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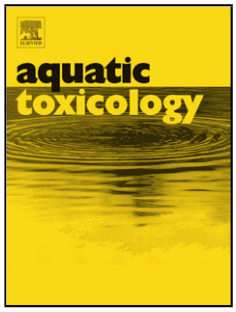

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Cellular transport of microcystin-LR in rainbow trout (*Oncorhynchus mykiss*) across the intestinal wall: possible involvement of multidrug resistance-associated proteins.

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Abstract

We studied Abcc mediated-transport in middle and posterior intestine of the rainbow trout, *Oncorhynchus mykiss*. Luminal and serosal transport were evaluated in everted and non-everted intestinal sacs, respectively, incubated with 1-chloro-2,4-dinitrobenzene (CDNB; 200 μM). CDNB enters the cells and is conjugated with glutathione via glutathione S-transferase (GST) to form 2,4-dinitrophenyl-*S*-glutathione (DNP-SG), a known Abcc substrate. DNP-SG concentration in the bath was recorded every 10 minutes, in order to calculate the mass-specific transport rate. For evaluating the possible involvement of Abcc proteins in microcystin-LR (MCLR) transport, 1.135 μM MCLR was added to the bath or inside the sacs, in everted or non-everted preparations, respectively. Both luminal and serosal DNP-SG efflux were significantly inhibited by MCLR. A concentration- response curve obtained using strips from middle intestine yielded an IC_{50} value of 1.33 μM MCLR. The Abcc inhibitor, MK571 produced concentration-dependent inhibition of DNP-SG

similar to that produced by MCLR. Since competition of MCLR and CDNB as GST substrates could bias the DNP-SG transport results, we evaluated the effects of MCLR on calcein efflux, which does not depend on GST activity. We applied the non-fluorescent, cell-permeant compound calcein-AM (0.25 μM) to middle intestinal strips and recorded the efflux of its hydrolysis product, the fluorescent Abcc substrate calcein. 2.27 μM MCLR and 3 μM MK571 inhibited calcein efflux (17.39 and 20.2 %, respectively). Finally, MCLR interaction with Abcc transporters was evaluated by measuring its toxic intracellular effects. Middle intestinal segments were incubated in saline solution with 1.135 μM MCLR (MC1), 2.27 μM MCLR (MC2), 3 μM MK571 (MK) or 1.135 μM MCLR + 3 μM MK571 (MC1/MK). After one hour, GSH concentration, protein phosphatase 1 and 2A (PP1, PP2A) and GST activities were measured in each segment. MC1did not produce significant effect while MC1/MK and MC2 significantly inhibited PP1and PP2A in similar proportions (34 - 49 %). MK alone significantly increased PP2A activity (40 %) with no effect in any other variable. GST activity and GSH concentration were not affected by any treatment. Concentration-response curves for MCLR (1.135 to 13.62 μM) alone or plus 3 or 6 μM MK571 were obtained using PP1 activity as response variable. The IC_{50} values were 1.0, 0.52, and 0.37 μM, respectively. Our results suggest that *O. mykiss* enterocytes are capable of eliminating MCLR by GST-mediated conjugation and luminal excretion through an Abcc-like apical transporter. This mechanism would prevent toxic effects and reduce the toxin uptake into the blood, which is likely mediated by basolateral Abccs.

Keywords: Cyanotoxin; trout intestine; intestinal sacs; detoxification; Abcc transporter, *Oncorhynchus mykiss*.

Abbreviations: ABC/Abc, ATP-binding cassette; BSA, bovine seroalbumine; calcein-AM, calcein acetoxymethyl ester; CDNB, 1-chloro-2,4-dinitrobenzene; DNP-SG, 2,4-dinitrophenyl-*S*-glutathione; DTNB, 5,5´-Dithiobis (2-nitrobenzoic acid); DTT, dithiothreitol; GSH, reduced glutathione; GSSG, oxidized gluthatione; GST, glutathione-S-transferase; GS-X, ATP-dependent glutathione S-conjugate exported; MC, microcystin; MCLR, microcystin with Leucine and Arginine in the variable amino acid positions; MCRR, microcystin with Arginine in the variable aminoacid positions; MCYR,

microcystin with Tyrosine and Arginine in the variable aminoacid positions; Mrp, multidrug resistance proteins; MXR, multixenobiotic resistance; Oatps, organic anion transporting polypeptides; Pgp, P-glycoprotein; *p*NPP, *p*-nitrophenyl phosphate disodium salt; PP, protein phosphatase.

1. Introduction

In freshwaters worldwide, it is common to find cyanobacteria as important components of the ecosystem. This group includes many species, which produce a variety of toxins (cyanotoxins) with different chemical structures and mechanisms of toxicity. The common feature among different cyanotoxins is that they can affect water quality and represent an important risk for animal and human health. Within this group, microcystins (MCs) are the most frequent and have been related to numerous intoxication cases (Dietrich and Hoeger, 2005; Dörr et al., 2010). MCs are cyclic heptapeptides of 900-1100 Daltons with more than 70 variants, being microcystin-LR (MCLR) the best known and one of the most toxic. These compounds are very stable and persistent in water, resisting important physicochemical changes in the environment, such as pH and temperature variations (Sivonen and[0] Jones, 1999). This means that once released in the water, MCs remain as a threat to the aquatic biota for long periods of time.

The characteristic toxic effect of MCs is the inhibition of protein phosphatases (PP) 1 and 2A, which leads to increased protein phosphorylation related to cytotoxic effects and tumor-promoting activity (Carmichael, 1994; Dawson, 1998; Hooser, 2000). Additionally, abundant evidence suggests that oxidative stress is important in MC toxicity (Amado and Monserrat, 2010 for a review). Although the liver is considered as the main target organ of MCs, toxic effects and MC accumulation have also been observed in intestine, kidney, muscle, brain, and gills of a broad spectrum of vertebrates and invertebrates (Kotak et al., 1996; Ito et al., 2000; Pinho et al., 2003; Botha et al., 2004; Wiegand and Pflugmacher, 2005, for a review).

The main route of MCs intake in fish is the gastrointestinal tract (Tencalla et al., 1994; Bury et al., 1998). It has been proposed that differences in sensitivity to MCs among fish species can be explained by differences in toxin absorption through this organ (Fischer and

Dietrich, 2000). These authors have reported that the omnivorous *Cyprinus carpio* has a longer intestine and is more severely affected than the carnivorous *Oncorhynchus mykiss*, when MCLR is administered by oral gavage, although both species have similar sensitivity to intraperitoneal injection. In contrast, Xie et al. (2004) have reported that *Hypophthalmichthys molitrix*, a phytoplanktivorous carp with a long intestine, is able to feed on toxic cyanobacteria with no toxic consequences. According to these authors, *H. molitrix* has a reduced intestinal absorption of MCLR and is highly tolerant to MCRR. MCs cannot readily diffuse through plasma membrane due to their high molecular weight and moderate hydrophobicity. Several reports have shown that the uptake of these toxins into the cells is mediated by transport proteins of the biliary system, namely the organic anion transporting polypeptides (Oatps). This transport has been studied in mammals, in cell lines, and in fish tissues (Eriksson et al., 1990; Runnegar et al., 1995; Fischer et al., 2005; Boaru et al., 2006; Meier-Abt et al., 2007; Lu et al., 2008). In aquatic animals, laboratory and field studies demonstrate that tissue accumulation of MC is followed by metabolization and elimination, limiting MC accumulation and transfer along the food chain (Williams et al., 1997a, b; Amorim and Vasconcelos, 1999; Soares et al., 2004; Ibelings and Chorus, 2007; Bieczynski et al., 2013). However, the mechanistic events implied in MCLR detoxification and elimination have not been elucidated so far. Detoxification processes comprise a system of enzymes and membrane transporters acting coordinately to avoid the accumulation of toxic compounds in the cells. These processes have been summarized in four phases (Omiecinski et al., 2011, for a review), which are not always consecutive. Briefly, phase 0, involves the uptake of xenobiotics, which can be exported unmodified, by an efflux pump of the ABC superfamily (ABCB1), or it could continue to phase I/II. Phase I involves chemical transformation through oxidation, epoxidation, hydroxylation, etc. Products of this phase can continue to phase II or can be excreted by phase III membrane transporters. Phase II comprises conjugation of phase 0/I products with glucuronic acid, glutathione (GSH) or sulphates, catalyzed by transferases (Zamek-Gliszczynski et al., 2006, for a review). Finally, phase III involves the export of toxic compounds, previously transformed through phase I/II reactions, through specialized membrane transporters such as those of the ABC superfamily (Ishikawa, 1992; Klaassen et al., 2010). ABC transporters extrude a wide spectrum of compounds against a concentration

gradient at the expense of energy from ATP, mediating the multixenobiotic resistance (MXR) (Kurelec, 1992; Bard, 2000).

In the intestine, P-glycoprotein (Pgp, ABCB1), multidrug resistance proteins (MRPs, ABCC) and the breast cancer resistance protein (BCRP; ABCG2) are part of the ABC members involved in the elimination of toxic compounds absorbed from the diet. ABCB1 is located at the apical membrane and shows broad substrate specificity, with a tendency towards moderate hydrophobicity, small sized and cationic compounds. Non-metabolized toxic compounds are common substrates of this transporter, although phase I products could also be eliminated by this way (Bard et al., 2000; Chan et al., 2004; Takano et al., 2006). The ABCC family has 9 members (ABCC1-9) with ABCC2 being localized at the apical membrane in several tissues, including the intestinal epithelium, while other ABCCs are basolateral or their localization is still poorly known (Borst et al., 1999; Chan et al., 2004; Klaassen and Aleksunes, 2010; Russel et al., 2010). ABCC2 exports phase II products, conjugated and unconjugated anionic compounds, such as LTC4 and 2,4-dinitrophenyl-*S*-glutathione (DNP-SG), and bilirubin glucuronides. This transporter can also export oxidized and reduced gluthatione (GSSG and GSH) and a number of drugs and conjugated drug metabolites (Fardel et al., 2005). Besides, ABCC2 has been spatially and functionally associated with phase I and II enzymes (Mottino et al., 2000; Catania et al., 2004; Chan et al. 2004). ABCC 1/3 share some substrate affinity with ABCC2 but are localized at the basolateral membrane in enterocytes (Chan et al., 2004). Finally, ABCG2 is mainly at the apical membrane of enterocytes and has similar substrate affinity to ABCB1 (Chan et al., 2004).

It is well known that MCLR is biotransformed through conjugation with GSH, catalyzed by glutathione-S-transferase (GST), before being excreted (Kondo, 1996; Pflugmacher et al., 1998; Ito et al., 2002). The latter authors and Malbrouck and Kestmont (2006) have suggested that, in rat hepatocytes, MC can be exported through GS-X pumps (ATP-dependent glutathione S-conjugate exporters) and MRPs, respectively.

However, besides two reports suggesting the participation of Abcb1 (Contardo-Jara et al., 2008; Amé et al., 2009), the nature of the phase III proteins responsible for MC transport is still unclear. To our knowledge, in spite of the availability of detailed functional and molecular characterization of fish Abccs (Zaja et al., 2008; Sauerborn Klobučar et al.,

2010; Fischer et al., 2011; Long et al., 2011), nothing is known about the possible function of these proteins in MC export. Considering that Abccs have been characterized as transporting GSH conjugates, it becomes interesting to analyze the involvement of these transporters in both apical and basolateral transport of MC in fish intestine. Accumulation and toxicity of MC in aquatic animals implies an important risk for environmental and human health. The knowledge of the possible effects of MC on Abcc-mediated transport would also facilitate the understanding of the interaction of MC with other important environmental toxicants, such as arsenic, which are likely eliminated by Abcc proteins (Miller et al., 2007).

Based on these antecedents, we hypothesize that fish are able to limit MCLR absorption through GST-catalyzed conjugation of this toxin with GSH followed by Abcc-mediated export to the intestinal lumen. If this absorption-limiting function is not completely efficient, MCLR could be transported to the serosal side by basolaterally localized Abccs. In this study, we analyze luminal and serosal MCLR transport through Abcc-like proteins in *ex vivo* intestinal preparations from *O. mykiss.* This fish has been introduced in many countries worldwide, including Argentina, both for fishing and for aquaculture purposes. The ABC proteins of this species, in particular Abcc, have been molecularly and functionally characterized in tissue samples and in cell lines from different tissues, such as the intestinal epithelium (Zaja et al., 2008; Fischer et al., 2011). Additionally, important toxicological studies on MC effects have been performed on *O. mykiss* (i.e. Kotak et al., 1996; Tencalla and Dietrich, 1997; Bury et al., 1998) and other salmonids (Williams et al., 1997a,b). Intracellular effects of MCLR are also studied in these preparations, with or without blocking Abcc-mediated transport. Fig. 1 shows part of the phase 0, I, II and III effectors present in the intestinal epithelium and the way in which the model substrates for ABC transport (CDNB and calcein-AM) are used in this study. The hypothetical pathways followed by MCLR are depicted by dotted lines.

2. Materials and methods

2.1. Chemicals

Reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), bovine serum albumine (BSA), 5,5´-Dithiobis (2-nitrobenzoic acid) (DTNB), MK571 and dithiothreitol (DTT) were purchased from Sigma-Aldrich (St Louis, MO). P-nitrophenyl phosphate disodium salt (*p*NPP) was from Merck (Germany). Calcein acetoxymethyl ester (calcein-AM, Calbiochem, San Diego, CA) was a generous gift from Dr. Amro M. Hamdoun (Scripps Institution of Oceanography, UCSD). Microcystin-LR, YR and RR were purchased from Alexis Biochemicals (San Diego, CA). All used chemicals and reagents were commercial products of analytical grade purity and were used as supplied. CDNB and Calcein-AM were previously dissolved in DMSO, MK571 was dissolved in distilled water. Final concentration of these solvents was always below 0.3%.

2.2. Fish

O. mykiss (n = 95; 182 ± 13 g body mass) obtained from the Centro de Ecología Aplicada del Neuquén (CEAN), Junín de los Andes, Argentina, were held in aquaria (8 kg/m^3) with freshwater obtained from Chimehuin river. The aquaria were set up with a continuous system of water filtration and aeration. Temperature was kept at 20 ± 5 °C. Fish received 1 % body mass ratio of commercial trout feed per day. Before each experiment, fish were fasted for 24 h and then sacrificed by a blow on the head followed by decapitation. Intestine was immediately removed and rinsed in ice-cold Cortland saline, pH 7.4 plus 5 mM HCO_3Na and 5.55 mM Glucose. This saline solution was used for all the experimental preparations. The anterior part with pyloric ceca was discarded. The middle and posterior intestine were used for measuring transport activity because the anatomy of these sections was more adequate for the experimental setup. Pieces of 15 mm length from these sections were thoroughly washed to eliminate gut content with saline solution and then intestinal sacs or strips were prepared as described below. Intestinal preparations were kept at 20 °C with constant aeration throughout the duration of the experiments. All the experimental protocols were approved by the Bioethics Committee, Faculty of Biochemical and Pharmaceutical Sciences, National University of Rosario, Argentina (6060/116).

2.3. Microcystin LR extract

MCLR was purified from a natural bloom of *Microcystis sp.* Cyanobacterial cells were disrupted by three cycles of freezing-thawing and subsequently centrifuged at 2,500 x *g* for 10 min. A volume of 450 mL of sample was applied to an 8.5 g C18 column (SPE C18, Phenomenex, Torrance, CA). Column support was prepared by passing 170 mL of methanol and the same volume of water. The sample was washed with 150 mL of water followed by 150 mL of 90:10 water:methanol mixture and eluted with 20 mL of methanol. The MC extract was quantified by HPLC/UV (Waters 1525) using a Phenomenex Luna column (5µm C18 250 x 4.6 mm). The analytical column was eluted with a two-step acetonitrile gradient (Brena et al., 2006). The outcome was followed by absorbance at 238 nm and the peaks corresponding to MCRR, MCYR, MCLR, were quantified using appropriate standards. The extract showed only MCLR presence, at a concentration of 1800 mg L^{-1} (methanolic extract) (Fig. 2). The presence of MCLR and the absence of other MC variants were confirmed by MALDI-TOF using a Microflex LR (Bruker Daltonics, Billerica, MS, USA) with a 337 nm nitrogen laser, operated in positive ion reflectron mode (Welker et al., 2002).

2.4. Transport activity in intestinal sacs and strips

For detection of Abcc-like activity, a specific transport substrate, CDNB (Evers et al., 2000; Gotoh et al., 2000, Mottino et al., 2001) was used alone or in combination with MCLR or MK571, an Abcc transport inhibitor (Gekeler et al., 1995). CDNB is a cell permeant substrate that once inside the cell is conjugated with GSH via GST and exported as DNP-SG by Abcc proteins (Gotoh et al., 2000). Similarly, we expected MCLR to enter the cell were it would be conjugated with GSH via GST (Kondo et al., 1992, 1996; Pflugmacher et al., 1998). The conjugate MC-SG would be exported by Abcc proteins. Alternatively, we studied Abcc-mediated transport of MCLR with the calcein efflux technique, which is not affected by GST activity. Calcein-AM is cell permeant and not fluorescent. Once inside the cell, esterases hydrolyze the ester bond producing calcein, which is a fluorescent substrate of Abccs (Evers et al., 2000; Takano et al., 2006). Everted and non-everted sacs were used for measuring intracellular to luminal and intracellular to serosal efflux transport, respectively. For preparation of everted sacs, pieces of intestine were put in a Petri dish with ice-cold saline and slid onto a crochet needle to expose the mucosal surface. For non-everted sacs, pieces of intestine were cut, placed in

ice-cold saline and rinsed for eliminating gut content. Immidiately after being weighed, both kinds of preparations were ligated at both ends and filled with saline solution (63 ± 4.6 µL) through a Teflon cannula.

For part of the experiments, the middle intestine was cut into longitudinal strips. Each strip was weighed and randomly placed in glass vessels or microcentrifuge tubes with CDNB or calcein-AM solutions. This kind of preparation is useful for working with small volumes, reducing quantity of chemicals and the number of fish needed. The results obtained reflect the sum of basolateral and apical effluxes.

2.4.1. Effect of MCLR on DNP-SG transport

Middle and posterior intestinal sections from five fish were separated and cut into halves for preparing paired everted and non-everted sacs (0.186 ± 0.010 g). Control sacs were kept submerged in small glass vessels with 5 mL saline solution with 200 µM CDNB (bath) for 1 hour. Treated sacs were prepared in the same way but adding 5.65 µg MCLR (1.135 µM final concentration) to the bath for everted sacs, or adding 5.65 µg MCLR, dissolved in saline solution, in the lumen of non-everted sacs. These MCLR concentrations were chosen in order to apply about 30 µg MCLR per gram of intestine, similar to the concentrations applied in previous *in vivo* experiments (Williams et al., 1997a; Bieczynski et al, 2013), in which ca. 30% of the applied dose has been detected in liver and intestine tissue, respectively.

Every ten minutes, 2 mL aliquots of bath solution were collected and DNP-SG was determined by reading absorbance at 340 nm with a spectrophotometer. After measurement, aliquots were returned to the bathfor keeping a constant volume. The DNP-SG transport rate of each preparation was calculated from the slope of cumulative absorbance *vs*. time, an extinction coefficient of 9.6 mM^{-1} cm^{-1} and 1cm pathlength, and referred to wet tissue mass. Results from each treated sac were expressed as percentage of the corresponding control sacs.

To confirm that the detected product was DNP-SG and to check for the presence of any subsequent metabolite, bath samples from 6 preparations (3 everted and 3 non-everted intestinal sacs) were prepared for HPLC analysis. Samples were treated with 70 % $HClO_4$ (50 µL mL^{-1}) and centrifuged at 3,500 x *g* for 5 min. Supernatants were injected into a

Waters M-6000 HPLC system (Waters, Milford, MA). Isocratic elution was performed with a 5 μm C18 column (Phenomenex, Torrance, CA) with a mobile phase of acetonitrile: 0.01% H_3PO_4 (1:3, v/v) at a flow rate of 1.0 mL min^{-1} (Hinchman et al., 1991). DNP-SG was detected at 365 nm and was quantified by the external standard method by the height of the peak.

The concentration-dependent effect of MCLR on DNP-SG transport was evaluated in middle intestinal strips from other four individuals, bathed with 0, 0.57, 1.135, 2.27, 3.4, 6.81 or 11.35 μM MCLR, final concentration, and the DNP-SG excreted to the bath was registered for one hour. Mass specific DNP-SG transport rate of each treated strip was expressed as percentage of the respective control. A nonlinear regression curve, log (inhibitor concentration) *vs*. response, was fitted and the IC_{50} calculated.

2.4.2. Effects of MK571 on DNP-SG transport

MK571 was applied on everted and non-everted middle and posterior intestinal sacs. Each intestinal section was cut into halves so to prepare paired control and treated sacs from each individual. A total of 18 everted sacs (0.301 ± 0.014 g) were incubated for 10 min with MK571 (at 0, 0.3, 1.5 or 3 μM) added to the bath and then transferred to 200 μM CDNB solution plus the corresponding inhibitor concentration. Non-everted sacs were treated with MK571 at 0, 3 or 10 μM added to the bath (n = 12, 0.406 ± 0.03 g) following the same experimental protocol as for everted sacs. Mass specific DNP-SG transport rate was measured as in 2.4.1 and expressed as percentage of the corresponding control sac. These MK571 concentrations were selected considering that in a previous paper on cell lines from *O. mykiss* (Fischer et al., 2011), the maximum effects on calcein transport were obtained at 3-10μM. Higher concentrations were avoided, in order to reduce unspecific effects.

2.4.3. Transport activity in intestinal strips using calcein-AM

Middle intestinal strips (6 from each of seven individuals, 0.080 ± 0.006 g) were weighed and randomly placed in microcentrifuge tubes (two strips *per* tube). In control tubes, the strips were bathed with 300 μL of saline solution containing 0.25μM calcein-AM (non-fluorescent form) (Zaja et al., 2008). MCLR and MK571 were added to the bath at final concentrations of 2.27 μM and 3 μM, in the respective test tubes. During one hour, 200 μL

aliquots were taken every ten minutes, and calcein fluorescence was read with a Qubit fluorometer (Invitrogen - Molecular Probes, Oregon) at excitation / emission wavelengths of 485 / 530 nm. Immediately after each reading, the aliquots were returned to the tube. Tubes were protected from light during the experiment. Calcein transport rate was calculated as fluorescent units *per* min and referred to tissue mass.

2.5. Toxic effects of MCLR in middle intestinal segments

Everted middle intestinal segments from 30 individuals (0.05 ± 0.002 g), with their extremes open to allow the contact of solutions with both sides of the epithelium, were placed in 5 mL of saline solution + 1.135 µM MCLR (MC1), 2.27 µM MCLR (MC2), 3 µM MK571(MK) or 1.135 µM MCLR + 3 µM MK571 (MC1/MK). MC1/MK segments were incubated for 10 minutes with the inhibitor before adding MCLR. After 1 hour of incubation in the corresponding solutions, intestinal segments were washed with ice-cold homogenization buffer (40 mM Tris-HCl, 20 mM KCl, 20 mM $MgCl_2$, pH 8.6) and homogenized with a teflon-glass homogenizer, 30 strokes (1:4 w/v). Homogenates were centrifuged at 11,000 x *g* for 15 min and the supernatants were used for enzyme assays, and GSH and protein quantitation.

Another nine fish were used to further study concentration dependent effects of MCLR on PP1. The middle intestine was cut into strips and each strip treated with a different MCLR concentration (repeated measures). Each strip was placed in 1 mL bath with 0, 1.135, 2.27, 4.54, 9.08 and 13.62 µM MCLR, in the presence of 0, 3 or 6 µM MK571. Strips were incubated for 10 minutes with MK571 before adding MCLR. After 1 hour of incubation in the corresponding solutions, intestinal segments were washed with ice-cold homogenization buffer and homogenized as described above. The supernatants were used for PP1 assays. Additionally, we have measured PP1 activity in intestinal strips incubated only with 6 µM MK571 (MK571 control).

2.5.1. Protein phosphatases

PP1 and PP2A activities were measured in a microtiter plate reader using *p*NPP as substrate, according to Carmichael and An (1999) and Heresztyn and Nicholson (2001),

respectively. For both enzymes, absorbance at 405 nm was read in triplicate, every 5 min during 40 min at 25 °C. Enzyme activity was expressed as nkatal mg protein^{-1}.

2.5.2. Glutathione-S-transferase (GST, EC1.11.1.9)

GST activity was measured at 340 nm following the methodology described by Habig et al., (1974). Each sample was measured in duplicate, using 100 mM GSH and 100 mM CDNB as substrates and GST activity was expressed as nkatal mg protein^{-1}.

2.5.3. Reduced glutathione (GSH) content

GSH was measured according to Ellman (1959) modified by Venturino et al., (2001). A calibration curve was performed with 0.1 mM GSH in 5 % sulfosalicylic acid. Supernatants were mixed with 15 % sulfosalicylic acid, centrifuged at 10,000 x *g* for 10 min and the resultant supernatants added to a microtiter plate with 1.5 mM DTNB in phosphate buffer, pH 8. After 5 minutes of incubation, absorbance was read at 405 nm. Each sample was measured in triplicate and GSH concentration was expressed as nmol GSH mg protein^{-1}.

2.5.4. Protein content

Total soluble protein content was measured in a Qubit fluorometer with the Quanti-iT^{TM} Q33211 Assay kit (Invitrogen - Molecular Probes, Oregon). Results were expressed as mg protein mL^{-1}.

2.6. Statistical analysis

Data are presented as mean ± SD. Comparisons among groups were performed by unpaired or paired Student t-tests; one-way analysis of variance (ANOVA) for independent or repeated measures or two-way ANOVA with repeated measures, when appropriate. We considered a value of $p < 0.05$ as statistically significant (Zar, 1999). P*ost hoc* Fischer LSD comparisons were used when ANOVA was significant. When the assumptions of homogeneity of variance (Bartlett test) or normality (Kolmogorov-Smirnov test) were not met, data were transformed by Log_{10}.

3. Results

3.1. Effects of MCLR on DNP-SG transport

HPLC analysis showed a single significant peak for DNP-SG at a retention time of 7 min. No metabolites from DNP-SG degradation were detected in significant quantity, indicating that the conversion of DNP-SG to other metabolites, e.g. those mediated by ɣ-glutamyl transferase, was minimal. Fig. 3 shows a representative chromatogram.

Both in everted and in non-everted intestinal sacs, transport of DNP-SG was affected by MCLR. In everted sacs MCLR inhibited DNP-SG transport with respect to control values, from 4.28 ± 0.99 to 3.11± 0.6 nmol DNP-SG g^{-1} min^{-1}, in middle intestine and from 7.11± 3 to 4.85 ± 2.6 nmol DNP-SG g^{-1} min^{-1} in posterior intestine (paired Student's T test, $p < 0.05$ for both sections). In non-everted sacs, transport rate was inhibited from 8.29 ± 2.6 to 6.15 ± 1.8 nmol DNP-SG g^{-1} min^{-1}, in middle intestine and from 5.78 ± 1.3 to 4.86 ± 0.7 nmol DNP-SG g^{-1} min^{-1}, in posterior intestine (paired Student's T test, $p < 0.05$ for both sections). Fig. 4a shows the results of this experiment as percentage of control transport rate. Comparison of percentage inhibition between middle and posterior intestine yielded not significant differences for both everted and non-everted sacs (paired Student's T test, $p > 0.05$).

MCLR inhibited DNP-SG transport in middle intestinal strips in a concentration-dependent fashion. The non-linear regression analysis of log MCLR concentration *vs.* percentage transport rate yielded an IC_{50} of 1.33 µM ($R^2 = 0.76$, Fig. 4b).

3.2. Effects of MK571 on DNP-SG transport

The Abcc inhibitor, MK571 inhibited DNP-SG transport in middle and posterior intestine in both, everted and non-everted sacs (Fig. 5). In everted middle intestinal sacs MK571 produced a clear concentration-dependent effect (one way ANOVA and Fischer LSD *post hoc* comparisons $p < 0.05$; Fig. 5a) with percentage inhibition of 25.6 %, 51.1% and 76.2 %, for 0.3, 1.5 and 3 µM, respectively. Everted posterior intestinal sacs also showed significant inhibition of DNP-SG transport with the same MK571 concentrations (5.1 %, 23.4 % and 37.8 %, respectively; Fig. 5b) but differences among concentrations were not significant. In non-everted sacs, 3 and 10 µM MK571 produced significant DNP-SG

transport inhibition (22.4 % and 35.3%, respectively, for middle intestine, and 20.2 % and 40.6 %, respectively, for posterior intestine; Fig. 5c-d).

3.3. Transport activity in intestinal strips as measured with calcein efflux

Calcein transport was significantly reduced by 2.27 μM MCLR and 3 μM MK571 respect to control strips (repeated measures ANOVA $p < 0.05$). There was no significant difference between strips treated with MCLR and MK571. Percentage inhibition was 17.39 % for MCLR and 20.2 % for MK571 (Fig. 6).

3.4. Toxic effects of MCLR in middle intestinal segments

Final concentrations of MCLR (in μmol g^{-1}) applied in each treatment are shown in Fig. 7a. PP1 and PP2A activities showed significant differences among treatments (one-way ANOVA, $p < 0.05$, for both protein phosphatases; Fig.7d-e) while GST and GSH were not affected (Fig. 7b-c). 1.135 μM MCLR (MC1) did not produce any significant effects with respect to the control in any of the studied variables, while 2.27 μM MCLR (MC2) caused significant inhibition of PP1 and PP2A activity (41% and 49 %, respectively). Similarly, MC1/MK significantly inhibited protein phosphatases (34 % for PP1 and 38 % for PP2A). MK571 applied alone affected only PP2A activity with a 40 % increase.

Finally, MCLR inhibited PP1 activity in middle intestinal strips in a concentration-dependent fashion. The non-linear regression analysis of log MCLR concentration *vs.* percentage of PP1 activity yielded an IC_{50} of 1.04 μM ($R^2 = 0.74$). A shift of this dose-response curve was observed in the presence of 3 and 6 μM MK571, with IC_{50} values of 0.52 and 0.37 μM ($R^2 = 0.86$ and 0.74), respectively (Fig. 8). 6 μM MK571 alone did not affect PP1 activity.

4. Discussion

This study is focused on the involvement of Abcc proteins in excretion and/or absorption of MCLR in different sections of fish intestine. Abcc-mediated transport has been functionally studied using DNP-SG and calcein as model substrates in *ex vivo* preparations from *O. mykiss* intestine.

There is abundant information about gene expression and functional characterization of Abccs and other ABC transporters in several fish species including *O. mykiss* (Masereeuw et al 2000; Sturm et al., 2001; Miller, et al., 2002; Cai et al., 2003; Caminada et al., 2008; Zaja et al., 2008; Sauerborn Klobučar et al., 2010; Fischer et al., 2010, 2011; Long et al., 2011). Particularly, Abccs and Abcb1 have been characterized for intestinal tissue and for a permanent cell line derived from intestine of *O. mykiss* (Lončar et al., 2010; Fischer et al., 2011). However, the role of intestinal ABC transporters in the efflux of toxicants has received little attention in fish species.

In our experiments, MCLR significantly reduces luminal and serosal DNP-SG efflux in intestinal sacs, with concentration-dependent effect (measured in intestinal strips). These results suggest that MCLR is exported from the enterocytes by Abcc-like proteins. However, we have not directly measured MCLR or MCLR-SG transport.

The specific Abcc inhibitor, MK571 (Gekeler et al., 1995) shows its maximum effect and the most clear concentration-dependent effects on luminal DNP-SG efflux measured in middle intestinal sacs. This differential sensitivity to MK571 could respond to higher affinity for this inhibitor in apical than in basolateral transporters. Although, we cannot totally discard that transporters other than Abccs could be involved in DNP-SG efflux in fish intestine, this conjugate has not been reported as ABCB1, ABCG2, or ABCB11 substrate (Chan et al., 2004, for a review).

Since competition of MCLR and CDNB as GST substrates could bias the DNP-SG transport results, we have studied the effect of MCLR on calcein efflux, an Abcc substrate independent of GST activity. MCLR inhibits calcein transport in the same fashion as it affects that of DNP-SG. Furthermore, similar concentrations of MCLR and MK571 inhibit calcein transport in the same proportion. Abcb1, another possible MCLR transporter (Contardo Jara et al., 2008; Amé et al., 2009) exports the non-fluorescent calcein-AM rather than calcein (Takano et al., 2006). This activity tends to reduce the intracellular concentration of the fluorescent product calcein. Thus, inhibition of Abcb1 by MCLR would increase intracellular calcein concentration, which in turn, would favor Abcc-mediated calcein export, leading to underestimation of MCLR effects on Abcc-mediated transport. This means that, although MCLR transport through Abcb1 cannot be discarded, it cannot explain the reduction of calcein efflux recorded herein. In the same sense, Abcg2

does not transport calcein or calcein-AM (Chan et al., 2004, for a review) and has not been reported as transporting MC.

Very little is known about the identity of the proteins which transport MC out of the cells. Contardo-Jara et al. (2008) and Amé et al. (2009) have studied Abcb1 as a possible MCLR excretion transporter in aquatic animals. The former authors report higher Abcb1 mRNA expression, although not statistically significant, in the gills of the freshwater mussel *Dreissena polymorpha* previously exposed to MCLR; along with increased rhodamine B efflux by the whole animal, which they assign to Abcb1 activity. Likewise, Amé et al. (2009) have recorded Abcb1 induction in gills, brain and liver of the fish *Jenynsia multidentata* exposed to MCLR. Until now, Abccs have not been related to MCs transport, with the possible exception of Ito et al., (2002) and Malbrouck and Kestmont, (2006), who have suggested that MC-SG conjugates could be excreted via a GS-X pump or multi-drug-resistance-associated proteins, respectively.

For further analysis of the implication of Abccs in MCLR transport, we have studied intracellular effects in middle intestinal segments of MCLR alone or, in the presence of MK571. 1.135 µM MCLR (MC1), which inhibits DNP-SG transport in everted middle intestinal sacs by about 27 %, does not produce any significant effects on enzyme activity. A two-fold higher MCLR concentration (MC2) significantly inhibits PP1 and PP2A. These enzymes have been described as principal targets of MCLR toxicity (Carmichael, 1994; Dawson, 1998; Hooser, 2000). Interestingly, MC1 applied along with MK571 (MC1/MK), produces the same toxic effects as MC2. MK571 applied alone did not produce a significant effect in any of the studied variables with the exception of PP2A activity, which has been increased by 40% with respect to the control. We have not found any explanation for this result and, as far as we know, there is no information related to effects of MK571 on PPs activity in the literature.

The effects observed in the treatment MC1/MK suggest that MK571 blocks MCLR efflux through Abcc-like transporters, increasing the intracellular toxin concentration. The shift of the concentration-response curve (MCLR concentration *vs*. % PP1activity) produced by the addition of MK571 reinforces this idea. The slight difference in the shift produced by 3 and 6 µM of MK571, suggests that 3 µM is near the maximum effect in this system. Besides,

we have obtained less consistent results with 6 μM MK571 (data not shown), which suggests unspecific effects at high inhibitor concentrations.

An alternative explanation for these results is a possible positive effect of MK571 on uptake transporters, which would increase permeability to MCLR, leading to increased intracellular toxin concentration. However, MCLR uptake has been reported to be mediated by Oatps in different species, including *O. mykiss* (i.e. Boaru et al., 2006). As far as we know, there is no report on stimulation of Oatps by MK571. On the contrary, a recent paper on human cell lines (Karlgren et al., 2012) reports inhibition of three OATPs with this inhibitor at concentrations similar to the ones used here.

Since Abccs have also been detected in fish liver and kidney (Cai et al., 2003; Miller et al., 2007; Lončar et al., 2010), it can be speculated that these proteins would transport MCLR in those organs. Thus, differences in sensitivity among fish species could be explained, at least in part, by differences in Abcc abundance and/or affinity for this toxin in detoxifying organs.

The study of the Abcc-like transporters detected in this work should be completed by the identification of these proteins through molecular biology techniques. This knowledge would be important for evaluating and predicting the environmental risk posed by the interaction of MC with other environmental toxicants, such as arsenic, which have been reported as Abcc substrates in fish (Miller et al., 2007).

5. Conclusions

Considering that MCLR is detoxified by conjugation with GSH and the affinity of Abccs for GSH conjugates, together with the results obtained in this study, we suggest that in *O. mykiss* enterocytes MCLR is metabolized by conjugation with GSH, followed by luminal excretion through an Abcc-like apical transporter. Part of the metabolized toxin is transported to the serosal side compartment through basolateral Abcc-like proteins.

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FIGURE CAPTIONS

Figure 1. Detoxification and export pathways known for model Abcc substrates and proposed for MCLR. X, xenobiotic; CYPs, cytochrome P450 enzymes; CDNB, 1-chloro-2,4-dinitrobenzene ; MCLR, microcystin-LR; GSH, reduced glutathione; DNP-SG, 2,4-dinitrophenyl-S-glutathione; MCLR-SG, microcystin-LR conjugated with GSH; GST, glutathione S-transferase; C-AM, calcein acetoxymethyl ester (non-fluorescent) ; C, calcein (fluorescent); Es, esterases; Abcb1, P-glycoprotein (Pgp); Abcc2, multidrug resistance protein type 2 (Mrp2); Abccs, basolateral multidrug resistance proteins (Mrps); Oatp, organic anion transporting polypeptide. Dotted lines show hypothetical efflux pathways, solid lines indicate known transport pathways.

Figure 2. HPLC chromatogram for the microcystin extract in a 1/100 dilution (large peak) and 2 mg/L MCLR standard (small peak).

Figure 3. Representative HPLC (at 365 nm) DNP-SG peak detected in the bath solution samples from everted and non-everted sac preparations from *O. mykiss* intestine.

Figure 4.a) Effects of 1.135 µM MCLR on DNP-SG transport as percentage of respective controls, in everted and non-everted sacs from *O. mykiss* middle and posterior intestine. Values are mean ± SD (n = 5). Asterisks indicate significant differences between each group and its respective control at $p < 0.05$. **b)** Non-linear regression curve for MCLR concentration *vs.* DNP-SG transport rate (as percentage of control), in middle intestinal strips of *O. mykiss*. Values are mean ± SD (n = 4 for each MCLR concentration).

Figure 5. MK571 Inhibitory effect as percentage of control DNP-SG transport rate in **(a)** everted sacs from middle intestine, (**b)** everted sacs from posterior intestine, (**c)** non-everted sacs from middle intestine and (**d)** non-everted sacs from *O. mykiss* posterior intestine. Values are mean ± SD, n = 3 for each MK571 concentration. Asterisks indicate significant effects with respect to control (* $p < 0.05$ and ** $p < 0.01$). Different letters indicate significant differences between MK571 concentrations at $p < 0.05$.

Figure 6. Effects of 2.27 µM MCLR and 3 µM MK571 on calcein efflux measured in *O. mykiss* middle intestinal strips. Values are mean ± SD (n = 7). Letters indicate significant differences between treatments at $p < 0.05$).

Figure 7. Toxic effects of MCLR in *O. mykiss* middle intestinal segments. Treatments: Control, 1.135 µM MCLR (MC1), 2.27 µM (MC2), 1.135 µM MCLR + 3 µM MK571 (MC1/MK) and 3 µM MK571 (MK). **a)** Final MCLR concentration *per* tissue. Letters indicate significant difference among treatments, $p < 0.05$). **b)** GSH intracellular concentrations, **(c)** GST activity, **(d)** PP1 activity and **(e)** PP2A activity. Values are expressed as mean ± SD, n = 5-7. Letters indicate significant differences between treatments, $p < 0.05$ or 0.01.

Figure 8. Non-linear regression curve for MCLR concentration, with 0, 3 or 6 µM MK571*vs.* PP1 activity (as percentage of control) in *O. mykiss* middle intestinal strips. Values are mean ± SD (n = 3 for each point of the curve).

Figure 1

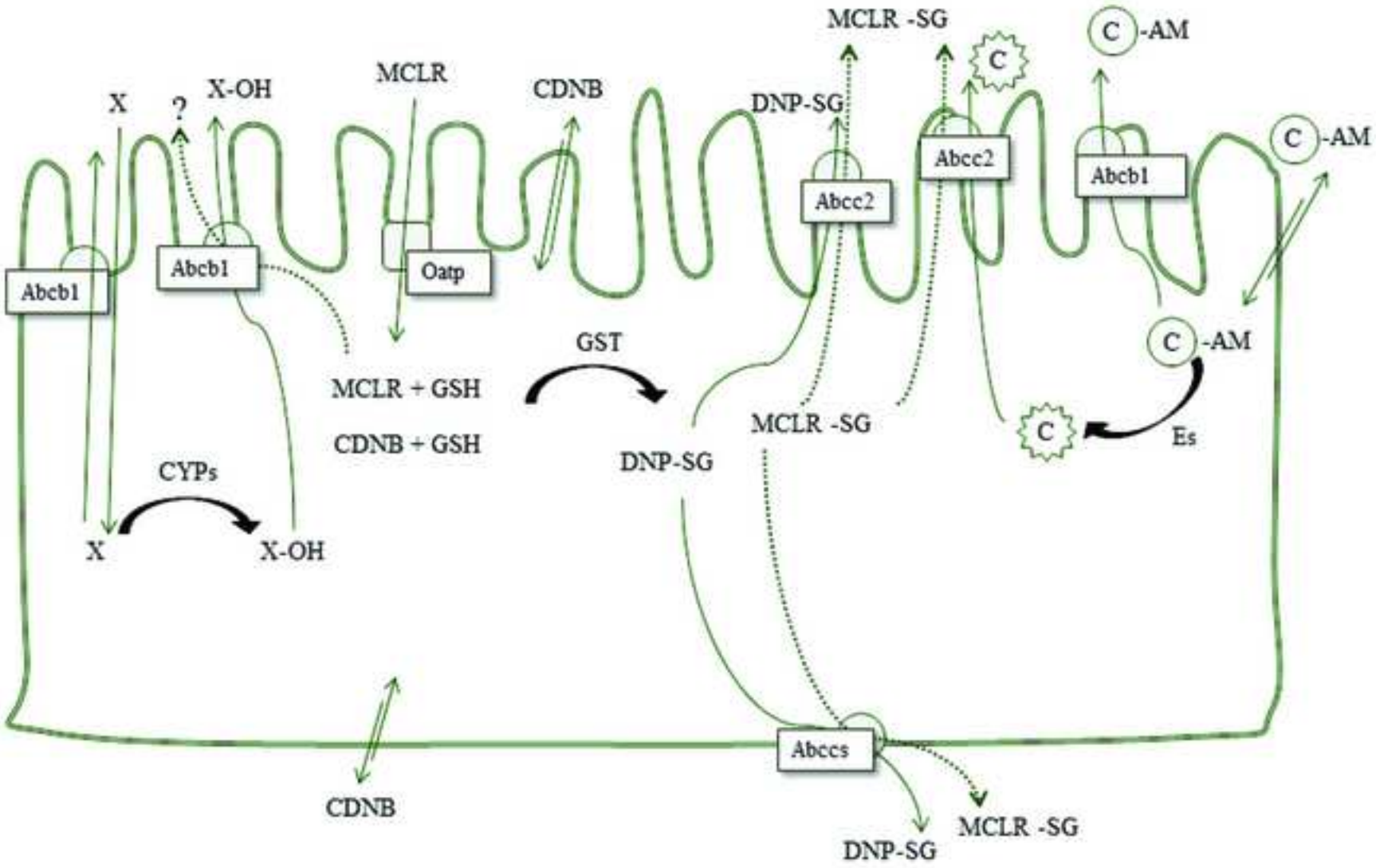

MCLR -SG
C
-AM
X
?
X-OH
MCLR
CDNB
DNP-SG
Abcc2
Abcb1
Abcb1
Abcb1
Oatp
Abcc2
C
-AM
GST
MCLR + GSH
CDNB + GSH
MCLR -SG
C
-AM
DNP-SG
C
Es
CYPs
X
X-OH
Abccs
CDNB
MCLR -SG
DNP-SG

Fig2

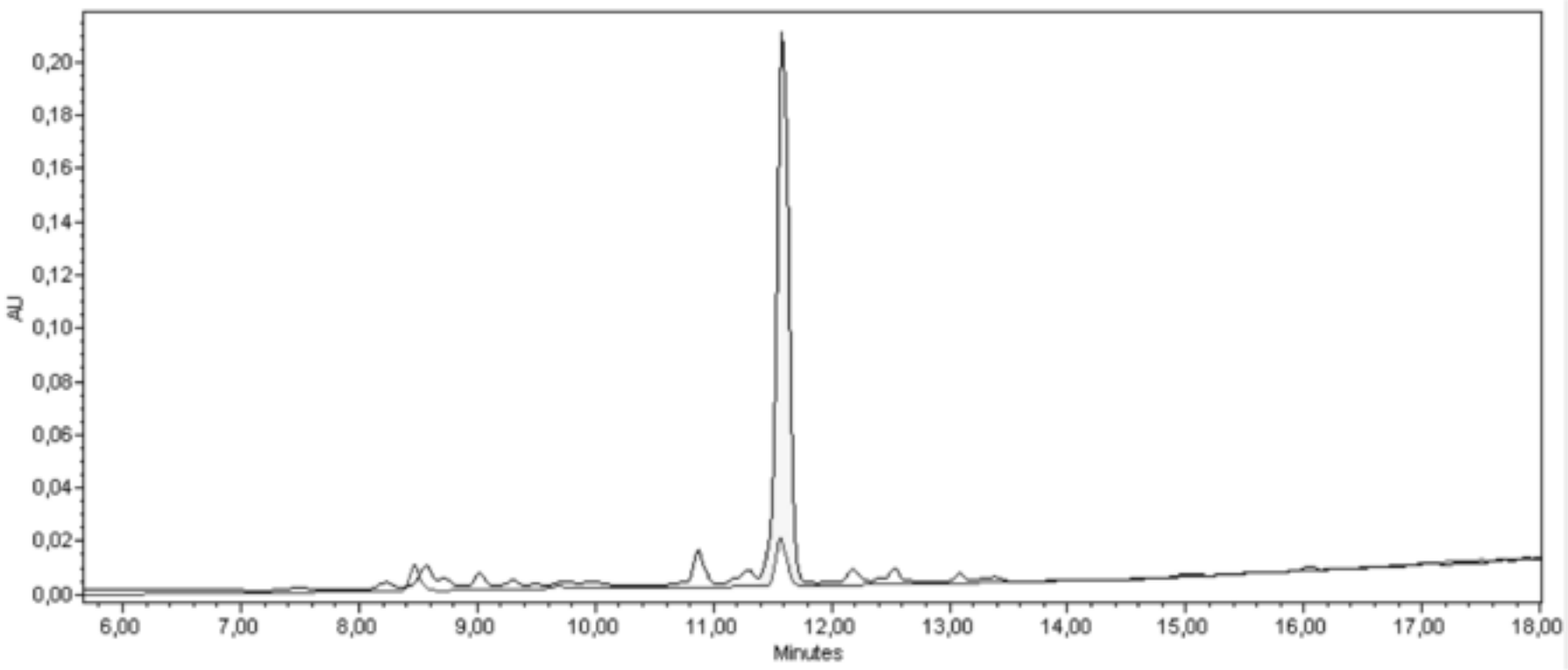

AU
0,20
0,18
0,16
0,14
0,12
0,10
0,08
0,06
0,04
0,02
0,00
6,00
7,00
8,00
9,00
10,00
11,00
12,00
13,00
14,00
15,00
16,00
17,00
18,00
Minutes

Fig3

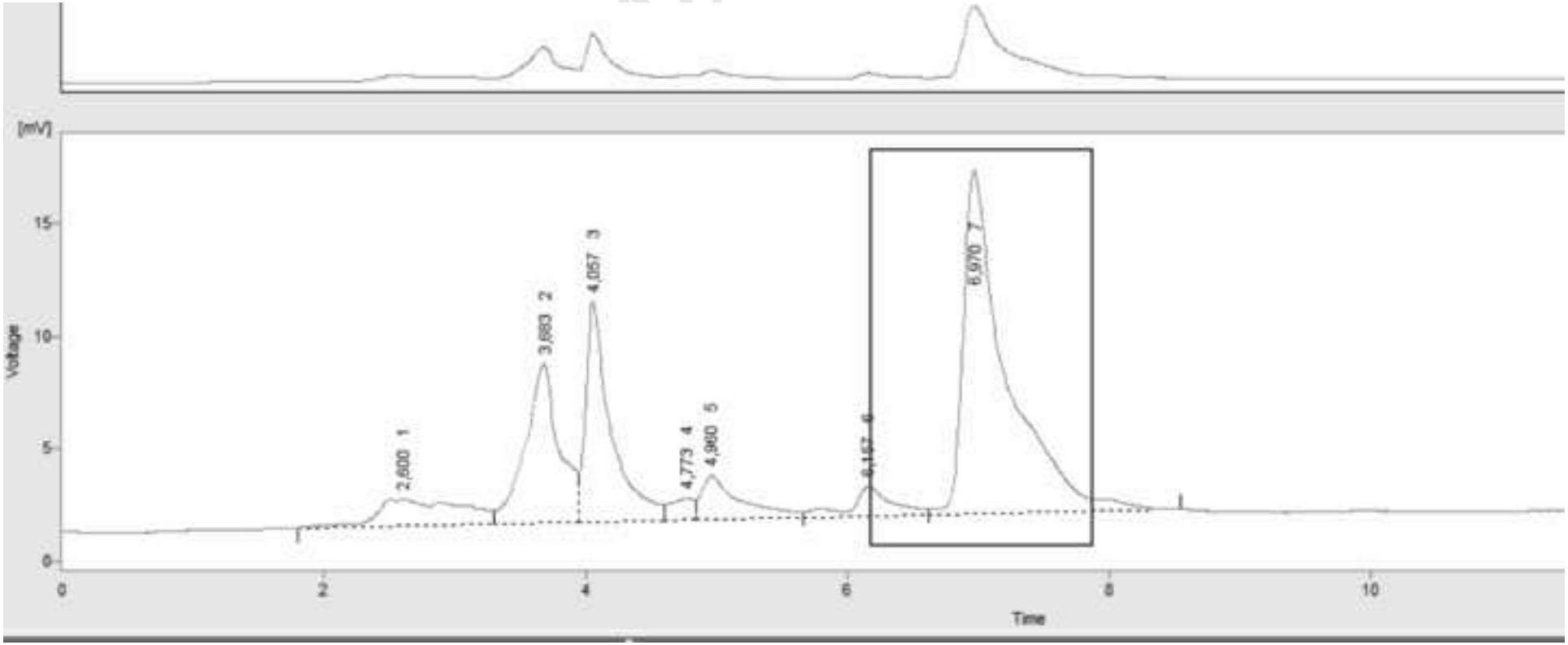
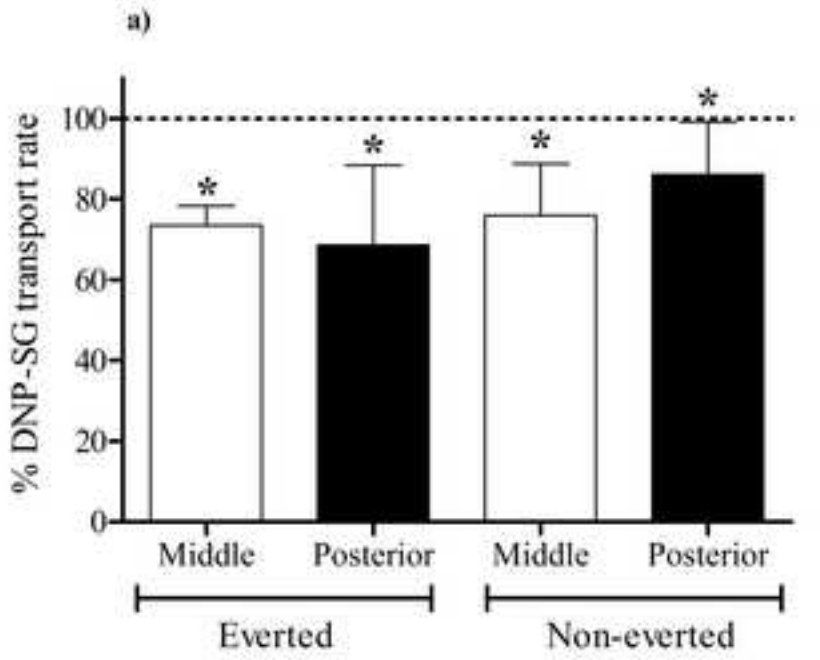

[mV]
15
10
5
0
Voltage
2,600 1
3,683 2
4,057 3
4,773 4
4,980 5
6,970 7
0
2
4
6
8
10
Time

Figure 4


a)
% DNP-SG transport rate
100
80
60
40
20
0
*
*
*
*
Middle
Posterior
Middle
Posterior
Everted
Non-everted

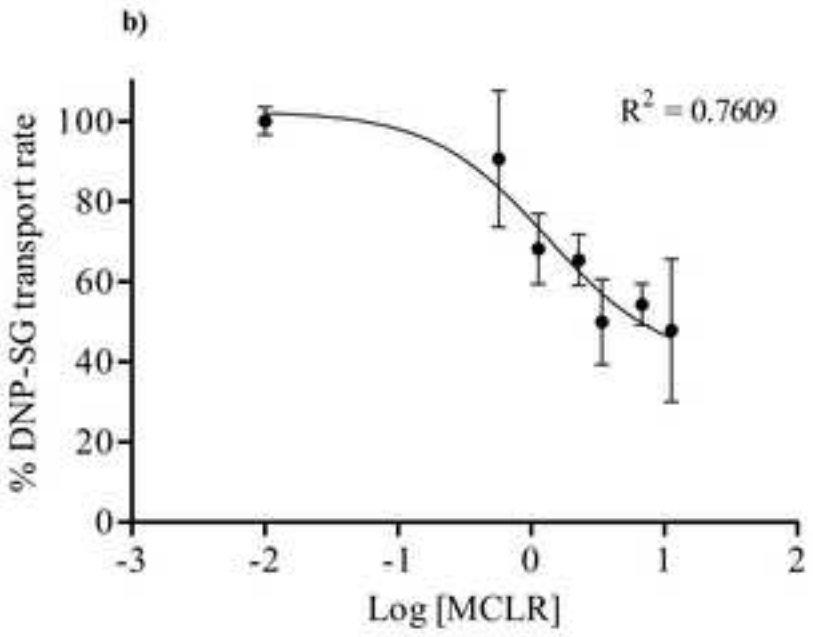

b)
% DNP-SG transport rate
100
80
60
40
20
0
$R^2 = 0.7609$
-3
-2
-1
0
1
2
Log [MCLR]

Figure 5

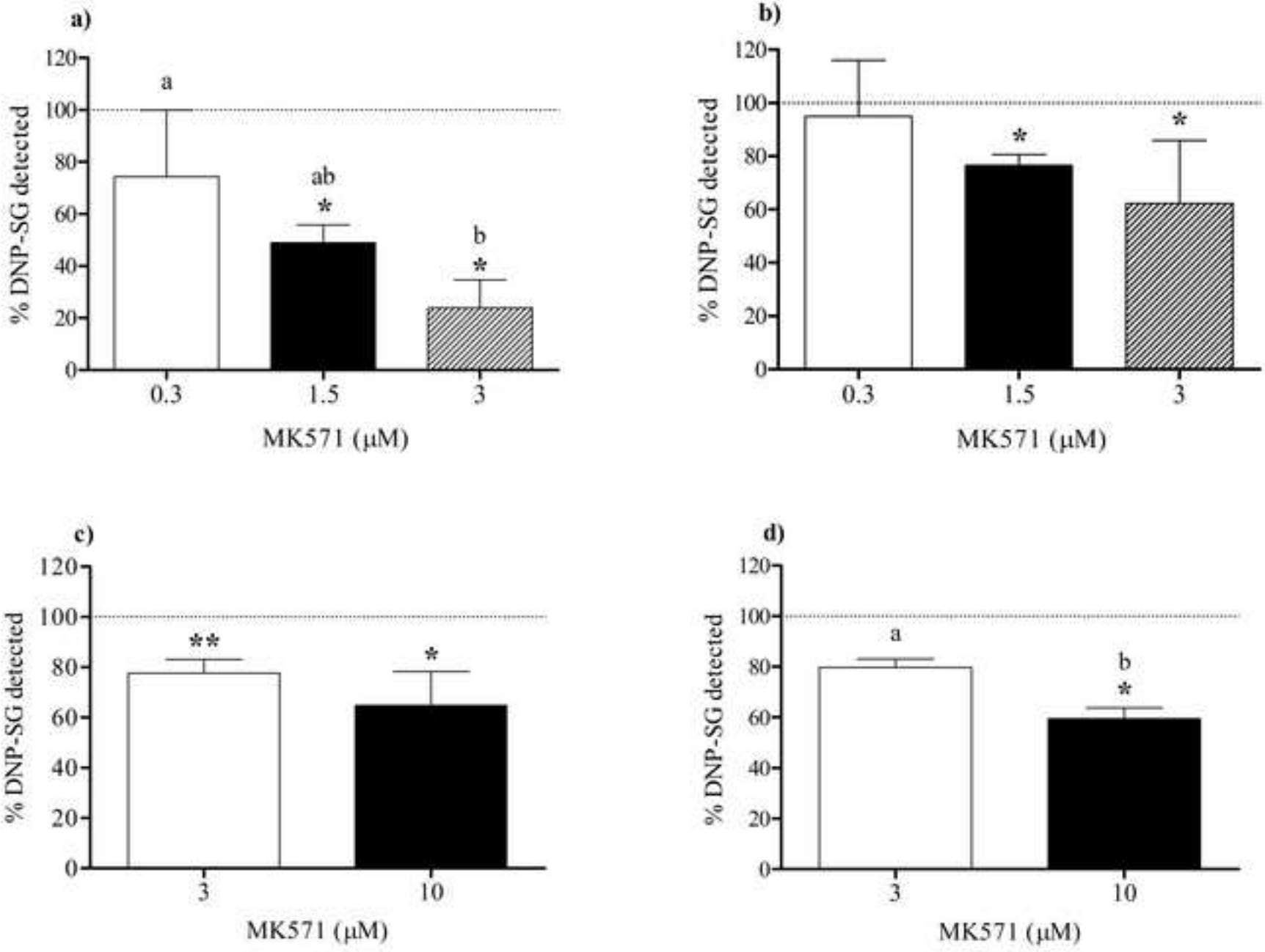

a)
% DNP-SG detected
120
100
80
60
40
20
0
a
ab
*
b
*
0.3
1.5
3
MK571 (μM)
b)
% DNP-SG detected
120
100
80
60
40
20
0
*
*
0.3
1.5
3
MK571 (μM)
c)
% DNP-SG detected
120
100
80
60
40
20
0
**
*
3
10
MK571 (μM)
d)
% DNP-SG detected
120
100
80
60
40
20
0
a
b
*
3
10
MK571 (μM)

Figure 6

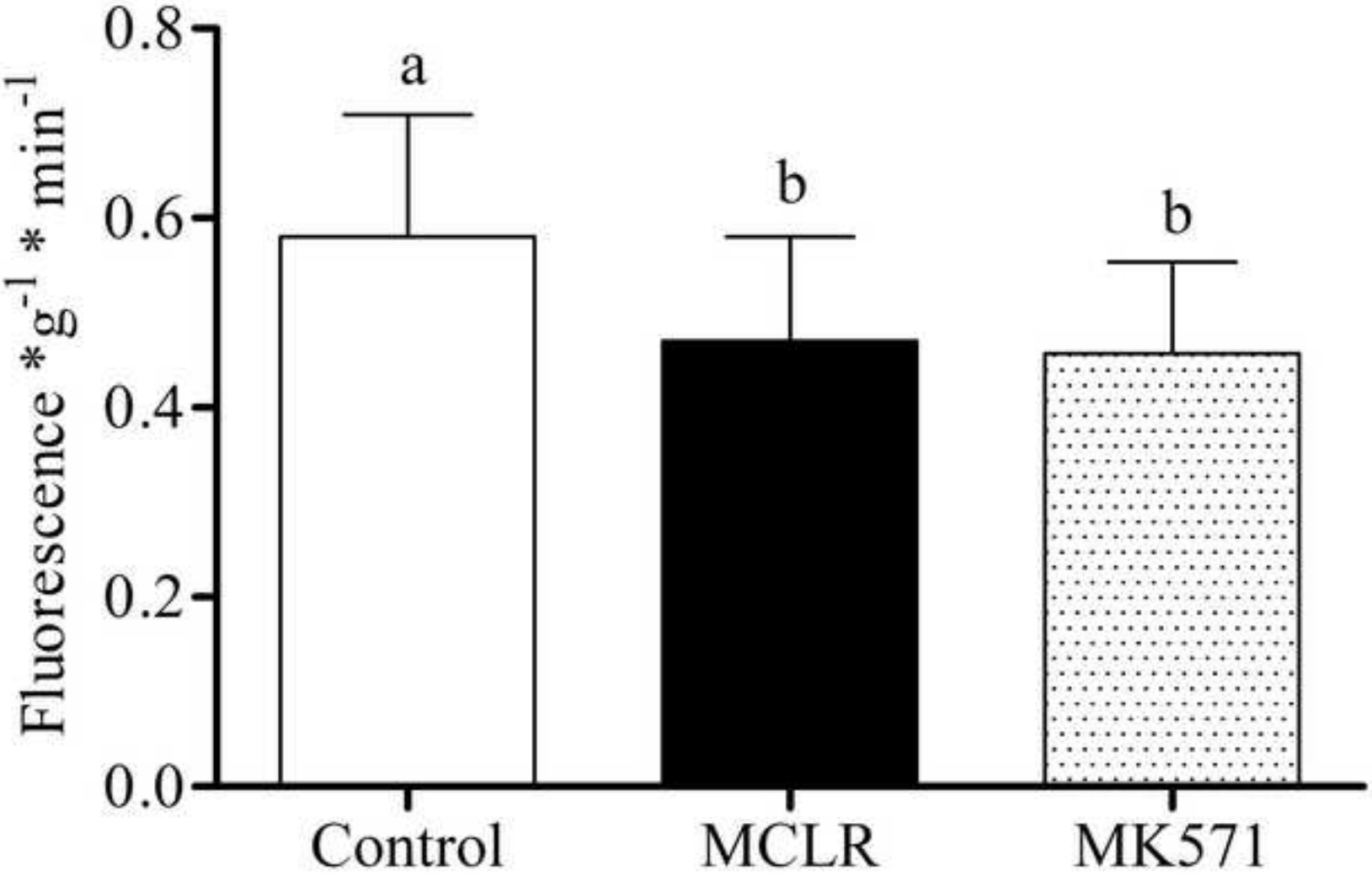


Figure 7

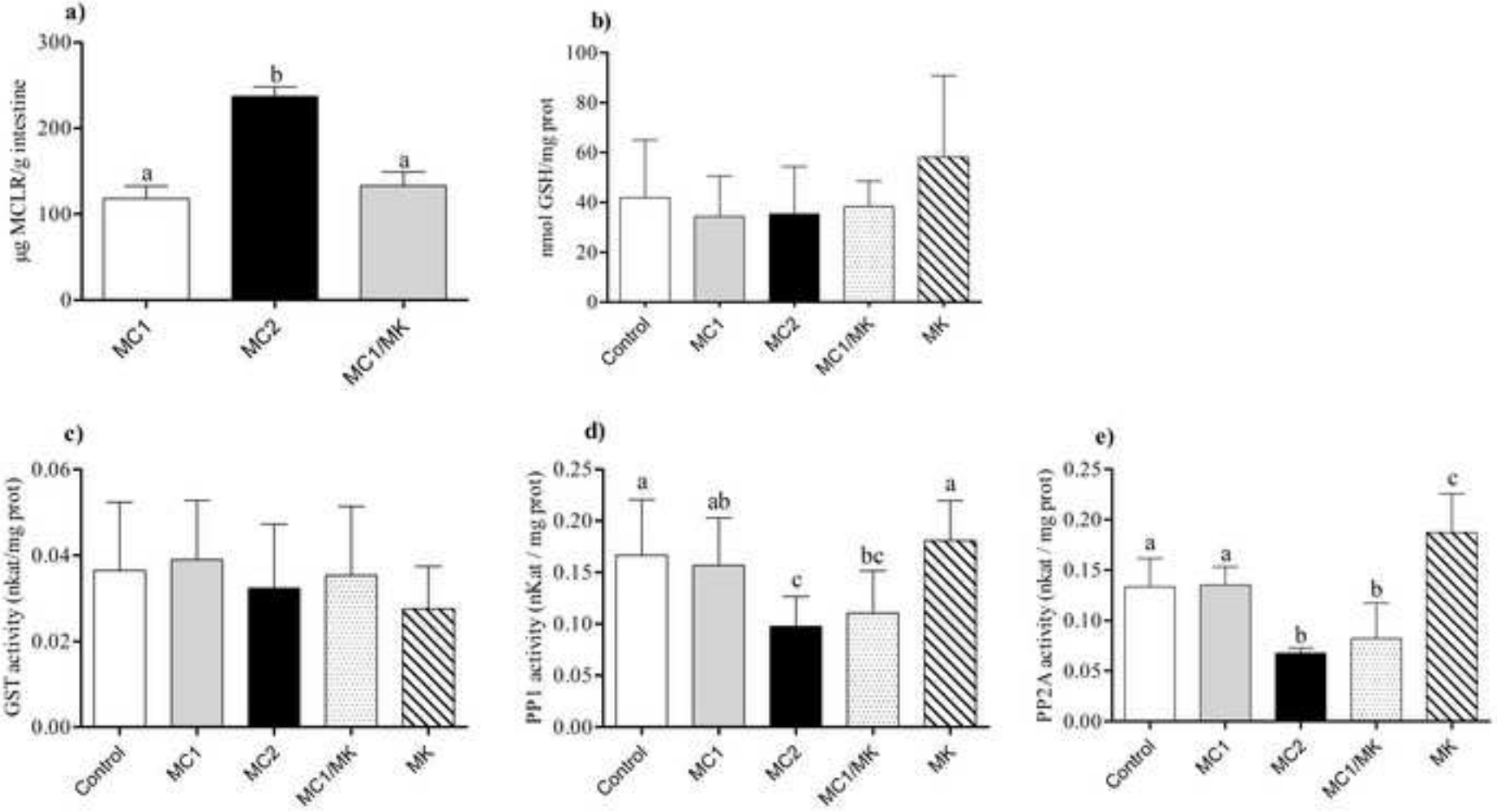
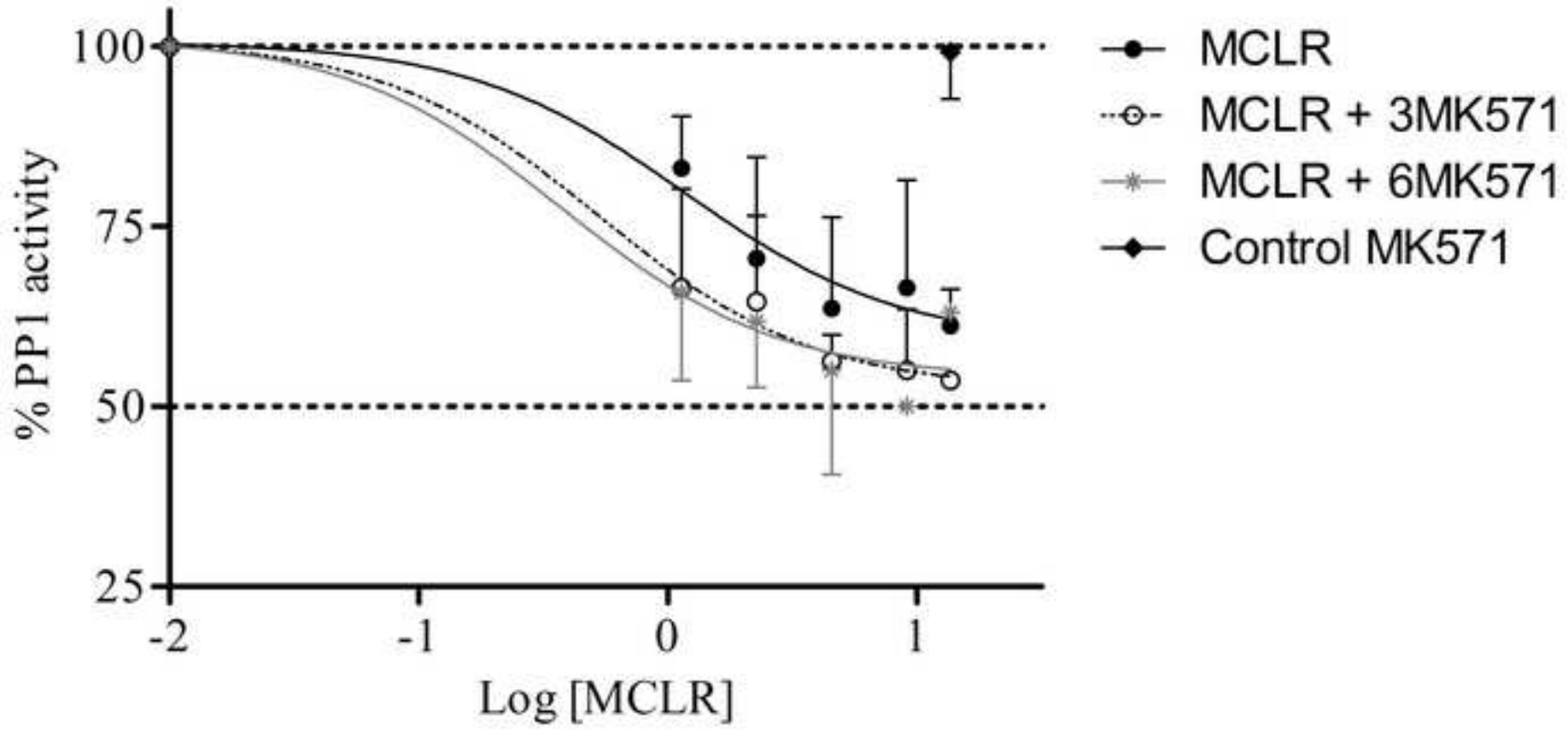

a)
µg MCLR/g intestine
300
200
100
0
a
b
a
MC1
MC2
MC1/MK
b)
nmol GSH/mg prot
100
80
60
40
20
0
Control
MC1
MC2
MC1/MK
MK
c)
GST activity (nkat/mg prot)
0.06
0.04
0.02
0.00
Control
MC1
MC2
MC1/MK
MK
d)
PP1 activity (nKat / mg prot)
0.25
0.20
0.15
0.10
0.05
0.00
a
ab
c
bc
a
Control
MC1
MC2
MC1/MK
MK
e)
PP2A activity (nkat / mg prot)
0.25
0.20
0.15
0.10
0.05
0.00
a
a
b
b
c
Control
MC1
MC2
MC1/MK
MK

Figure 8


100
75
50
25
% PP1 activity
-2
-1
0
1
Log [MCLR]
MCLR
MCLR + 3MK571
MCLR + 6MK571
Control MK571

Highlights

- *Ex vivo* preparations from *O. mykiss* intestine, for studying microcystin-LR transport.
- Microcystin-LR inhibits apical and basolateral transport of Abcc substrates.
- Microcystin-LR causes cell toxicity when Abcc-like transport is blocked.
- Microcystin-LR would be excreted and/or absorbed to the blood by Abcc proteins.