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Investigation of the Multixenobiotic Resistance Mechanism in the Freshwater Fishes Western Mosquitofish, *Gambusia affinis*, and Bluegill Sunfish, *Lepomis macrochirus*

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Abstract The purpose of the study was to evaluate multixenobiotic resistance mechanism expression as a biomarker for contaminant exposure in freshwater fishes. Exposure to a known mammalian inhibitor (verapamil, 10 μM) and inducer (rhodamine 123, 3 μM) on the transport protein *P*-glycoprotein (Pgp) was investigated in the liver of the western mosquitofish and bluegill sunfish. No differences in the activity or expression of Pgp were measured in either species using a fluorometric accumulation assay and western blot analyses. The preliminary results from this study indicate that this detoxification mechanism may not be a sensitive indicator of contaminant exposure in certain teleost species.

Keywords MXR · *p*-Glycoprotein · Mosquitofish · Bluegill sunfish

The phenomenon of multi-drug resistance (MDR), which is referred to as multixenobiotic resistance (MXR) in aquatic organisms, prevents the intracellular accumulation of potentially toxic compounds by extruding them from the plasma membrane before they are allowed entry into the cell. In aquatic organisms, phase I and II enzymes, as well as both Pgp and multi-drug resistance associated proteins (MRP) have been characterized in a variety of non-teleost species (Zaja et al. 2007). Substrates of Pgp may include pharmaceuticals such as anticancer drugs, antibiotics, antidepressants, antiepileptics and analgesics (Sarkadi et al. 2006). The concern with these emerging pollutants is their rapid introduction into the consumer market as well as their ubiquitous use and their relatively unknown fate in sewage systems (Daughton and Ternes 1999). Many of these compounds have the ability to modulate the MXR system through the inhibition or induction of Pgp and/or MRPs, which can be measured through a variety of methods, including transport studies and western blotting. Induction of Pgp transport activity would result in a decreased intracellular concentration of potentially toxic compounds whereas inhibition of Pgp activity would lead to the opposite effect. Thus, exposure to compounds that have the ability to modulate the MXR system could potentially alter toxicity. Therefore, the present study was conducted to evaluate the potential of various compounds to induce or inhibit the MXR system in two species of freshwater fishes.

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Materials and Methods

Rhodamine B (dye content ~95%), verapamil (purity 98%), and rhodamine 123 (purity ≥ 90%) were purchased from Sigma–Aldrich (St. Louis, MO). Western mosquitofish, *Gambusia affinis*, of mixed sex and age were collected from local surface waters in northeast Louisiana, all of which are free from point sources of pollution. Juvenile bluegill sunfish, *Lepomis macrochirus*, of mixed sex were purchased from Aquatic Research Organisms (Hampton, NH). Fish were maintained in 250–520 L tanks supplied with dechlorinated tap water with a 12/12 dark-light photoperiod at 23 ± 2°C. All animals were fed frozen brine shrimp (Ogden, UT) twice daily on weekdays and once daily on weekends.

A method modified from Smital and Sauerborn (2002) was used to determine Pgp transport activity in both fish species by measuring the accumulation and efflux of a

model Pgp fluorescent substrate, rhodamine B (RB). For the inhibition study, mosquitofish (n = 24) and bluegill (n = 35) were exposed to either the known Pgp inhibitor verapamil (VER, 10 μM; dissolved in dechlorinated tap water) or dechlorinated tap water for 3 h prior to the transport study. The animals were then exposed to RB for 3 h with or without concurrent exposure to VER (10 μM). At 0, 2, and 3 h exposure, mosquitofish (n = 3) and bluegill (n = 5) were sacrificed by pithing and livers were weighed and homogenized in deionized water. The homogenate was centrifuged (3,000×*g* for approximately 7 min) and the supernatant was plated in a 96-well dark microplate for fluorescence analysis (Biotek synergy 2, Winooski, VT) using a 535 and 620 nm filter for excitation and emission, respectively. After the 3 h accumulation, the remaining animals were placed in dechlorinated tap water with or without VER for 18 (bluegill sunfish) and 48 h (mosquitofish) to allow for the efflux of RB. During the 48 h efflux, mosquitofish (n = 3) were removed at 6, 9, 18, 24, and 48 h exposure and bluegill sunfish (n = 5) were removed at 3, 6, 9, and 18 h exposure for analysis as described above.

For the induction study, mosquitofish (n = 18) and bluegill (n = 35) were exposed to RB for 3 h with or without 7 days of prior exposure to rhodamine 123 (R123, 3 μM). Mosquitofish (n = 3) were removed at 0 and 3 h exposure and bluegill sunfish (n = 5) were removed at 0, 2, and 3 h exposure and sacrificed for analysis as described above. The remaining animals were placed in dechlorinated tap water for 18 h (bluegill sunfish) and 48 h (mosquitofish) to allow for the efflux of RB. During the efflux, animals were removed at 9, 18, and 48 h (mosquitofish, n = 3) and 3, 6, 9, and 18 h (bluegill sunfish, n = 5) for analysis as described above.

Additionally, western blotting was performed to quantify the expression of protein in the livers of both species with and without exposure to the inducing agent R123 (3 μM) for 7 days. Livers were homogenized in lysis buffer (RIPA buffer, Thermo Fisher Scientific, Inc., Rockford, IL) and centrifuged at 14,000*g* for 15 min to remove the cellular debris. Proteins were determined by BCA assay (Pierce Biotechnology, Rockford, IL). A total of 25 μg of protein was separated by electrophoresis using sodium dodecyl sulfate–polyacrylamide gels (8%) and transferred to Trans-Blot nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was then blocked with 2% BSA in PBS containing 0.05% tween-20 for 1 h. Pgp was detected by using mouse anti-human MDR1 monoclonal C219 as primary antibody (Singet, Dedham, MA) followed by goat anti-mouse horseradish peroxidase conjugated secondary antibody (Chemicon, Temecula, CA). The blots were then developed using a chemiluminescence detection kit (Pierce Biotechnology).

All statistical analyses were examined using JMP version 7.0 statistical software. Normality of data sets was tested using the Shaipro–Wilks test. Equal variances were checked for all data sets using Levene's test. When data was distributed normally and exhibited equal variances, a Student's *t*-test was used to detect significant differences between groups at each time point. If the variances were not equal, or the data was not distributed normally, a non-parametric Wilcoxon's test was conducted to determine significant differences between groups at each time point. When comparing more than two groups at a particular time point, a Dunnett's test was used to determine significant differences between groups.

Results and Discussion

VER, a known mammalian inhibitor of the MXR mechanism, did not appear to have any effects in mosquitofish or bluegill sunfish. In mosquitofish, the only significant difference observed between controls and VER treated animals (10 μM) in the accumulation of RB was at 2 h. Controls accumulated 41.43 ± 7.1 FI/mg liver tissue and VER exposed animals accumulated 7.98 ± 4.4 FI/mg liver tissue (Fig. 1a). The only significant difference in the efflux of RB from the livers of mosquitofish was observed at 24 h efflux ($p \leq 0.05$). Controls exhibited a fluorescence of 29.33 ± 3.4 FI/mg liver tissue, which is actually higher than the 3 h accumulation of 24.48 ± 7.0 FI/mg liver tissue (Fig. 1b). VER treated animals retained 12.63 ± 3.0 FI/mg liver tissue, which relates to an efflux of 67.70% of the previously accumulated RB. Both control and treated animals effluxed the substrate within 48 h.

There were also few differences in RB accumulation in bluegill sunfish controls and VER treated animals. Control bluegill accumulated 58.20 ± 6.4 FI/mg liver tissue after 3 h of exposure to the substrate, whereas treated animals accumulated 37.94 ± 7.2 FI/mg liver tissue (Fig. 1c). The only significant difference found in the efflux of RB from the livers of bluegill was at 3 h efflux ($p \leq 0.05$). Controls retained 24.99 ± 1.4 FI/mg liver tissue, which relates to an efflux of 57.06% of the previsouly accumulated RB. VER exposed animals retained 14.88 ± 1.0 FI/mg liver tissue, which correlates to an efflux of 60.78% of the previously accumulated RB (Fig. 1d). However, both groups exhibited significant depuration of the substrate by 18 h. These results are in contrast to a study by Smital et al. (2000) and Smital and Sauerborn (2002) which showed that VER (10 μM) exhibited inhibitory activity on Pgp in the common carp and freshwater and marine mussels.

Rhodamine 123, a fluorescent dye similar to RB, was used to evaluate the induction potential of Pgp transport activity in mosquitofish. R123 was shown to induce Pgp

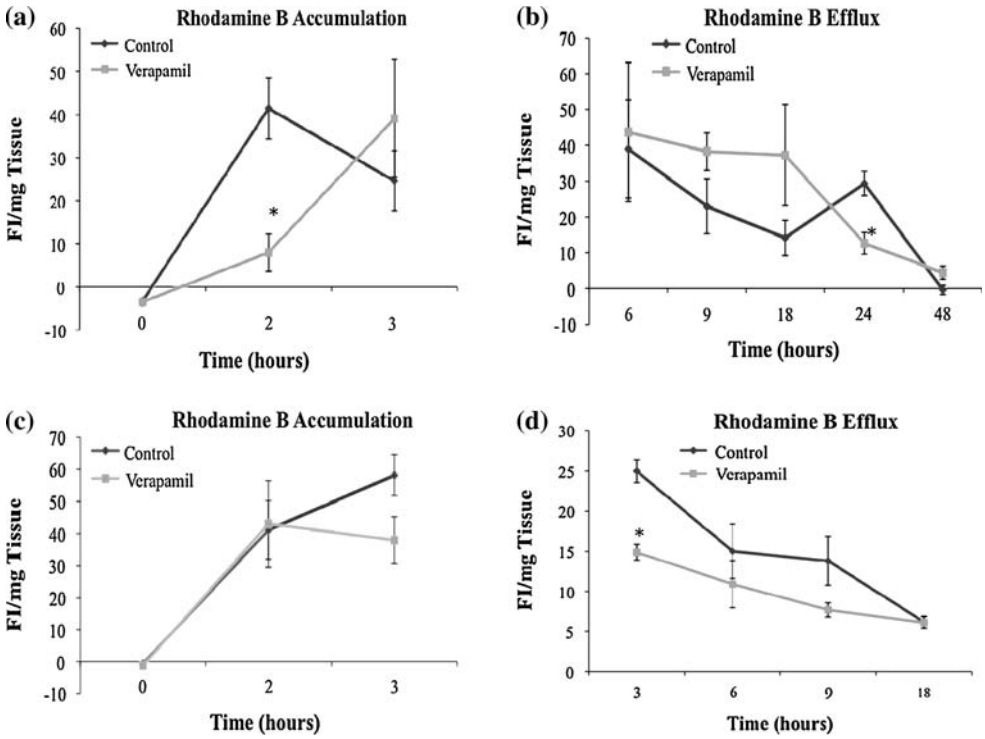

Fig. 1 Time dependent accumulation and efflux of RB from the livers of mosquitofish (**a**, **b**) and bluegill sunfish (**c**, **d**) with or without exposure to verapamil (10 μM)

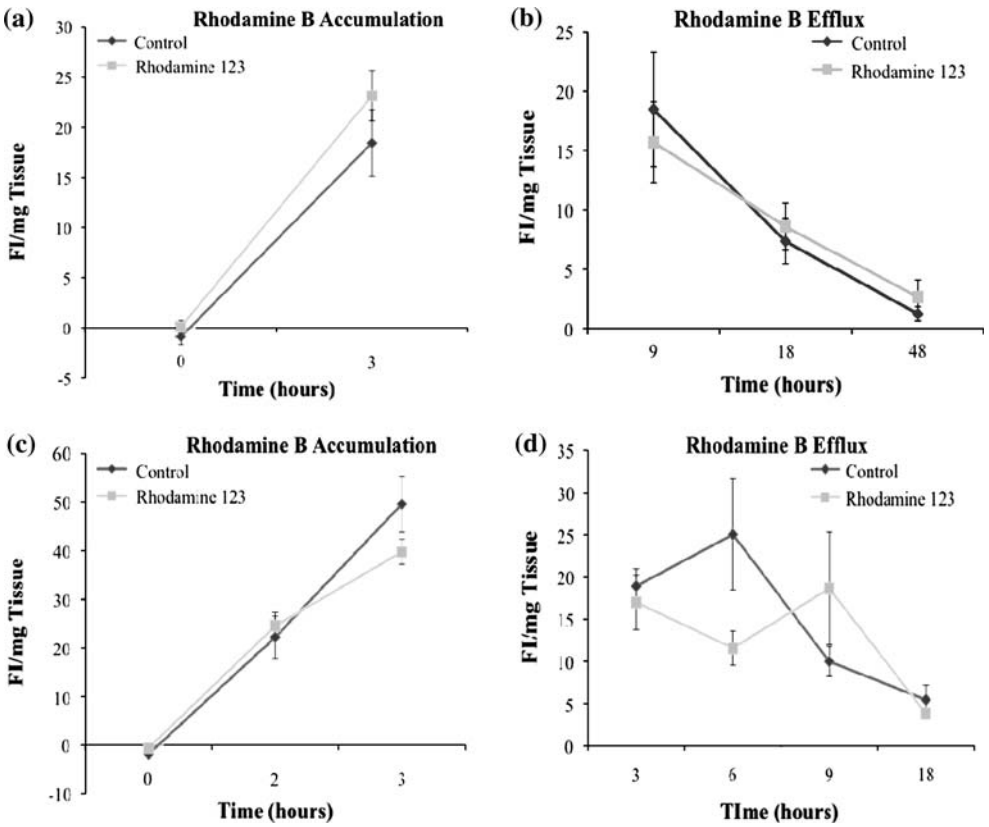

Fig. 2 Time dependent accumulation and efflux of RB from the livers of mosquitofish (**a**, **b**) and bluegill sunfish (**c**, **d**) to rhodamine 123 (3 μM)

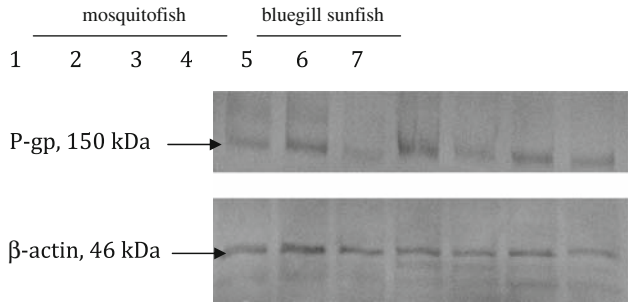

Fig. 3 Western blot analysis of Pgp content in mosquitofish and bluegill sunfish after R123 treatment for 7 days (*lanes 1, 2, 5, 6*) compared to control (without treatment, *lanes 3, 4, 7*). *β*-actin was used as loading control

transport activity in both marine and freshwater mussels in a study by Smital et al. (2003). However, in the present investigation no significant differences in accumulation or efflux of RB in R123 treated animals were found (Fig. 2a, b). Additionally, both groups significantly depurated RB within 48 h.

Rhodamine 123 was also investigated for its Pgp modulating potential in bluegill. Within 3 h, controls accumulated 49.59 ± 5.7 FI/mg liver tissue of RB, while treated animals accumulated 39.74 ± 2.5 FI/mg liver tissue (Fig. 2c). Therefore, there were no significant differences found in the accumulation of RB between controls and R123 treated animals. Additionally, there were no differences detected in the efflux of RB from the livers of bluegill with or without exposure to R123 for 7 days (Fig. 2d).

Western blot analysis confirmed the presence of Pgp and detected its expression in bluegill sunfish and mosquitofish. However, there was no difference in Pgp expression between controls and R123 exposed animals of either species (Fig. 3). This correlates with the results of the transport studies in that R123 was not capable of inducing Pgp transport activity or de novo synthesis in these animal models. Variability in the transport data is consistent with the variability in Pgp expression as supported by western blotting.

This is the first study that has detected the presence of Pgp in either mosquitofish or bluegill sunfish. However, under the present experimental conditions, a known mammalian inhibitor and inducer did not significantly modulate the MXR mechanism. These results should be interpreted with caution because the MXR system is not fully characterized in aquatic species. Further studies are needed to evaluate the importance of this system compared with other detoxification mechanisms in fish.

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