) (Table 1). Due to the very low basal activity in microsomes from untreated fish, MG effects on EROD and ECOD were examined only on BNF-microsomes. At both 50 and 100 μ M MG, EROD activity was undetectable, while at 25 μ M it was inhibited to more than 50% vs control microsomes; a concentration-dependent inhibition was recorded for ECOD, which exhibited statistically different values ($P < 0.05$) at 50 (- 35%) and 100 μ M (- 67%) MG concentrations only.

3.1.2. *Reduced glutathione and Phase II enzymes*

Results are depicted in Table 2. A moderate (up to -33%) but statistically significant and concentration-related decrease in GSH content was observed in both control and BNF cytosolic fractions. When incubated with MG, microsomes from BNF-treated animals showed a concentration-dependent inhibition of UGT activity reaching the statistical significance at 50- (-39%) or 100 μ M (-66%). A more remarkable effect was detected on GST, where already the lowest tested MG concentration (25 μ M) entailed a reduction of about 95% enzyme activity in both control and BNF cytosolic fractions.

3.2. *IC₅₀ and kinetic analysis*

The concentration-dependent changes in the activity of the enzymes most susceptible to MG-mediated inhibition, i.e. EROD and GST, were recorded in the presence of increasing dye concentrations. Both enzyme activities exhibited a concentration-related decrease with calculated IC₅₀ values of 7.1 μ M and 0.53 μ M for EROD and GST, respectively (Figure 2). In order to get further insight into the inhibition mechanisms, kinetic analysis was performed using both Michaelis-Menten non linear regression analysis and Lineweaver-Burk plots.

Kinetic parameters are depicted in Table 3. As regards EROD, MG significantly increased K_m from 0.61 ± 0.05 (0 μM MG) to $1.32 \pm 0.12 \mu\text{M}$ (8 μM MG), while V_{max} values did not display statistically significant changes. Data analysed by the non-linear Michaelis-Menten regression curve were best fitted to a competitive inhibition model, with a calculated K_i of $2.7 \pm 0.2 \mu\text{M}$; this model was confirmed by the Lineweaver-Burk plot, in which the nest of the generated lines intersected at the y-axis (Figure 3). In the case of GST, the incubation of a fixed CDNB concentration (1 mM) with different GSH concentrations (0.5 to 4mM) in the presence of increasing MG concentrations (0.175 to 1.4 μM) also resulted in a progressive increase of the K_m (up to 2.5-fold at the highest dye concentration) along with a limited, not statistically significant decrease in V_{max} values (Table 3). Both the Michaelis-Menten non linear regression analysis and the Lineweaver-Burk plot pointed to a competitive inhibition mechanism (Figure 4A and 4B) with a calculated K_i of $0.39 \pm 0.02 \mu\text{M}$. When GST activity was assayed at a fixed GSH concentration (1 mM) and varying CDNB concentrations (0.125 to 2 mM) in the presence of the same MG concentrations as above, there was a concentration-related fall in V_{max} , resulting in values amounting to about one third of those measured in the absence of the dye (1.44 ± 0.11 vs 0.54 ± 0.03); by contrast K_m values were substantially unaffected (Table 3). The results showed the best fit for a mixed type (competitive-non competitive) inhibition model (Figure 4C and 4D). The calculated K_i was $0.63 \pm 0.06 \mu\text{M}$.

Leucomalachite green

At all tested concentrations and in both control or βNF subfractions, LMG failed to alter either the content or the spectral properties of P450 as well as the examined phase I and phase II DME activities or the GSH content (data not shown).

4. Discussion

Results from this study indicate that MG is overall acting as a powerful in vitro inhibitor of liver drug metabolism in trout subfractions.

Using a stepwise approach, a general screening was first carried out using dye concentrations up to 100 μM . As regards the components of the monooxygenase chain, MG did not affect either CYP content or its spectral characteristics, with no apparent conversion into P420, which is unable to carry out biotransformation reactions (Ishikawa et al., 1967).

The CYP central haeme iron is bound to the apoprotein moiety via a cysteine residue, which is thought to play a key role in the maintenance of the hemoprotein spectral characteristics (White and Coon, 1980). Chemicals able to strongly react with –SH groups, like mercurials (Lamè and Segall, 1987) or organotins (Nebbia et al., 1999), or conditions like lipid peroxidation (Nebbia et al., 1997) are reported to cause CYP loss matched by a concomitant rise in P420. This was not detected in the present study, irrespective of the source of microsomes (i.e. from untreated or BNF-pretreated fish) and even when the dye was incubated with NADPH 1mM. Our findings suggest that, under the applied *in vitro* conditions, neither MG itself nor its NADPH-mediated metabolites behave as strong –SH reagents (see below for further discussion) or act as direct/indirect (per)oxidizing agents. Oxidative stress has been recently proposed as a common toxicity mechanism for triarylmethane dyes based on their property to accept and transfer electrons (Kovacic and Somanathan, 2014). Such a property may well explain the observed concentration-related fall (up to 67%) in both tested microsomal cytochrome *c* reductases, with no major differences related to either the microsome source or the nature of the cofactor (NADPH vs NADH). In the only published paper available for comparison, a less marked inhibition of NADPH cytochrome *c* reductase activity (30% vs. 67%) was reported by Beyhl (1981) upon the incubation of rat liver microsomes with MG 100µM, pointing to a possible higher sensitivity of trout reductases.

There were notable differences in the extent of inhibition of the two tested microsomal monooxygenases. While ECOD showed limited inhibition only at the two higher tested dye concentrations (50 or 100 µM), EROD was sharply depressed (~ 80%) already at the lowest one (25 µM). Similar results occurred in the cited paper of Beyhl (1981), where MG 100µM resulted in degrees of inhibition ranging from almost 90% (aminopyrine N-demethylase) to less than 10% (methylyapanine N-demethylase). In our study a general fall in the monooxygenase activity had to be expected in the light of the observed decrease in the NAD(P)H cytochrome *c* reductases activity and the role played by those flavoproteins in the CYP-mediated reactions. However, the wide discrepancy in the depression of the rate of the *in vitro* metabolism of different CYP substrates points to the occurrence of further inhibition mechanisms (see below for further discussion).

MG caused a limited (up to -33%) concentration-related decrease in liver cytosolic GSH content. A similar decline in tripeptide concentration was documented by Yonar and Yonar (2010) in livers from rainbow trout exposed for 60 min to 18 µM MG once a day during five

consecutive days. This finding may be related to the formation of MG adducts with GSH and other (protein) thiols, which has been shown to occur spontaneously in vitro (Debnam et al., 1993; Eldem and Özer, 2004). Although evidence has been provided of the biliary excretion of a MG-GSH conjugate in the rat (Debnam et al., 1993), the nature of such adducts/conjugates has yet to be confirmed by mass spectrometry.

As regards phase II enzymes, MG proved to act as a strong inhibitor of GST accepting CDNB as a substrate, with about 10% remaining activity at the lowest tested concentration (25 μM). Our results are in agreement with previous work by other investigators performed with purified rat liver cytosolic GSTs or purified human GSTs incubated with MG concentrations in the range 4-45 μM (Debnam et al., 1993) and up to 50 μM (Glanville and Clarke, 1997), respectively. Although, as mentioned before, several lines of evidence support the formation of GSH-adducts, the role of GST in such a phenomenon has never been assessed. Finally, the observed MG-mediated depression in the 1-naphthol glucuronidation rate is difficult to explain. Since, to the best of our knowledge, the formation of glucuronides of MG or its metabolites has never been documented either in vitro or in vivo (EFSA, 2016), a competitive mechanism could be reasonably excluded.

After a preliminary screening, the MG-mediated inhibition of EROD and GST were further characterized using a wider range of dye concentrations and BNF subfractions.

The remarkable degree of EROD inhibition was confirmed by concentration-response studies showing an IC_{50} in the micromolar range (7.1 μM). An analysis of the inhibition kinetics by both the non linear Michaelis Menten regression curve and a double-reciprocal (Lineweaver–Burk) plot revealed low K_i values corroborating the marked inhibitory potency of the dye; in addition, the observed competitive inhibition type suggests that MG may compete for the substrate binding site of the CYP involved in EROD activity. BNF is reported to specifically induce CYP1A gene family in trout liver (Lemaire et al., 1996) and it is generally accepted that EROD activity closely mirrors CYP1A expression in mammalian and piscine species (see Whyte et al., 2000 for a review). Our findings would therefore suggest that MG could be a substrate of CYP1A and possibly other CYPs, which has been so far demonstrated only in cultures of the fungus *Cunninghamella elegans* (Cha et al., 2001). Interestingly, the exposure of SHE primary cultures to MG concentrations in the range 0.07-0.27 μM for 24h triggered an up to three-fold induction of aryl hydrocarbon hydroxylase (Panandiker et al., 1992), whose activity is also associated with CYP1A (Whyte et al., 2000)

In line with the results of the screening studies, MG turned out as a powerful GST inhibitor with both IC₅₀ and K_i values in the submicromolar range. The inhibition kinetic studies, which were performed using the same approach followed for EROD, revealed a competitive inhibition with GSH at the G site, and a mixed type inhibition with respect to CDNB at the H site. The same inhibition mechanisms were observed in previous investigations on different human liver GST families upon the incubation with MG (0-50 μM) (Glanville and Clarke, 1997). Of note, the MG-mediated inhibition of purified rat liver GSTs performed with similar assay conditions was best characterized as partially non-competitive and partially competitive regarding GSH and CDNB, respectively (Debnam et al., 1993) pointing to species-related differences in the susceptibility to the dye (Glanville and Clarke, 1997). Clear evidence concerning the formation of MG-GSH adducts and their significant contribution to the overall enzyme inhibition was provided in both the human and the rat studies

Finally, unlike MG, LMG failed to depress both the NAD(P)H cytochrome c reductases and the tested oxidative and conjugative enzyme activities, and to deplete GSH as well. Since LMG lacks the iminium structure, capable of accepting/transferring electrons, it may be argued that this functional group is at least partially involved in the inhibitory properties displayed by MG. In this respect, it should be noted that cell proliferation and viability of HEp-2 and Caco-2 cells were unaffected by LMG concentrations up to 610 μM, while either function was remarkably depressed starting from MG concentrations as low as 1 μM (Stammati et al., 2005)

In conclusion, MG proved to act as an *in vitro* GSH depletor and a general inhibitor of trout liver DMEs, most notably EROD and GST, which exhibited IC₅₀ values in the micromolar and submicromolar range, respectively. Liver concentrations of 3-5 μM are reported to occur in trout liver in the 12-24 h following MG exposure at concentrations mimicking a therapeutic protocol (Kasuga et al., 1992; Alderman and Clifton-Hadley, 1993). Although care is needed in extrapolating *in vitro* results to *in vivo* outcomes, the present findings indicate the potential for an MG-mediated acute interference on the kinetics of drugs or environmental pollutants that are substrates of either enzyme. By contrast, this effect would not be expected to occur to a significant extent as MG is progressively converted to LMG. Additional *in vivo* studies are warranted to eventually confirm the present *in vitro* results and further research is needed to gain further insight into the involvement of CYP(s) in MG biotransformation.

Acknowledgements

This study was supported by a grant of Regione Piemonte (DGR 23-26314 -Problemi derivanti dall'impiego del verde malachite in acquacoltura)

References

- Alderman, D.J., 1985. Malachite green: a review. *J. Fish Dis.* 8, 289–298.
- Alderman, D.J., Clifton-Hadley, R.S., 1993. Malachite green: a pharmacokinetic study in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J. Fish Dis.* 16, 297–311.
- Bernhoft, A., Hektoen, H., Skaare, U.J., Ingebrigsten, K., 1994. Tissue distribution and effects on hepatic xenobiotic metabolising enzymes of 2,3,3',4,4'-pentachlorobiphenyl(PCB-105) in cod (*Gadus morhua*) and rainbow trout (*Oncorhynchus mykiss*). *Environ. Poll.* 85, 351–359.
- Beyhl, F.E., 1981. Interaction of organic dyes with hepatic microsomal drug-metabolizing monooxygenases in vitro. *Experientia.* 37, 943–945.
- Bock, W., Clausbruch, U.C.W., Kaufmann, R., Lilienblum, W., Oesch, F., Pfeil, H., Platt, L., 1979. Functional heterogeneity of UDP-glucuronyltransferase in rat tissues. *Biochem. Pharmacol.* 29, 495–500.
- Cha, C.J., Doerge, D.R., Cerniglia, C.E., 2001. Biotransformation of Malachite Green by the fungus *Cunninghamella elegans*. *Appl. Environ. Microb.* 67, 4358–4360.
- Clemmensen, S., Jensen, J.C., Jensen, N.J., Meyer, O., Olsen, P., Würtzen G., 1984. Toxicological studies on malachite green: a triphenylmethane dye. *Arch. Toxicol.* 56, 43–45.
- Culp, S.J., Beland, F.A. 1996. Malachite green: a toxicological review. *J. Am. Coll. Toxicol.* 15, 219–238.
- Culp, S.J., Blankenship, L.R., Kusewitt, D.F., Doerge, D.R., Mulligan, L.T., Beland, F.A., 1999. Toxicity and metabolism of malachite green and leucomalachite green during short-term feeding to Fischer 344 rats and B6C3F1 mice. *Chem.-Biol. Interact.* 122, 153–170.
- Culp, S.J., Mellick, P.W., Trotter, R.W., Greenlees, K.J., Kodell, R.L., Beland F.A., 2006. Carcinogenicity of malachite green chloride and leucomalachite green in B6C3F(1) mice and F344 rats. *Food Chem. Toxicol.* 44, 1204–1212.
- Debnam, P., Glanville, S., Clark A.G., 1993. Inhibition of glutathione S-transferases from rat liver by basic triphenylmethane dyes. *Biochem. Pharmacol.* 45, 1227–33.
- Doerge, D.R., Churchwell, M.I., Gehring, T.A., Pu, Y.M., Plakas, S.M., 1998a. Analysis of malachite green and metabolites in fish using liquid chromatography atmospheric pressure chemical ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* 12, 1625–1634.
- Doerge, R.D., Chang, C.H., Divi, L.R., Churchwell I.M., 1998b. Mechanism for inhibition of thyroid peroxidase by leucomalachite green. *Chem. Res. Toxicol.* 11, 1098–1104.
- Eaton, D.L., Bammler, T.K., 1999. Concise review of the glutathione S-transferases and their significance to toxicology. *Toxicol Sci.* 49, 156–164.
- EFSA, 2016. Malachite green in food. *EFSA J.* 14, 4530, 80 pp.
- Eldem, Y., Özer, I., 2004. Electrophilic reactivity of cationic triarylmethane dyes towards proteins and protein related nucleophiles. *Dyes Pigm.* 60, 49–54.
- Fent, K., Bucheli, T.D., 1994. Inhibition of hepatic microsomal monooxygenase system by organotins in vitro in freshwater fish. *Aquat. Toxicol.* 28, 107–126.

- Gergel', D., Cederbaum, A.I., 1997. Interaction of nitric oxide with 2-thio-5-nitrobenzoic acid: implications for the determination of free sulfhydryl groups by Ellman's reagent. *Arch Biochem Biophys.* 347, 282–288.
- Glanville, S.D., Clark, A.G., 1997. Inhibition of human glutathione S-transferases by basic triphenylmethane dyes. *Life Sci.* 60, 1535–44.
- Gooneratne, R., Miranda, C.L., Henderson, M.C., Buhler, D.R., 1997. β -naphthoflavone induced CYP1A1 and 1A3 proteins in the liver of rainbow trout (*Oncorhynchus mykiss*). *Xenobiotica.* 27, 175–187.
- Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione-S-transferase, the first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* 249, 7130–39.
- Henderson, A.L., Schmitt, C.T., Heinze, M.T., Cerniglia, C.E., 1997. Reduction of malachite green to leucomalachite green by intestinal bacteria. *Appl. Environ. Microbiol.* 10, 4099–4101.
- Hidayah, N., Abu Bakar, F., Mahyudin, N.A., Faridah, S., Nur-Azura, M.S., Zaman, M.Z., 2013. Detection of malachite green and leuco-malachite green in fishery industry. *Int Food Res J.* 20, 1511–1519.
- Ichikawa, Y., Yamano, T., 1967. Reconversion of detergent- and sulfhydryl reagent-produced P-420 to P-450 by polyols and glutathione. *Biochim Biophys Acta.* 131, 490–497.
- Kasuga, Y., Hishida, M., Tanahashi, N., Arai, M., 1992. Studies on disappearance of malachite green in cultured rainbow trout. *Shokuhin Eiseigaku Zasshi [Journal of the Food Hygienic Society of Japan].* 33, 539–542.
- Kietzmann, M., Hapke, H.J., Beeke, M., Sthele, S., 1990. Problems with malachite green use in fish. *Dtsch. Tierarztl. Wochenschr.* 97, 290–293.
- Kovacic, P., Somanathan, R., 2014. Toxicity of imine-iminium dyes and pigments: electron transfer, radicals, oxidative stress and other physiological effects. *J Appl Toxicol.*, 34, 825–834.
- Lame', M.W., Segall, H.J. 1987. In vitro effects of trans-4-hydroxy-2-alkenals on mouse liver cytochrome P-450. *Chem Biol Interact.* 62, 59–74
- Lemaire, P., Forlin, L., Livingstone, D. R., 1996. Responses of hepatic biotransformation and antioxidant enzymes to CYP1A-inducers (3-methylcholanthrene, β -naphthoflavone) in sea bass (*Dicentrarchus labrax*), dab (*Limanda limanda*), and rainbow trout (*Oncorhynchus mykiss*), *Aquat. Toxicol.*, 36, 141–160.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- Machova, J., Svobodova, Z., Svobodnik, J., 1996. Persistence of malachite green in tissues of rainbow trout after a long term therapeutic bath. *Acta Vet. Brno.* 65, 151–159.
- Nebbia, C., Dacasto, M., Topi, B., Burdino, E., Ugazio, G., 1997. Zinc ethylene-bis-dithiocarbamate (Zineb)-mediated inhibition of monooxygenases and lipid peroxidation in bovine liver microsomes. *Vet Hum Toxicol.*, 39, 272–275.
- Nebbia, C., Ceppa, L., Dacasto, M., Carletti, M., 1999. Triphenyltin acetate-mediated in vitro inactivation of rat liver cytochrome P450. *J. Toxicol. Environ. Health.* 56, 443–447.
- Nebbia, C., 2001. Biotransformation enzymes as determinants of xenobiotic toxicity in domestic animals. *Vet. J.* 161, 238–252.
- Nebbia, C., Dacasto, M., Rossetto Giaccherino, A., Giuliano Albo, A., Carletti, M., 2003. Comparative expression of liver cytochrome P450-dependent monooxygenases in the horse and in other agricultural and laboratory species. *Vet. J.* 165, 53–64.
- Novi, S., Pretti, C., Cognetti, A., Longo, V., Marchetti, S., Gervasi, P.G., 1998. Biotransformation enzymes and their induction by β -naphthoflavone in adult sea bass (*Dicentrarchus labrax*). *Aquat. Toxicol.* 41, 63–81.

- NTP (National Toxicology Program), 2005. Toxicology and carcinogenesis studies of malachite green chloride and leucomalachite green. (CAS NOS. 569-64-2 and 129-73-7) in F344/N rats and B6C3F1 mice (feed studies). National Toxicology Program technical report series, 0888-8051, 1–312.
- Panandiker, A., Fernandes, C., Rao K.V.K., 1992. The cytotoxic properties of malachite green are associated with the increased demethylase, aryl hydrocarbon hydroxylase and lipid peroxidation in primary cultures of Syrian hamster embryo cells. *Cancer Lett.* 67, 93–101.
- Rao, K.V., 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, 280–286.
- Schlenk, D., Celander, M., Gallagher, E., George, S., James, M., Seth Kullman, S., van den Hurk, P., Willett, K., 2008. Biotransformations in fishes, in: Di Giulio, R.T., Hinton, D.E. (Eds.), *The toxicology of fishes*. CRC Press, Boca Raton, pp. 153–234.
- Stammati A, Nebbia C, De Angelis I, Albo AG, 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, 853–858.
- Sundarajan, M., Prabhudesai, S., Krishnamurthy, S.C., Rao, K.V., 2001. Effect of metanil yellow and malachite green on DNA synthesis in N-nitrosodiethylamine induced preneoplastic rat livers. *Ind. J. Exp. Biol.* 39, 845–852.
- Tacal, O., Özer, I., 2004. Adduct-forming tendencies of cationic triarylmethane dyes with proteins: metabolic and toxicological implications. *Journal of Biochemical and Molecular Toxicology*, 18, 253–256.
- White, R.E., Coon, M.J., 1980. Oxygen activation by cytochrome P-450. *Annu Rev Biochem.* 49, 315–356.
- Whyte, J.J., Jung, R.E., Schmitt, C.J., Tillitt, D.E., 2000. Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure. *Crit Rev Toxicol.*, 30, 347–570.
- Yonar, M.E., Yonar, S.M., 2010. Changes in selected immunological parameters and antioxidant status of rainbow trout exposed to malachite green (*Oncorhynchus mykiss*, Walbaum, 1792). *Pestic Biochem Physiol.*, 97, 19–23.
- Zhan, T., Braunbeck, T., 1995. Cytotoxic effects of sublethal concentrations of malachite green in isolated hepatocytes from rainbow trout (*Oncorhynchus mykiss*). *Toxicol. in vitro.* 9, 729–741.

Table 1

Effect of malachite green (MG) on cytochrome P450 and phase I enzymes in trout liver microsomes from untreated or pre-treated with β -naphthoflavone (BNF) individuals.

Parameter	MG concentrations (μ M)		
	0	25	50
	100		
Cytochrome P450 0.14 \pm 0.01 (nmol/mg protein)	0.18 \pm 0.01	-	0.16 \pm 0.01
Cytochrome P450 BNF 0.30 \pm 0.02 (nmol/mg protein)	0.32 \pm 0.01	-	0.29 \pm 0.01
Cytochrome P450 + NADPH 0.18 \pm 0.01 (nmol/mg protein)	0.19 \pm 0.01	-	0.19 \pm 0.01
Cytochrome P450 BNF + NADPH 0.28 \pm 0.02 (nmol/mg protein)	0.32 \pm 0.01	-	0.31 \pm 0.01
NADPH cyt.c reductase 25 \pm 3* (nmol/min/mg protein)	74 \pm 1	60 \pm 2*	56 \pm 2*
NADPH cyt.c reductase BNF 33 \pm 1* (nmol/min/mg protein)	77 \pm 1	50 \pm 1*	37 \pm 1*
NADH cyt.c reductase 193 \pm 1* (nmol/min/mg protein)	289 \pm 9	242 \pm 3*	228 \pm 2*
NADH cyt.c reductase BNF 158 \pm 1* (nmol/min/mg protein)	299 \pm 8	270 \pm 2*	221 \pm 11*
ECOD BNF 158 \pm 1* (pmol/min/mg protein)	177 \pm 16	184 \pm 5	121 \pm 5*
EROD BNF ND (pmol/min/mg protein)	183 \pm 1	37 \pm 2*	ND

ECOD, 7-ethoxycoumarin O-deethylase; EROD, 7-ethoxyresorufin O-deethylase; -, not determined; ND, not detectable; * P<0.05 vs. control values. Data are expressed as mean \pm SEM for three independent experiments performed in triplicate.

Table 2

Effect of malachite green (MG) on phase II enzymes and reduced glutathione (GSH) in trout liver subfractions from untreated or pre-treated with β -naphthoflavone (BNF) individuals.

Parameter	MG concentrations (μ M)			
	0	25	50	100
GST 34 \pm 4* (mol/min/mg protein)	703 \pm 9	49 \pm 5*	27 \pm 6*	
GST BNF 22 \pm 2* (nmol/min/mg protein)	584 \pm 6	60 \pm 3*	52 \pm 2*	
UGT BNF 1.8 \pm 0.2* (nmol/min/mg protein)	4.6 \pm 0.1	3.9 \pm 0.2	3.1 \pm 0.3*	
GSH 3.3 \pm 0.1* (μ mol/g liver)	4.6 \pm 0.1	4.3 \pm 0.1*	3.9 \pm 0.1*	
GSH BNF 4.1 \pm 0.1* (μ mol/g liver)	5.0 \pm 0.1	4.5 \pm 0.1*	4.4 \pm 0.1*	

UGT, uridindiphosphoglucuronyl-transferase; GST, GSH S-transferase; * P<0.05 or less vs. blank values. Data are expressed as mean \pm SEM for three independent experiments performed in triplicate.

Table 3

Enzyme kinetic analysis of EROD and GST inhibition by MG in trout liver subfractions from BNF-pretreated individuals.

Enzyme	Parameter	MG concentrations (μM)				
		0	2	4	8	
EROD	Vmax (pmol/mg protein/min)	0.58 \pm 0.05	0.53 \pm 0.06	0.48 \pm 0.05	0.46 \pm 0.06	
	Km (μM)	0.61 \pm 0.05	0.80 \pm 0.05	0.87 \pm 0.07	1.32 \pm 0.12*	
GST vs GSH ^a	Vmax (nmol/mg protein/min)	0	0.175	0.35	0.7	1.4
	Km (μM)	2.24 \pm 0.11	2.19 \pm 0.09	2.08 \pm 0.15	1.70 \pm 0.20	1.65 \pm 0.18
GST vs CDNB ^b	Vmax (nmol/mg protein/min)	1.20 \pm 0.06	1.73 \pm 0.07	2.03 \pm 0.11*	1.98 \pm 0.20*	3.03 \pm 0.25*
	Km (μM)	1.44 \pm 0.11	1.33 \pm 0.07	1.19 \pm 0.09	0.89 \pm 0.07*	0.54* \pm 0.03
	Km (μM)	0.64 \pm 0.04	0.77 \pm 0.04	0.88 \pm 0.05	0.76 \pm 0.07	0.84 \pm 0.09

EROD, 7-ethoxyresorufin O-deethylase; GST, glutathione S-transferase; GSH, reduced glutathione; CDNB, 1,2 dichloronitrobenzene; ^aCDNB held constant; ^bGSH held constant; * P<0.05 vs. control values. Data are expressed as mean \pm SEM for three independent experiments performed at least in triplicate.

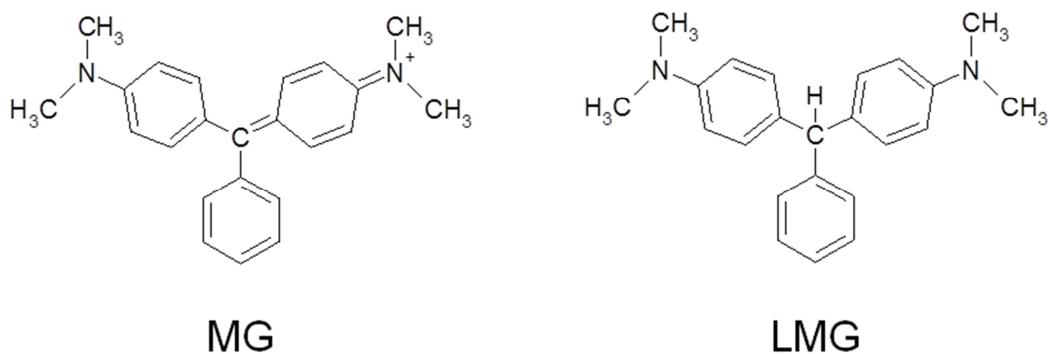


Fig. 1. Chemical structures of malachite green (MG) and its reduced metabolite leucomalachite green (LMG)

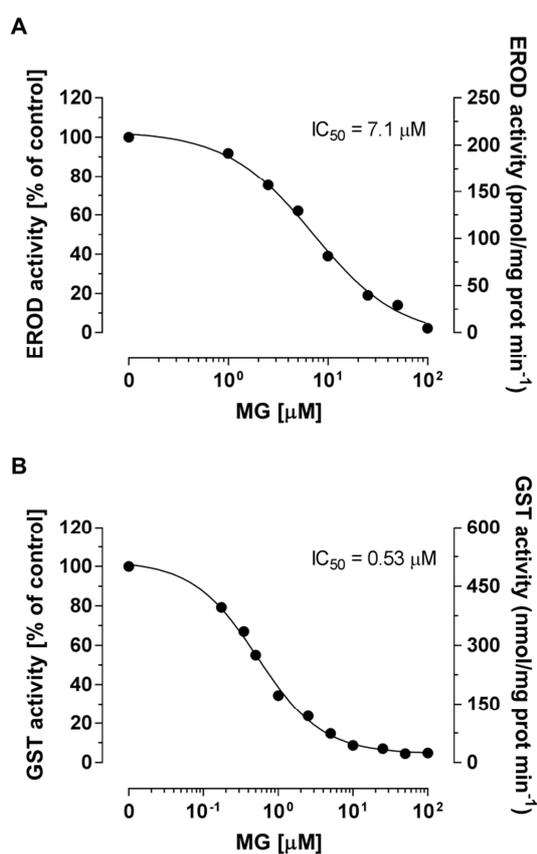


Fig. 2. Sigmoid dose response curve of the percent of the remaining enzyme activity vs. the logarithm of graded MG concentrations for the determination of IC₅₀. A) 7-ethoxyresorufin O-deethylase (EROD); B) glutathione S-transferase (GST). Each point represents the mean \pm SEM of three independent experiments performed in triplicate.

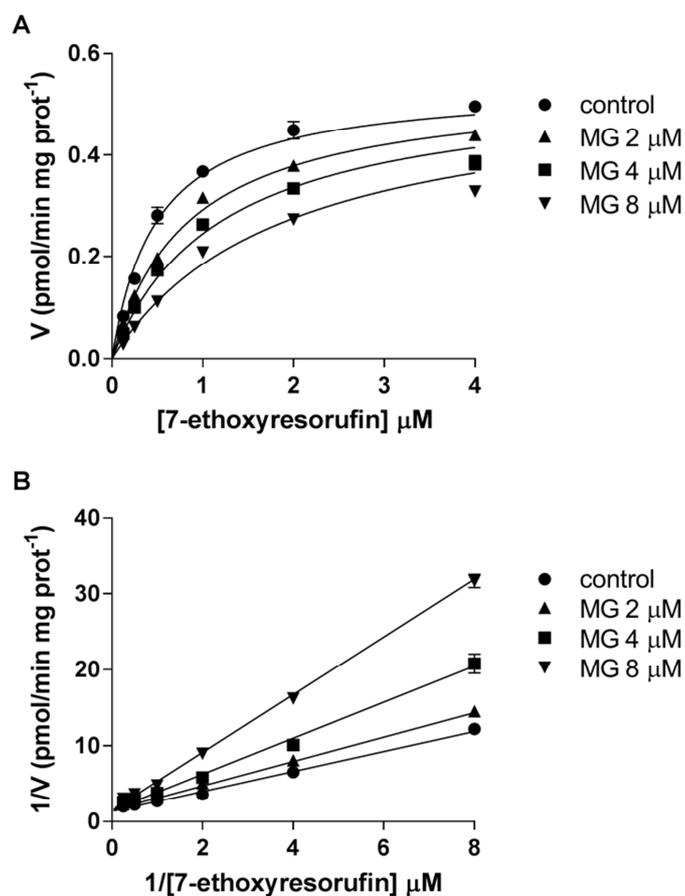


Fig. 3. Inhibition kinetics of 7-ethoxyresorufin O-deethylase (EROD) by MG. A) non-linear Michaelis-Menten plot; B) Lineweaver-Burk plot. The solid lines represent the best fit determined by Graph Pad Prism software. Each point represents the mean \pm SEM of three independent experiments performed at least in triplicate.

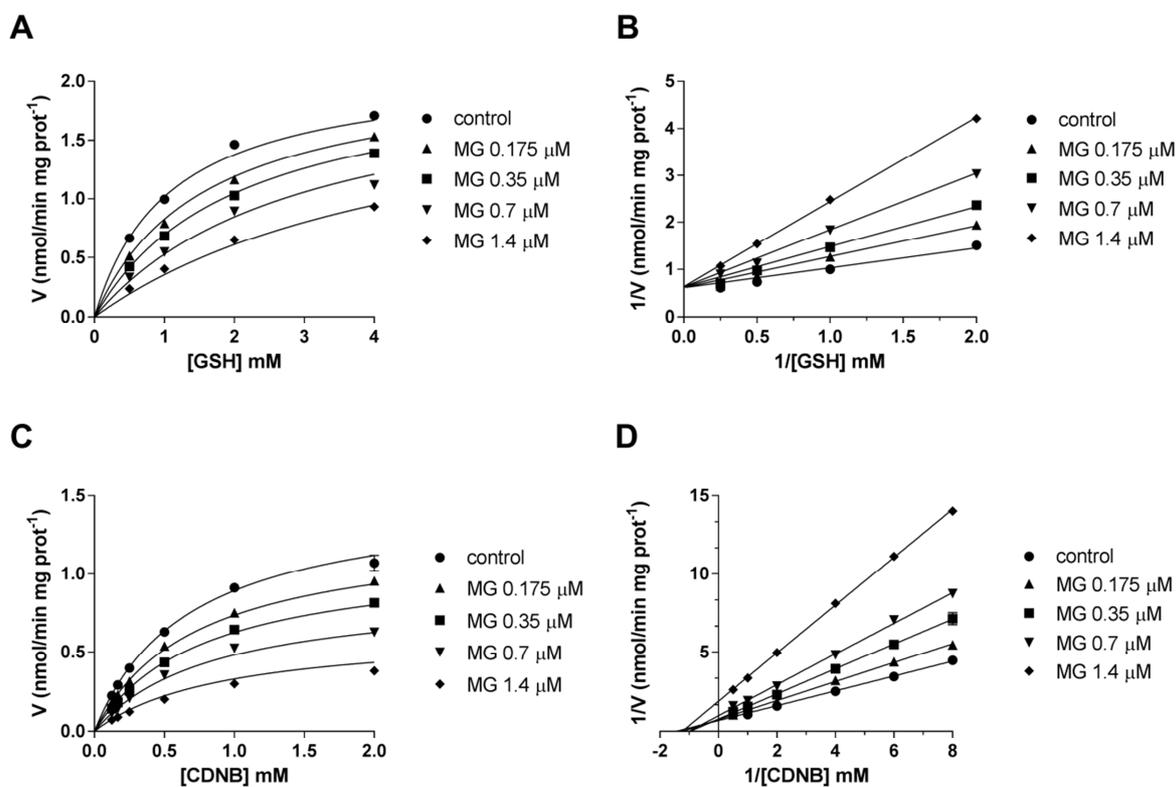


Fig. 4. Inhibition kinetics of glutathione-S transferase (GST) by MG. A) non-linear Michaelis-Menten plot and B) Lineweaver-Burk plot, with respect to varying GSH concentrations; C) non-linear Michaelis-Menten plot and D) Lineweaver-Burk plot, with respect to varying CDNB concentrations. The solid lines represent the best fit determined by Graph Pad Prism software. Each point represents the mean \pm SEM of three independent experiments performed at least in triplicate.