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Assessing neuroprotective P-glycoprotein activity at the blood-brain barrier

in killifish (*Fundulus heteroclitus*) using behavioural profiles

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Abstract

The neuroprotective role of P-glycoprotein, a multixenobiotic resistance transporter (ABCB1/MDR1), in the blood-brain barrier in fish was examined using behavioural toxicological assays. P-glycoprotein acts as cellular efflux pump to prevent substrates from accumulating in the brain, including environmental contaminants such as ivermectin, a common aquaculture pesticide and mammalian anti-parasitic drug. The behavioural toxicological assays were developed to determine the neuropathological effect of ivermectin in killifish (*Fundulus heteroclitus*). P-glycoprotein function and thus blood-brain barrier integrity can be compromised by chemosensitizers that inhibit transport activity. Fish treated with ivermectin and the P-glycoprotein inhibitor cyclosporin A were significantly more sensitive and succumbed more rapidly to tilting, lethargy, slowing of pectoral fin movement and loss of haptic reactivity compared to fish treated with ivermectin only. P-glycoprotein inhibition is associated with significantly earlier onset and increased mortality in ivermectin-exposed fish. Our results suggest that P-glycoprotein confers resistance against ivermectin-induced behavioural neuropathology and mortality in fish. This assay provides us with a non-invasive tool to study P-glycoprotein function in the blood-brain barrier and evaluate the behavioural effects of potential environmental neurotoxins.

Keywords: P-glycoprotein (P-gp/mdr1/ABCB1); multixenobiotic resistance; multidrug resistance; blood-brain barrier; environmental toxicology; fish behaviour; ivermectin; cyclosporin A; *Fundulus heteroclitus*; Halifax, Nova Scotia, Canada

P-glycoprotein (P-gp) is a multidrug resistance transporter within the ATP-binding cassette transport protein superfamily (ABCB1/MDR1). This multixenobiotic efflux pump is predominantly expressed in organs that have an excretion (liver, kidney), absorption (intestine) or barrier function, such as the blood-brain barrier. P-glycoprotein facilitates the transmembrane excretion of numerous xenobiotics and endogenous compound that vary widely in structure and function. P-glycoprotein substrates include diverse moderately hydrophobic environmental contaminants, including the aquaculture/veterinary pesticide and anti-parasitic drug ivermectin (IVM) (Schinkel et al., 1994). Many environmental toxicants induce P-gp expression *in vivo* (Bard, 2000; Bard, Woodin & Stegeman, 2002; Bard, Bello, Hahn & Stegeman, 2002). The P-gp gene was identified in killifish (*Fundulus heteroclitus*) (Cooper, 1996; Cooper et al., 1998) and high levels of P-gp protein has been detected by immunohistochemistry in killifish organs involved in a barrier function (e.g. periphery of intestinal mucosa) and detoxification (e.g. bile canaliculi in the liver) (Bard, Bello, Hahn & Stegeman, 2002.) Assays of P-gp function in multiple organs *in vivo* in killifish, sensitively measured P-gp-mediated xenobiotic excretion at the blood-brain barrier (Bard & Stegeman, 2002). To further investigate the *in vivo* neuroprotective role of P-gp at the blood-brain barrier in real time, we developed sensitive behavioural toxicology assays in killifish. Behavioural toxicology is a burgeoning sub-discipline of toxicology that examines the impact of environmental contaminants on neurophysiology, pharmacokinetics, and behaviour. These behavioural toxicology assays were used to measure the potential neuropathological effect of IVM, a possible environmental neurotoxin. P-glycoprotein function can be compromised by chemosensitizers such as cyclosporin A (CsA) that inhibit P-gp-mediated xenobiotic transport activity. We examined whether inhibition of P-gp activity and thus potentially increased cerebral accumulation of IVM, would correlate with IVM-toxicity induced neuropathological

behaviour. To detect subtle effects of IVM, we developed a scoring technique to quantify one State (Posture [tilting]), and three Behaviours (General-Motor-Activity, Pectoral-Fin-Activity, and Haptic-Reactivity [escape response]). Detailed analyses of single movements and action sequences are very sensitive to developmental, neuropathology-related and drug-induced changes and often have a high temporal resolution (Fentress and Gadbois, 2001). Our objectives were to investigate P-gp function in the blood-brain barrier, develop behavioural assays to evaluate neuropathology, determine effects of IVM on fish behaviour, and evaluate the role of P-gp in resistance against IVM.

We examined 18 male and 31 female killifish collected in October, 2004 from Conrad Beach, Halifax, Nova Scotia, Canada. Pre-experiment, fish were depurated for 6 months in clean aquaria at Dalhousie University. All fish were habituated to test tanks (9.5 litres, 30.5x12.7x17.8 cm) for 10 minutes prior to treatment and scored for each behavioural parameter to establish a baseline. A pilot study (data not shown) determined that untreated fish (no injections) did not differ in each scored behavioural parameter from either control fish that received injections of vehicle solutions (i.v. saline and i.p. dimethyl sulfoxide [DMSO]), or fish injected with 10 µg/g i.v. CsA, the P-gp inhibitor. Thus the vehicle treatments were selected as controls for the present experiment. Control fish (N=6 males, 11 females) received injections of vehicle solutions (i.v. saline and i.p. [DMSO]); IMV-only fish (N=6 males, 10 females) received injections of 5µg/g i.p. IVM and i.v. saline; and P-gp-inhibited fish (IVM+CsA) (N=6 males, 10 females) received 5µg/g i.p. IVM and 10 µg/g i.v. CsA, a sufficient dose to inhibit P-gp function (Bard & Stegeman, 2002). Post-injection, fish were observed in test tanks for 2 hours. For every fish, five behavioural parameters were scored by eye (see Figure 1) every 10 minutes for the first hour and every 5 minutes over the second hour. This experimental time frame was selected because maximal IVM penetration into fish brain peaks within 2 hours (Katharios,

Pavlidis, & Iliopoulou-Georgudaki, 2004.) Inferential statistics included repeated measures ANOVAs ($p < 0.05$) for each behavioural parameter. Tukey-Kramer post hoc tests (tolerant of missing cells due to subject mortality) identified specific differences between groups.

IVM+CsA fish were unresponsive to the touch test, indicating imminent mortality, earlier (starting at 20 minutes post-injection) than IVM fish (onset at 70 minutes post-injection) while all control fish were fully responsive to the touch test throughout the experiment. At the termination of the experiment at the end of 2 hours, >70% of the IVM+CsA fish exhibited symptoms of imminent mortality compared to 25% of the IVM fish. No control fish showed symptoms of imminent mortality.

We found no effect of sex on any State or Behaviour. Posture (tilting) was more pronounced in IVM+CsA fish (~2-fold greater) than in IVM fish (Figure 1a). IVM+CsA fish exhibited lower scores (~50% depressed) compared to IVM fish for the behavioural measures of General-Motor-Activity, Pectoral-Fin-Activity and Haptic-Reactivity (Figures 1b, 1c, and 1d respectively).

In conclusion, fish treated with IVM and the P-gp-inhibitor CsA were significantly more sensitive and succumbed more rapidly to Postural changes, lethargy and loss of Haptic-Reactivity compared to IVM fish. P-glycoprotein inhibition is associated with significantly earlier onset and increased imminent mortality in IVM-exposed fish. The postural changes and activity measures may be explained by agonist effects within the GABA-ergic system and possible disruption of the cholinergic system. Our results suggest that P-gp activity confers resistance against IVM-induced behavioural neuropathology and mortality in fish. This study is a first step in devising a robust observational assay based on simple and quickly assessed State (Posture) and Behaviour measures (general and specific activity). This assay provides us with a non-invasive tool to study P-gp function in the blood-brain barrier and to evaluate the

behavioural effects of potential environmental neurotoxins.

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References

- Bard, S.M., & Stegeman, J.J. (2002). Inhibition of rhodamine B transport in liver, brain, and ovary by cyclosporin A: development of an *in vivo* model for analysis of P-glycoprotein function. *Marine Environmental Research*, 54, 461-463.
- Bard, S.M., Bello, S.M., Hahn, M.E., & Stegeman, J.J. (2002). Expression of P-glycoprotein in killifish (*Fundulus heteroclitus*) exposed to environmental xenobiotics. *Aquatic Toxicology*, 59, 237-251.
- Bard, S.M., Woodin, B.R., & Stegeman, J.J. (2002). Expression of P-glycoprotein and cytochrome P450 1A in intertidal fish (*Anoplarchus purpureus*) exposed to environmental contaminants. *Aquatic Toxicology*, 60, 17-32.
- Bard, S.M. (2000). Multixenobiotic resistance as a cellular defense mechanism in aquatic organism. *Aquatic Toxicology*, 48, 357-389.
- Cooper, P.S., 1996. Expression of the xenobiotic transport P-glycoprotein in the mummichog (*Fundulus heteroclitus*), in School of Marine Science, College of William and Mary: Williamsburg, VA. p. 208.
- Cooper, P.S., Hardison, J.L., Reece, K.S., and Van Veld, P.A., 1998. P-glycoprotein-related sequences from mummichog (*Fundulus heteroclitus*) liver and intestine. in NIEHS Workshop on Unique Marine/Freshwater Models for Environmental Health Research.
- Fentress, J.C. & Gadbois, S. (2001). In E.M. Blass (Ed), *Handbooks of Behavioral Neurobiology*, Volume 13: Developmental Psychobiology, Developmental Neurobiology and Behavioral Ecology: Mechanisms and Early Principles. New York: Kluwer Academic Publishers.

Katharios, P., Pavlidis, M., & Iliopoulou-Georgudaki, J. (2004). Accumulation of ivermectin in the brain of sea bream, *Sparus aurata* after intraperitoneal administration.

Environmental Toxicology and Pharmacology, 17, 9-12.

Schinkel, A.H., Smit, J.J., van Tellingen, O., Beijnen, J.H., Wagenaar, E., van Deemter, L., Mol, C.A., van der Valk, M.A., Robanus-Maandag, E.C., and te Riele, H.P., et al., 1994. Disruption of the mouse *mdr1a* P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell* 77, 491-502.

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Figure Captions

Figure 1: Effect of P-glycoprotein inhibition on ivermectin neuropathology.

Control fish (N=6 males, 11 females) received injections of vehicle solutions (i.v. saline and i.p. dimethyl sulfoxide [DMSO]); ivermectin-only fish (IVM) (N=6 males, 10 females) received injections of 5µg/g i.p. IVM and saline (i.v.); and P-gp-inhibited fish (IVM+CsA) (N=6 males, 10 females) received 5µg/g i.p. IVM and 10 µg/g i.v. cyclosporin A (CsA). Every fish was assessed on a scale from 0 to 3 for one State [Posture (1a)] and three Behaviours [General-Motor-Activity (1b), Pectoral-Fin-Activity (1c), and Haptic-Reactivity (1d)]. For Posture, or the general body orientation of the fish, a score of 0 represented normal upright posture and a score of 3 represented a strong longitudinal or lateral tilting (90°). General-Motor-Activity was defined as general movement in water, including movements of all body parts relevant to swimming (all fins, tail, etc), with a score of 0 given to immobile fish and a score of 3 indicating normal activity. Pectoral-Fin-Activity was rated with a score of 0 for no pectoral fin movement and a score of 3 for normal pectoral fin movement. The Haptic-Reactivity assay evaluated if fish touched with a stylus were able to react to tactile stimuli by exhibiting an escape response. Reaction to touch was defined by a score of 0 for being unresponsive and 3 for being very responsive and showing a normal escape response. Error bars represent standard errors. Overall, Tukey-Kramer post hoc tests found significance in all pairwise comparisons.

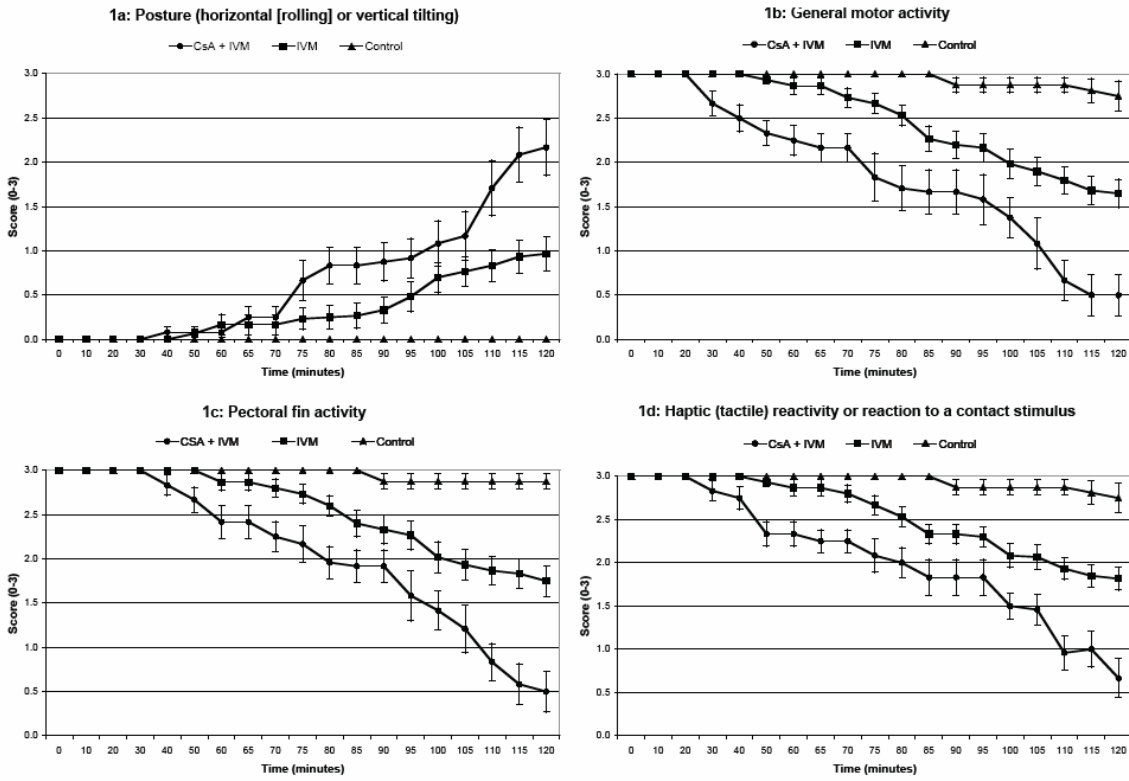


Figure 1