Function and regulation of P-glycoprotein in the liver of teleost fish

by

Christina Ursula Johnston

MSc (Physiology), University of Calgary, 2015

BSc (Biological Sciences), University of Calgary, 2011

BSc (Chemical Engineering), University of Calgary, 2006

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	Vicki L. Marlatt Committee Member Associate Professor, Biological Sciences
	Tony D. Williams Committee Member Professor, Biological Sciences
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	Lynn P. Weber External Examiner Professor, Veterinary Biomedical Sciences University of Saskatchewan

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Abstract

P-glycoptotein (P-gp), an active transmembrane exporter, transports various substances across cell membranes, sharing a similar set of substrates with other transporters in a coordinated system that maintains cellular chemical homeostasis. Increased expression of these transporters in aquatic animals results in the multi-xenobiotic resistance (MXR) phenotype, increasing survival and reproduction in contaminated environments. Known as chemosensitizers, inhibitors of these proteins enable toxic substrates to accumulate in tissues, heightening sensitivity and causing adverse effects at lower external concentrations. While extensively studied in mammals, the physiological functions and environmental relevance of fish P-gp are still emerging fields. To gain insight into the function and regulation of P-gp in fish, a series of studies was conducted examining the interaction of P-gp with its substrates and inhibitors at the biochemical, cellular, tissue, and whole animal levels in rainbow trout (*Oncorhynchus mykiss*). In cultured trout hepatocytes, experiments showed that four well-known mammalian chemosensitizers (cyclosporin A [CsA], quinidine, valspodar, and verapamil) competitively inhibited rainbow trout P-gp transport of rhodamine 123 (R123) and cortisol, at similar concentrations as reported for mammalian P-gp. Accumulation and efflux assays in trout hepatocytes showed that trout P-gp has similar binding sites and affinities for the known mammalian P-gp substrates doxorubicin, R123, and vinorelbine, while trout P-gp has a higher affinity for cortisol. P-gp inhibition by CsA caused small but significant changes in ivermectin distribution in trout blood and brain, suggesting that P-gp plays a smaller role in xenobiotic protection in fish than it does in mammals. Fish P-gp functions very similarly to mammalian P-gp, but with lineage-specific adaptations due to different chemical exposures and divergent evolutionary histories.

Keywords: P-glycoprotein; chemosensitizer; substrate; rainbow trout; hepatocyte; xenobiotic

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List of Acronyms

^{3}H	Hydrogen-3, tritium
ABC	ATP binding cassette
ADME	Absorption, distribution, metabolism, elimination
AhR	Aryl hydrocarbon receptor
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AUC	Area under the curve
BBB	Blood-brain barrier
BCRP	Breast cancer resistance protein
CAR	Constitutive androstane receptor
Cl	Clearance
C_{max}	Maximal concentration
CNS	Central nervous system
CsA	Cyclosporin A
CYP	Cytochrome P450
DDD	Dichlorodiphenyldichloroethane
DDT	Dichlorodiphenyltrichloroethane
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPM	Disintegrations per minute
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FSH	Follicle-stimulating hormone
GR	Glucocorticoid receptor
GST	Glutathione S-transferase
hpf	Hours post-fertilization
HSD	Honestly significant difference
HSS	Hanks balanced salt solution

HSSB	Hanks balanced salt solution + Bovine serum albumin
IC50	Inhibitory concentration 50%, half-maximal inhibitory concentration
iv	Intravascular
IVM	Ivermectin
K_{OW}	n-Octanol-water partition coefficient
LH	Luteinizing hormone
Log	Logarithm
LSC	Liquid scintillation counting
MATE	multidrug and toxin extrusion protein
MDR	Multi-drug resistance
MPP+	1-methyl-4-phenylpyridinium
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
MRP	Multidrug resistance protein
MRT	Mean residence time
MXR	Multi-xenobiotic resistance
N	Number
NBD	Nucleotide binding domain
Nrf2	Nuclear factor erythroid 2-related factor 2
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
P-gp	Permeability-glycoprotein, P-glycoprotein
PCN	Pregnenolone 16α-carbonitrile
PK	Pharmacokinetics
PXR	Pregnane X receptor
R123	Rhodamine 123
RO	Reverse osmosis
rpm	Revolutions per minute
RSSN	Remote sensing and signalling network
SDS	Sodium dodecyl sulphate

SE	Standard error
SEM	Standard error of the mean
SLC	Solute carrier
$T_{1/2}$	Half life
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
TK	Toxicokinetics
TMD	Transmembrane domain
UDP	Uridine diphosphate
VD_{SS}	Volume of distribution at steady state

Chapter 1. A review of P-glycoprotein function and regulation in fish

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Christina U. Johnston and Christopher J. Kennedy†

Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia, Canada

Abstract

The teleost ATP Binding Cassette (ABC) transporter P-glycoprotein (P-gp) is an active transmembrane transporter that plays a pivotal role in facilitating the movement of both endogenous and xenobiotic substrates (moderately hydrophobic and amphipathic compounds) across cell membranes. P-gp exhibits substrate specificity often shared with other ABC transporters and solute carrier proteins, thereby ensuring the maintenance of chemical homeostasis within cells. These transporters are integral to chemical defense systems in fish, as they actively expel a wide range of substrates, primarily unmodified compounds, from cells. This transport process assists in preventing chemical absorption (e.g., intestine), safeguarding sensitive tissues (e.g., brain and gonads), and effectively excreting substances (e.g. liver and kidney). Upregulated P-gp export activity in aquatic animals results in the multi-xenobiotic resistance (MXR) phenotype that plays an essential protective role in survival in contaminated environments. Pollutants inhibiting P-gp are termed chemosensitizers, and heighten fish sensitivity to toxic P-gp substrates. While the known intrinsic functions of P-gp in fish encompass steroid hormone and bile acid processing, relatively little attention has been given to endogenous substrates and inhibitors. Fish P-glycoprotein regulation is orchestrated by pivotal nuclear transcription factors, including pregnane X receptor (PXR), and nuclear factor erythroid 2-related factor 2 (Nrf2). This comprehensive review provides profound insights into P-gp's significance across diverse fish species, contributing to an enhanced understanding of fish physiology, evolution, and toxicology, and provides information with potential applications, such as environmental monitoring.

Keywords: P-glycoprotein, chemosensitizer, multixenobiotic resistance, fish

1.1. Introduction

Cell membranes house a diverse array of transporters essential for maintaining cellular homeostasis and functionality. These transporters are categorized into distinct classes based on their mechanism of action and substrate specificity. Solute carrier (SLC) transporters encompass a large superfamily that facilitates the transport of nutrients, ions, and metabolites. Ion channels permit the passive flux of ions across membranes, critical for electrical signaling and osmoregulation. Symporters and antiporters, categorized as secondary active transporters, utilize electrochemical gradients to transport substrates simultaneously in the same direction or in opposite directions, respectively. Finally, vesicular transporters mediate the intracellular trafficking of molecules through vesicle formation and fusion. These transporters control the movement of molecules across membrane barriers and serve to maintain chemical homeostasis. They also export endogenous molecules from sites of production to where they are needed, such as the transport of morphogenic signaling molecules during embryonic development (Nigam, 2015), the movement of gut microbiome metabolites into host intestinal tissue (Wikoff et al., 2011; Wu et al., 2013), and the export of fragrant volatile organic compounds from flowers (Adebesin et al., 2017). ATP-binding cassette (ABC) transporters utilize energy from ATP hydrolysis to efflux a wide range of substrates, including lipids, ions, and xenobiotics. These transporters maintain concentrations of endogenous molecules at appropriate physiological levels, and serve to reduce xenobiotic accumulation to prevent potential toxicity as part of the cell's chemical defense system (Nigam, 2015).

Cells have evolved intricate defense mechanisms against the accumulation of xenobiotic compounds, and process these xenobiotics through a sequence of four phases: (phase 0) unmodified efflux, (phase I) functionalization reactions, (phase II) conjugation reactions, and (phase III) metabolite elimination. Phases I and II are biotransformation reactions that convert hydrophobic compounds into more hydrophilic metabolites with reduced membrane permeability. In phase III, hydrophilic metabolites are actively exported from the cell by transmembrane transport proteins, including breast cancer resistance protein (BCRP) and the multidrug resistance proteins (MRPs) (Iversen et al., 2022). In phase 0, unmodified parent compounds are actively exported from the

cell by transmembrane transporters that include the ABC protein P-glycoprotein (Epel et al., 2008; Ferreira et al., 2014; Kroll et al., 2021).

The ATP Binding Cassette (ABC) family of proteins facilitate the translocation of an extensive array of substrates. These transporters are engaged in critical cellular processes such as nutrient uptake, lipid trafficking, and antigen presentation, and play a major role in limiting the toxic potential of xenobiotics. Their structure is modular, and consists of highly conserved nucleotide binding domains (NBDs) which bind and hydrolyze ATP, and structurally diverse transmembrane domains (TMDs) which anchor the protein in the membrane and bind and transport substrates (Pierman et al., 2018; Rice et al., 2014). Full transporters include four components (two NBDs and two TMDs) in one protein, while half transporters include one NBD and one TMD, and must dimerize to function (Pierman et al., 2018). Eukaryotic ABC proteins are categorized into ten different families: ABCA to ABCJ (Bieczynski et al., 2021; Pierman et al., 2018). The ABCA and ABCC families consist entirely of full transporters, the ABCB family includes both full and half transporters, and the ABCD, ABCG, and ABCH families are all half transporters (Dean and Annilo, 2005). Transporters in the ABCI family are composed of four separate proteins, with each NBD and TMD encoded by a different gene (Pierman et al., 2018). The recently-proposed ABCJ family consists of DNA repair enzymes. These proteins are composed of half-enzymes, with one NBD and one substrate-interacting domain each, that form dimers around one or two DNA strands (Figueira-Mansur et al., 2020; Hopfner and Tainer, 2003). ABCE and ABCF proteins contain only a pair of NBDs without any TMDs, and function in the assembly of sub-cellular components by NBD dimerization (Dean and Annilo, 2005).

ABC transporters are represented in all branches of life; prokaryotes have both importers and exporters, while eukaryotic ABC transporters are mostly exporters with only a few examples of importers (Pierman et al., 2018; Rice et al., 2014). Plants have an especially broad set of ABC transporters, with more than 120 members, compared to about 60 in fish and mammals (Bieczynski et al., 2021; Pierman et al., 2018). This diversity of plant ABC transporters appears to be related to secondary metabolism, as well as the increased importance of stress responses and detoxification for sessile organisms compared to motile organisms (Pierman et al., 2018). The physiological functions of ABC transporters are an area of active research, and they tend to fall into the broad categories of homeostasis, signaling, and detoxification.

Permeability glycoprotein (P-glycoprotein [P-gp]) is a transmembrane exporter in the ABCB family. It transports a broad range of substrates, including both endogenous and xenobiotic molecules, that tend to be mildly hydrophobic (Luckenbach et al., 2014; Silva et al., 2015). Its expression in excretory (e.g., liver, kidney), absorptive (e.g., intestine), and barrier (e.g., blood-brain, blood-eye, blood-gonad) tissues allows it to modulate its substrates' absorption, tissue distribution, and elimination (Leslie et al., 2005; Lončar et al., 2010; Love et al., 2021). P-gp's main identified function is xenobiotic resistance, but its role in regulating endogenous molecules is being increasingly recognized (Bieczynski et al., 2021; Nigam, 2015).

The discovery of P-gp marked a significant milestone in the understanding of cellular drug resistance mechanisms. P-gp was first identified in Chinese hamster ovary cells resistant to colchicine, revealing its role in the efflux of drugs from cells (Juliano and Ling, 1976). A major impetus for investigating P-gp stems from its role in multi-drug resistance (MDR) observed in some forms of cancer. Tumor cells that overexpress P-gp exhibit an enhanced ability to efflux chemotherapy drugs, rendering them less susceptible to the cytotoxic effects of these agents. This phenomenon, often accompanied by overexpression of other transporters and metabolic enzymes, poses a significant challenge in cancer treatment (Catalano et al., 2022). The ability of P-gp (sometimes called multidrug resistance protein 1 [MDR1]) to mediate MDR highlights its clinical relevance and the need for a deeper understanding of its underlying mechanisms.

Research efforts on P-gp have been primarily directed towards unraveling the molecular basis of MDR and exploring strategies to circumvent this resistance. The study of mammalian P-gp, in particular, has garnered attention due to its involvement in drug interactions, bioavailability, and treatment outcomes. Investigations into the structural and functional aspects of P-gp have revealed insights into its substrate specificity, drug-binding sites, and the conformational changes associated with substrate transport (Gottesman and Ling, 2006). These studies provide a foundation for designing targeted interventions aimed at modulating P-gp activity to enhance drug efficacy.

P-gp has received relatively little attention in the context of cancer in fish, although the MDR phenotype has been observed in several fish cancers (Koehler et al., 2004; Machado et al., 2014; Vogelbein et al., 1999). The focus of research on fish and

other aquatic animals originated in the discovery that animals living in contaminated aquatic environments over-express the same set of proteins that are overexpressed in MDR cancers, resulting in the multi-xenobiotic resistance (MXR) phenotype that allows them to survive and reproduce despite continuous chemical exposure. Specifically, resistant animals display higher levels of expression and activity of both efflux transporters (especially ABCB, ABCC and ABCG transporters) and biotransformation enzymes (including cytochromes and transferases) (Bard et al., 2002; Paetzold et al., 2009). The MXR phenotype was first identified in marine and freshwater mussels (Kurelec and Pivčević, 1991, 1989), and reported in fish soon after (Kurelec, 1992).

The evolution of fish populations towards pollution tolerance or resistance underscores the intricate interplay between natural selection and environmental pressures. Anthropogenic pollution is a recent and novel stressor in aquatic systems, and in response, fish populations have exhibited adaptive changes in chemical defense, that over generations enhances their ability to reduce the potential for adverse effects (Hamilton et al., 2017). Over time, the frequency of these beneficial genetic variations can increase within a population. For example, stickleback populations exposed to pulp mill effluent over many generations show significant changes in genotype compared to populations from uncontaminated sites (Lind and Grahn, 2011). Pollution-driven selection can lead to shifts in the allele frequencies of certain genes associated with stress response mechanisms. In two North Atlantic eel species, allele frequencies of genes related to sterol regulation and the response to oxidative stress differed significantly between populations exposed to varying contaminant profiles (Laporte et al., 2016). Fish populations exposed to pollutants may experience increased selection pressure on genes that code for transporters and enzymes involved in biotransformation and detoxification (Laporte et al., 2016; Williams and Oleksiak, 2011; Wirgin et al., 2011). This can result in the evolution of fish with improved abilities to reduce accumulation, biotransform, and eliminate contaminants.

In addition to multi-generational genetic changes, rapid responses, such as phenotypic plasticity, allow fish to adjust their traits within their lifetime in response to pollution-induced stressors. This can include changes in behavior, morphology, or physiology that improve their chances of survival in polluted habitats, including induction of P-gp and other genes that lead to the MXR phenotype (Bard et al., 2002; Paetzold et al., 2009). These plastic responses can provide an initial buffer against pollution impacts

while genetic adaptations accumulate over longer timeframes. Inhibitors of MXR proteins including P-gp are an emerging class of environmental pollutant (Kurelec, 1995; Smital and Kurelec, 1998) called chemosensitizers, making resistant organisms sensitive to toxic MXR protein substrates by reducing toxic concentration thresholds.

While most research on P-gp function in fish has focused on its role in xenobiotic defense and reducing chemical accumulation, the physiological roles of P-gp in fish are also beginning to be explored. This review aims to collect and synthesize the research on P-gp in fish to date, for the purpose of identifying knowledge gaps and providing a solid background for future research.

1.2. P-glycoprotein genes in fish species

In humans, P-glycoprotein is encoded by the ABCB1 gene on chromosome 7 (Dean et al., 2022; Fischer et al., 2013). Human ABCB4 has a high sequence homology to ABCB1 and is located next to it on the same chromosome due to a gene duplication (Moitra et al., 2011). ABCB5 also has a high sequence homology to ABCB1 due to an earlier gene duplication, and is located farther away on the same chromosome (Moitra et al., 2011). All fish species whose P-glycoprotein genes have been investigated thus far have Abcb4 or an Abcb4-like gene (Fischer et al., 2013; Kropf et al., 2020; Liu et al., 2013; Nicklisch et al., 2021). In zebrafish, the protein encoded by Abcb4 is functionally the most similar to the mammalian P-glycoprotein encoded by Abcb1 (Fischer et al., 2013). Thus, Abcb4 appears to be the primary functional P-glycoprotein in fish. Fish Abcb4 is an ortholog to the gene that gave rise to mammalian Abcb1 and Abcb4 (Luckenbach et al., 2014). Multidrug transport is the ancestral function of this gene, which was retained in mammalian Abcb1 (Luckenbach et al., 2014). All fish P-gp genes were initially labelled Abcb1 (or a derivation thereof) after their mammalian counterpart, and this nomenclature persists in the literature (Fischer et al., 2013).

Most fish species investigated to date, including Atlantic cod (*Gadus morhua*), Nile tilapia (*Oreochromis niloticus*), medaka (*Oryzias latipes*), and three-spined stickleback (*Gasterosteus aculeatus*), lack Abcb1 and Abcb5 genes (Fischer et al., 2013; Luckenbach et al., 2014). Two pufferfish species (*Takifugu rubripes* and *Tetraodon nigroviridis*) have Abcb1 in addition to Abcb4 (Fischer et al., 2013), while African coelacanth (*Latimeria chalumnae*), channel catfish (*Ictalurus punctatus*), rainbow trout

(*Oncorhynchus mykiss*), and zebrafish (*Danio rerio*) have Abcb5 in addition to Abcb4 (Fischer et al., 2013; Kropf et al., 2020; Liu et al., 2013). The Abcb1 gene present in pufferfish likely arose from a separate family-specific duplication of Abcb4 (Fischer et al., 2013; Luckenbach et al., 2014). The P-gp genes that have been identified in fish species are shown in Table 1.1.

Fish Abcb5 is an ortholog of mammalian Abcb5 (Luckenbach et al., 2014). Its function is not entirely clear, but it provides some multidrug transport function in fish and mammals (Chartrain et al., 2012; Fischer et al., 2013; Luckenbach et al., 2014). It also appears to have endogenous transport functions, perhaps related to biliary excretion, epithelial cell adhesion, and stem cell maturation (Fischer et al., 2013; Kropf et al., 2020; Luckenbach et al., 2014).

Table 1.1 P-glycoprotein genes in fish species

Species	Abcb1	Abcb4	Abcb5	References
Danio rerio (Zebrafish)	-	+	+	(Fischer et al., 2013)
Gadus morhua (Atlantic cod)	-	+	-	(Fischer et al., 2013; Luckenbach et al., 2014)
Gasterosteus aculeatus (Three-spined stickleback)	-	+	-	(Fischer et al., 2013; Luckenbach et al., 2014)
Ictalurus punctatus (Channel catfish)	-	+	+	(Liu et al., 2013; Luckenbach et al., 2014)
Latimeria chalumnae (African coelacanth)	-	+	+	(Fischer et al., 2013; Kropf et al., 2020)
Oncorhynchus mykiss (Rainbow trout)	-	+	+	(Kropf et al., 2020)
Oreochromis niloticus (Nile tilapia)	-	+	-	(Kropf et al., 2020)
Oryzias latipes (Medaka)	-	+	-	(Fischer et al., 2013; Luckenbach et al., 2014)
Takifugu rubripes (Japanese pufferfish)	+	+	-	(Fischer et al., 2013; Luckenbach et al., 2014)
Tetraodon nigroviridis (Green spotted pufferfish)	+	+	-	(Fischer et al., 2013)

Determined by synteny analysis. + indicates that the gene is present in the species, - indicates that the gene is absent in the species.

1.3. P-glycoprotein tissue expression and function

P-gp tends to be expressed in tissues with excretory (e.g., liver, kidney), absorptive (e.g., intestine), and barrier (e.g., skin, blood-brain, blood-eye and blood-gonad barriers) functions in both fish and mammals (Leslie et al., 2005; Lončar et al., 2010; Love et al., 2021). P-gp activity in excretory and absorptive tissues eliminates P-gp substrates from the body through excretory fluids (urine, bile, feces), while P-gp activity in blood-tissue barriers protects sanctuary tissues from the accumulation of P-gp substrates. P-gp mRNA and protein expression reported in fish tissues are shown in Table 1.2.

Many studies of P-gp expression used samples of only a single tissue, almost exclusively the liver (Assef et al., 2019; Damaré et al., 2009; Klobučar et al., 2010; Zucchi et al., 2010). Studies of this nature confirm the presence of a P-gp gene in a species without examining tissue expression in any detail. When P-gp expression is measured in multiple tissues, expression is usually highest in the kidney, followed by the liver and intestine (Lončar et al., 2010; Saeed et al., 2022; R. Tutundjian et al., 2002; Yuan et al., 2014). P-gp expression tends to be moderate in the brain, eye, and gonads, while gill, muscle, skin, and other tissues generally have low P-gp expression (Lončar et al., 2010; Love et al., 2021; Lu et al., 2015; Saeed et al., 2022; Yuan et al., 2014). This expression pattern suggests that the primary role of P-gp in fish is elimination of substrates from the body, and that among sanctuary tissues, the brain, eye, and gonads are the most protected by P-gp.

In fish, as well as in mammals and invertebrates, P-gp can play a role in preventing the absorption of chemicals from the intestinal tract, protecting the organism from potentially harmful substances (Romersi and Nicklisch, 2022). P-gp is expressed in the apical membrane of enterocytes, where it actively pumps substrates back into the intestinal lumen, limiting their uptake into the bloodstream (Bieczynski et al., 2021b). P-gp expression increases along the intestinal tract, allowing for increasing rates of active transport as the lumen contents become more concentrated (Bieczynski et al., 2021b; Lončar et al., 2010; Love et al., 2021). This efflux mechanism significantly contributes to the overall reduced accumulation and elimination of potentially harmful compounds.

Table 1.2 Expression of P-glycoprotein mRNA or protein (Abcb1, Abcb4, Abcb5, P-glycoprotein not otherwise specified) in fish tissues

Species	Tissue	References
Anoplarchus insignis (Slender cockscomb blenny)	Liver	(Bard, 2000)
Anoplarchus purpurescens (High cockscomb blenny)	Liver	(Bard et al., 2002b)
Barbus barbus (Barbel)	Liver	(Klobučar et al., 2010)
Carassius auratus gibelio (Silver Prussian carp)	Liver	(Klobučar et al., 2010)
Chelon labrosus (Thicklip grey mullet)	Liver, brain	(Diaz de Cerio et al., 2012)
Chondrostoma nasus (Sneep)	Liver	(Klobučar et al., 2010)
Cyprinodon variegatus (Sheepshead minnow)	Liver, kidney, intestine, exocrine pancreas	(Hemmer et al., 1998)
Cyprinus carpio (Common carp)	Liver	(Klobučar et al., 2010)
Danio rerio (Zebrafish)	Liver, kidney, intestine, ovary, testis, gill, skin, brain, brain vasculature, eye, muscle, heart, embryo (72 hpf), embryonic ionocytes	(Bieczynski et al., 2021; Gordon et al., 2019; Lu et al., 2015; Robey et al., 2021)
Fundulus heteroclitus (Killifish, mummichog)	Liver, distal intestine, brain capillaries	(Bard et al., 2002a; Cooper et al., 1999; Miller et al., 2002)
Gambusia affinis (Western mosquitofish)	Liver	(Damaré et al., 2009)
Gobiocypris rarus (Chinese rare minnow)	Liver, kidney, intestine, skin, brain, spleen	(Yuan et al., 2014)
Hatcheria macraei (Patagonian catfish)	Liver, gill	(Assef et al., 2019)

Species	Tissue	References
Ictalurus punctatus (Channel catfish)	Liver, intestine	(Doi et al., 2001)
Jenynsia multidentata (Killifish, onesided livebearer)	Liver, gill, brain	(Amé et al., 2009)
Lepomis macrochirus (Bluegill sunfish)	Liver	(Damaré et al., 2009)
Oncorhynchus mykiss (Rainbow trout)	Liver, kidney, intestine, gonad, gill, skin, brain, larval abdominal viscera, larval skin, larval head, larval muscle, larval yolk sac	(Assef et al., 2019; Kropf et al., 2020, 2016; Lončar et al., 2010; Love et al., 2021; Shúilleabháin et al., 2005; Sturm et al., 2001b, 2001a; Zaja et al., 2008)
Oreochromis niloticus (Nile tilapia)	Liver, intestine, zygote, embryo, larva	(Costa et al., 2012a, 2012b)
Poecilia reticulata (Guppy)	Liver, kidney, intestine, gill, muscle, ovary, esophagus, pancreas, branchial blood vessels, adrenal cortical tissue, gas gland	(Hemmer et al., 1995; Saeed et al., 2022)
Salmo trutta (Brown trout)	Liver	(Assef et al., 2019)
Scophthalmus maximus (Turbot)	Liver, kidney, intestine, gill, brain, muscle, esophagus, heart	(Tutundjian et al., 2002)
Squalius cephalus (European chub)	Liver	(Klobučar et al., 2010)
Thunnus albacares (Yellowfin tuna)	Liver, gill, brain, gonad	(Nicklisch et al., 2021)
Trematomus bernacchii (Emerald rockcod)	Liver	(Zucchi et al., 2010)

P-gp plays a pivotal role in the excretion of chemicals by both the liver and the kidney, contributing to the elimination of xenobiotics. In the liver, P-gp is expressed in the canalicular membrane of hepatocytes, facilitating the transport of various substances, including drugs and environmental contaminants, from the hepatocyte cytoplasm into the bile canaliculus (Sturm et al., 2001b). This excretion into the bile ultimately leads to the elimination of these substances from the body *via* feces. Studies using fish hepatocytes have demonstrated the active export of xenobiotic compounds, and its impairment by P-gp inhibitors (Bains and Kennedy, 2005; Hildebrand et al., 2009; Johnston and Kennedy, 2024; Sturm et al., 2001b).

In the kidney, P-gp is expressed in the proximal tubules, where it is involved in the active secretion of chemicals from the blood into the renal filtrate (Miller, 1995; Schramm et al., 1995). This process aids in the elimination of xenobiotics, contributing to their excretion in urine. By preventing the reabsorption of these substances from the filtrate, P-gp assists in reducing their systemic exposure and potential toxicity. Isolated renal proximal tubules from flounder (*Paralichthys lethostigma, Pseudopleuronectes americanus*) and killifish (*Fundulus heteroclitus*) were an important early model system for studying xenobiotic transport in fish (Miller, 1987; Schramm et al., 1995). These studies provided evidence for P-gp-mediated transport of numerous xenobiotics, including cyclosporin A, daunomycin, rapamycin, and ivermectin, into the renal tubule lumen (Fricker et al., 1999; Miller, 1995; Miller et al., 1997; Schramm et al., 1995), as well as an understanding of the relationship between SLC importers and ABC exporters expressed on opposite sides of the same epithelial cells (Fricker et al., 1999; Miller, 2014, 1995).

The blood-brain barrier (BBB) in fish, much like that in mammals, plays a pivotal role in regulating the exchange of substances between the bloodstream and the brain in order to maintain an environment optimal for neural function. Central to this barrier's function is the presence of efflux transporters, notably P-gp, which actively transport molecules out of brain endothelial cells, limiting their entry into the brain. The evidence for P-gp's role in the fish BBB is supported by studies demonstrating its presence in brain capillary endothelial cells, where it contributes to restricting the passage of xenobiotics and potential neurotoxicants into the brain parenchyma (Miller et al., 2002b). This is particularly important as fish species are frequently exposed to aquatic

contaminants that act as neurotoxicants (Azevedo and Kennedy, 2022; Bard and Gadbois, 2007; Kennedy et al., 2014).

The importance of the BBB in fish varies among species and is influenced by ecological and evolutionary factors. Evidence for a strong BBB in certain fish species is shown in studies where P-gp actively prevents the entry of lipophilic compounds. For example, in isolated killifish (*Fundulus heteroclitus*) and dogfish shark (*Squalus acanthias*) brain capillaries, the fluorescent analogues of the P-gp substrates verapamil and cyclosporin A were actively exported into the capillary lumen; this transport was inhibited by P-gp inhibitor PSC-833 (valspodar) (Miller et al., 2002b). Similarly, exposure of rainbow trout to the P-gp substrate ivermectin revealed limited brain accumulation due to P-gp-mediated efflux, indicating a robust barrier function (Johnston et al., 2023). Conversely, studies on some fish species suggest a more permeable BBB (Cserr and Bundgaard, 1984). Evidence for a weak BBB can be seen in the relatively higher brain uptake of certain molecules or drugs, possibly due to lower P-gp expression levels or less restrictive tight junctions between endothelial cells (Cserr and Bundgaard, 1984). The variability in BBB properties across fish species is likely linked to their habitats and ecological niches, as well as prior chemical exposures.

In addition to the blood-brain barrier, P-gp is expressed with other transmembrane transporters in barriers protecting sensitive tissues. In the mammalian eye, P-gp transports its substrates from the endothelium of ocular capillaries into the blood as part of the blood-retina or blood-aqueous barrier (Chen, 2020; Kajikawa et al., 1999). Fish also possess a blood-retina barrier, but the role of P-gp in this barrier has not been investigated in detail (Chen, 2020). Mammalian gonads are protected by blood-testis and blood-follicle barriers, which include P-gp (Guerreiro et al., 2018; Su et al., 2009). P-gp is also expressed in fish gonads (Lončar et al., 2010; Love et al., 2021; Lu et al., 2015; Nicklisch et al., 2021; Saeed et al., 2022), but the existence of blood-gonad barriers has not been investigated in fish.

Conversely, P-gp plays a relatively small role in xenobiotic transport in gills. Fish gills form an important interface between the animal and its environment, allowing for the exchange of gasses, ions, and organic molecules between the water and the blood (Evans et al., 2005). P-pg expression is typically lower in the gills than in liver, kidney, intestine, brain, and gonad (Lončar et al., 2010; Love et al., 2021). In rainbow trout gills,

the main xenobiotic transporter form of P-gp (Abcb4) is nearly absent, while Abcb5 is expressed only in interlamellar progenitor cells in contact with neither the water nor the blood (Kropf et al., 2020). Gills express high levels of biotransformation enzymes, similar to or sometimes higher than expression in liver and intestine (Costa et al., 2012b; Kropf et al., 2020). Perhaps due to the prevalence of biotransformed metabolites, phase III transporters are expressed at much higher levels in fish gills than P-gp (Costa et al., 2012b; Kropf et al., 2020; Lončar et al., 2010).

1.4. P-glycoprotein in the chemical defense system

Proteins perform a wide spectrum of chemical defense functions, and collaboratively direct the movement of endogenous and xenobiotic molecules across bodily barriers, their distribution within an organism, and their subsequent elimination. Playing a key role in this chemical defense system in fish is P-gp, which operates in tandem with other defense components. Fish tissues commonly express P-gp alongside other transporters and enzymes including phase I functionalization enzymes (e.g., cytochrome 450 oxidases), phase II conjugating enzymes (e.g., glutathione S-transferase, sulfotransferase, UDP-Glucuronosyltransferase) (Bao et al., 2018b; Cunha et al., 2016; Karmakar et al., 2022; Kropf et al., 2016; Meinan et al., 2022), phase III exporters (e.g., multidrug resistance proteins [MRPs], breast cancer resistance protein [BCRP]) (Cunha et al., 2016; Kropf et al., 2020, 2016; Vannuccini et al., 2015)), and solute carrier (SLC) transporters (e.g., organic anion transporting polypeptides [OATPs], organic anion transporters [OATs], organic cation transporters [OCTs], and multidrug and toxin extrusion proteins [MATEs]) (Bolten et al., 2022; Muzzio et al., 2014; Romersi and Nicklisch, 2022). Although the ABCB (MDR, P-gp) and ABCC (MRP) families of transporters are both referred to as “multidrug resistance proteins”, they are distinct classes of transporters that perform different functions in the chemical defense system. The interrelation of these enzymes and transporters in epithelial cells is illustrated in Fig. 1.1.

In fish, the liver, kidney, and gill express high levels of biotransformation (phase I and II) enzymes (Costa et al., 2012b; Kropf et al., 2020). In these tissues, biotransformation enzyme mRNA expression is 10- to 150-fold higher than ABC transporter expression (Costa et al., 2012b; Kropf et al., 2020). These tissues also have higher expression of phase III transporters (MRPs [Abccs], BCRP [Abcg]) than P-gp, as

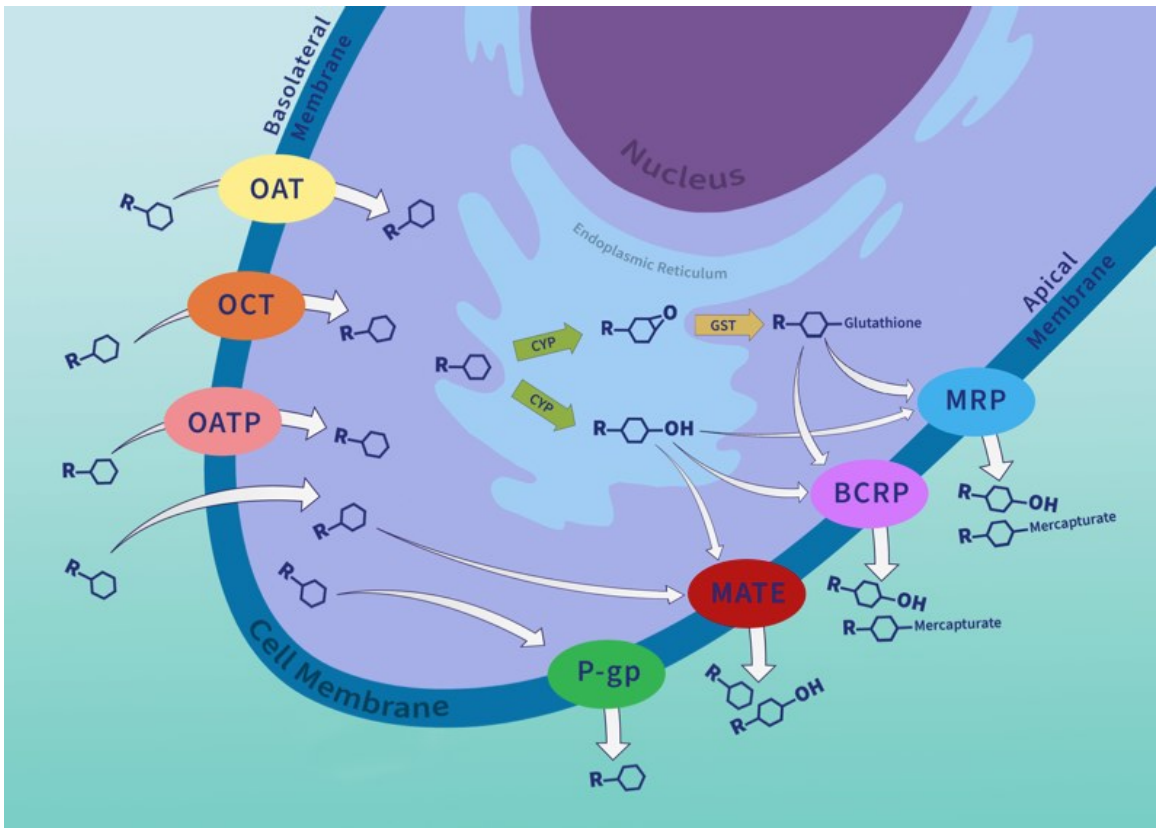

Figure 1.1 The chemical defense system in epithelial cells
A schematic representation of the relationships between transmembrane transporters and biotransformation enzymes in epithelial cells. The apical membrane faces the lumen of the vessel, which is in contact with blood in blood-tissue barriers (eg. blood-brain, blood-eye, blood-gonad), and is in contact with excretory fluid (urine, bile, feces) in absorptive (eg. intestine) and excretory (eg. liver, kidney) tissues. Substrate entry on the basolateral membrane is represented by membrane diffusion, as well as importer proteins: the SLC21 family (organic anion transporter proteins [OATP]) and the SLC22 family (organic cation transporters [OCT], organic anion transporters [OAT]). Chemical biotransformation occurs inside the cell by phase I functionalization enzymes (represented by cytochrome monooxygenases [CYP]), and phase II conjugation enzymes (represented by glutathione-S-transferase [GST]). Glutathione conjugates are further processed through the mercapturic acid pathway, and exported as mercapturate conjugates. Substrate efflux on the apical membrane is performed in phase 0 (unmodified substrates) by the ABCB family (p-glycoprotein [P-gp]), and in phase III (biotransformed substrates) by the ABCC family (multidrug resistance proteins [MRP]) and the ABCG family (breast cancer resistance protein [BCRP]). The SLC47 family (multidrug and toxicant extrusion proteins [MATE]) exports substrates, but its specificity for transport of unmodified or biotransformed substrates (and therefore its phase) is unknown in fish.

expected for tissues that have a high concentration of biotransformed metabolites (Costa et al., 2012b; Kropf et al., 2020; Lončar et al., 2010). Conversely, the fish intestine displays relatively modest biotransformation enzyme expression, with nearly equivalent levels of biotransformation enzymes and ABC transporters (Costa et al., 2012b). In the proximal intestine, phase III Abcc and Abcg transporters are expressed at similar levels

to P-gp, but the distal intestine expresses much more P-gp compared to phase III transporters (Costa et al., 2012b; Lončar et al., 2010). This transporter expression pattern favours the transport of unmodified parent compounds, which will be the predominant form (as opposed to biotransformed metabolites) due to the lower expression of biotransformation enzymes in this tissue.

Importer proteins tend to be expressed in the same cells as exporters, usually on the opposite sides of barrier tissues. The SLC21 (OATP) and SLC22 (OAT, OCT) families of secondary active transporters in particular are commonly expressed with P-gp in fish tissues (Bolten et al., 2022; Muzzio et al., 2014; Romersi and Nicklisch, 2022). These transport proteins import both membrane permeable (lipophilic) and non-membrane permeable (hydrophilic) molecules. Membrane-permeable molecules require import proteins for the same reasons that they require export proteins including P-gp: active transport moves these molecules across barriers much faster than passive diffusion, and allows the organism to maintain substrate concentrations that are substantially different from passive equilibrium concentrations (Adebesin et al., 2017; Pierman et al., 2018; Widhalm et al., 2015). These import proteins share a very similar set of substrates and inhibitors with P-gp (Mihaljević et al., 2017; Nigam, 2015; Popovic et al., 2014; Willi and Fent, 2018). This common substrate base is expected for an integrated system that controls the movement of compounds across barriers: each substrate molecule needs to be sequentially imported then exported in order to cross an epithelial cell, thus the importers and exporters in the same cell will transport a shared set of substrates.

Regulation of these defense mechanisms is orchestrated by various signaling pathways and transcription factors. Nuclear receptors, including the aryl hydrocarbon receptor (AhR) and pregnane X receptor (PXR), play a crucial role in coordinating the expression of biotransformation enzymes, P-gp, and MRPs. Upon binding to their ligands, these receptors initiate the transcription of target genes involved in xenobiotic metabolism and transport. For example, AhR activation in fish induces the expression of phase I enzymes (Bard et al., 2002b, 2002a), while PXR activation upregulates both biotransformation enzymes and efflux transporters including P-gp and MRPs (Bresolin et al., 2005; De Anna et al., 2021; Jackson and Kennedy, 2017; Reschly et al., 2007).

P-gp performs essential chemical defense functions in different tissues, but it is not essential for life until faced with specific chemical challenges. For example, the dog and cat genomes both include alleles coding for non-functional P-gp proteins, but affected individuals show no adverse health effects unless exposed to a toxic P-gp substrate (e.g., ivermectin) (Mealey et al., 2023). Similarly, P-gp knockout mice are viable and generally indistinguishable from the wild-type, but are much more sensitive to drugs that are P-gp substrates (Borst and Schinkel, 2013). In fish, zebrafish embryos subjected to morpholino knockdown of Abcb4 and Abcb5 are viable and develop normally, but accumulate higher levels of P-gp substrate molecules compared to untreated embryos (Fischer et al., 2013).

1.5. P-glycoprotein structure and transport mechanism

P-gp is a full transporter with two transmembrane domains (TMDs) and two nucleotide binding domains (NBDs) (Fig. 1.2). In both fish and mammals, each TMD contains 6 transmembrane helices (Costa et al., 2012b; Fischer et al., 2013; Kropf et al., 2020; Silva et al., 2015). The NBDs contain highly conserved motifs that are a hallmark of all ABC transporters: A-loop, Walker A, Walker B, and ABC signature (Kropf et al., 2020; Pierman et al., 2018). P-gp has an atomic mass of 170 kDa in both fish and mammals (Amé et al., 2009; Cooper et al., 1999; Nicklisch et al., 2021; Silva et al., 2015). The 3-dimensional structure has been determined for human and mouse P-gp (Aller et al., 2009; Nosol et al., 2020), but the structure of fish P-gp has not yet been fully elucidated. There is a 64% amino acid sequence identity between zebrafish Abcb4 and human ABCB1, while zebrafish Abcb5 shares 57% of its amino acid sequence with human ABCB1 (Fischer et al., 2013). Thus, fish and mammalian P-gp are expected to share most aspects of their structure and function (Fischer et al., 2013; Robey et al., 2021). The differences in P-gp function between these animal lineages are found in the finer points of their operation, such as substrate specificities and post-translational regulation (Löscher and Gericke, 2020; Robey et al., 2021). These structural disparities might be attributed to differences in evolutionary history, ecological niches, and physiological requirements between mammals and fish.

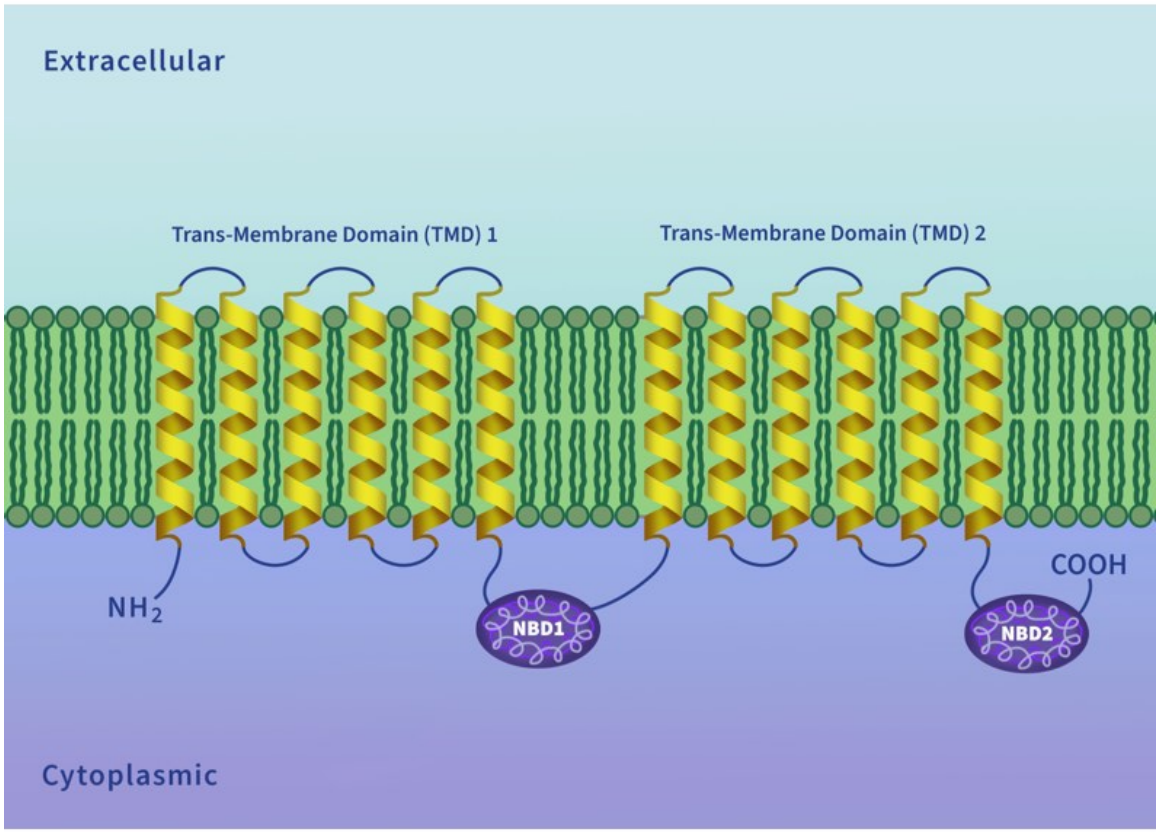

Figure 1.2 Structure of fish P-glycoprotein
The protein is composed of two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs). Each transmembrane domain contains six transmembrane helices (Kropf et al., 2020; Robey et al., 2021).

Substrates enter the drug-binding pocket of P-gp either from the cytoplasm, or directly from the hydrophobic region of the plasma membrane (Aller et al., 2009; Nosol et al., 2020). The binding pocket is surrounded by the 12 α-helices of the two TMDs, and is lined with hydrophobic, amphipathic, polar, and charged amino acid side chains (Aller et al., 2009). Substrate molecules interact with a subset of these amino acid side chains, forming the molecule's binding site (Aller et al., 2009). While the amino acid composition of human and zebrafish Abcb4 and Abcb5 is similar, there are different amino acids in their TMD α-helices, leading to different substrate specificities and affinities (Robey et al., 2021).

The resting state of the P-gp molecule is the inward-facing configuration (Srikant and Gaudet, 2019). In this state, the outside ends of two TMDs are in contact with each other (closed) on the outer surface of the cell membrane. The intracellular ends of the two TMDs are separated (open) on the cytoplasmic side of the cell membrane, and the

two NBDs are separated from each other in the cytoplasm (Srikant and Gaudet, 2019). When a substrate is bound in the drug binding pocket and an ATP molecule is bound to each of the two NBDs, the transport cycle begins (Fig. 1.3) (Srikant and Gaudet, 2019). First, the cytoplasmic ends of the TMDs come together, and the NBDs dimerize with the two ATP molecules sandwiched between them. One of the bound ATP molecules is then hydrolyzed, and the P-gp molecule transitions to an outward-facing configuration. The outer ends of the TMDs separate, changing the shape of the drug binding site, and releasing the substrate into the extracellular media. Finally, the second ATP molecule is hydrolyzed, and the two NBDs separate. The P-gp molecule transitions back to the inward-facing configuration, from which it is able to begin a new transport cycle (Srikant and Gaudet, 2019).

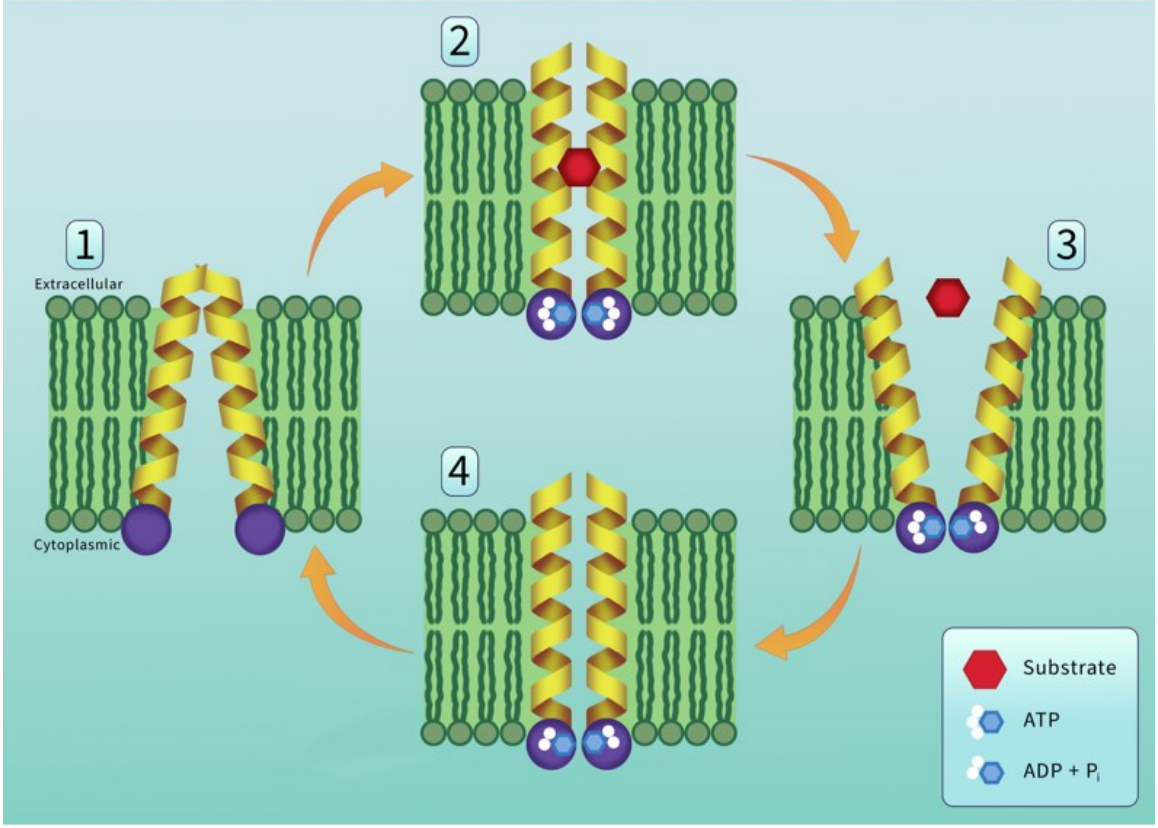

Figure 1.3 The P-glycoprotein transport cycle
1. P-gp has an inward-open conformation, with no substrate or ATP bound. 2. A substrate binds in the drug binding pocket between the transmembrane domains (TMDs), and one ATP molecule binds to each of the two nucleotide binding domains (NBDs). The NBDs dimerize with the two ATP molecules sandwiched between them. 3. One of the ATP molecules is hydrolyzed. The P-gp molecule transitions to an outward-facing conformation and releases the substrate into the extracellular space. 4. The second ATP molecule is hydrolyzed, allowing P-gp to return to an inward-facing conformation (Srikant and Gaudet, 2019).

1.6. P-glycoprotein substrates

Substrates of fish P-gp tend to be moderately hydrophobic, amphipathic, and often have a positively charged nitrogen atom and aromatic rings, closely matching substrates of mammalian P-gp (Silva et al., 2015). The known substrates of fish P-glycoprotein are shown in Table 1.3.

In studies comparing the substrates of fish P-gp to mammalian P-gp directly, the sets of molecules that are substrates in each animal lineage are similar but not identical. In a high-throughput study of 90 known substrates of human P-gp, zebrafish abcb4-expressing cells closely matched human P-gp-expressing cells in cytotoxicity resistance assays, while zebrafish abcb5-expressing cells showed resistance to fewer substrates in these assays (Robey et al., 2021). In a study comparing human P-gp to P-gp from the livebearer fish *Poeciliopsis lucida*, 16 substrates were common to both species, while 5 substrates were exclusive to fish P-gp and 7 substrates were exclusive to human P-gp (Zaja et al., 2011).

The majority of research efforts have concentrated on substrates of anthropogenic origin, which are materials that have become pervasive environmental contaminants due to human activities, such as fluorescent dyes, pharmaceuticals, pesticides, and industrial chemicals. This emphasis can be attributed to the origins of P-gp's discovery, which revolved around drug resistance in mammalian cancers and resistance to xenobiotic pollutants in aquatic organisms. Xenobiotic substrates present in the environment throughout evolutionary history likely included such chemicals as bacterial toxins (e.g., doxorubicin, microcystin-LR, lipopolysaccharides) and plant metabolites (e.g., quinidine, nerol, isoeugenol).

P-gp has played an important role in chemical homeostasis in fish predating the onset of anthropogenic pollution, but its physiological roles have received relatively little attention. Few studies have investigated endogenous substrates in fish to any great extent; only two steroid hormones (cortisol and testosterone) have been identified as substrates to date (Johnston and Kennedy, 2023; Zaja et al., 2011). Mammalian P-gp actively transports a diverse array of endogenous substrates including steroid hormones (e.g., cortisol, aldosterone, testosterone), lipids (e.g., phospholipids, cholesterol), and peptides (e.g., enkephalins) (Abulrob and Gumbleton, 1999; Dagenais et al., 2001;

Table 1.3 Substrates of fish P-glycoprotein

Substrate	Species	References
Hormones		
19-methyl testosterone	*Poeciliopsis lucida*	(Zaja et al., 2011)
Cortisol	*Oncorhynchus mykiss*	(Johnston and Kennedy, 2023)
Octreotide (synthetic somatostatin)	*Fundulus heteroclitus*	(Gutmann et al., 2000)
Bacterial Toxins		
Lipopolysaccharides	*Danio rerio*	(Mottaz et al., 2017)
Microcystin-LR	*Danio rerio*	(Lu et al., 2015)
Fluorescent Dyes		
BCECF-AM	*Danio rerio*	(Gordon et al., 2019)
Calcein-AM	*Danio rerio, Oncorhynchus mykiss, Poeciliopsis Lucida*	(Caminada et al., 2008; Fischer et al., 2011, 2013; Gordon et al., 2019; Zaja et al., 2007, 2008b)
DiOC6	*Danio rerio*	(Gordon et al., 2019)
Rhodamine 123	*Oncorhynchus mykiss, Poeciliopsis Lucida*	(Caminada et al., 2008; Johnston and Kennedy, 2023; Sturm et al., 2001b; Uchea et al., 2015; Zaja et al., 2007, 2008b, 2011)
Rhodamine B	*Hatcheria macraei, Salmo trutta, Oncorhynchus mykiss, Oncorhynchus tshawytscha, Danio rerio, Cyprinus carpio*	(Assef et al., 2019; Bieczynski et al., 2021a; Fischer et al., 2013; Smital and Sauerborn, 2002)
Metal Compounds		
Arsenic trioxide (As_2O_3)	*Poeciliopsis Lucida*	(Zaja et al., 2011)
Cadmium chloride ($CdCl_2$)	*Danio rerio*	(Hu et al., 2019)
Silver nitrate ($AgNO_3$)	*Danio rerio*	(Hu et al., 2019)
Polycyclic Aromatic Hydrocarbons		
2-aminoanthracene	*Leuciscus idus melanotus*	(Kurelec, 1992)
Benzo[a]pyrene	*Cyprinus carpio*	(Chen et al., 2016)
Phenanthrene	*Danio rerio*	(Fischer et al., 2013)

Substrate	Species	References
Fragrance Ingredients		
α-amylcinnamaldehyde	*Danio rerio*	(Cunha et al., 2017)
α-hexylcinnamaldehyde	*Danio rerio*	(Cunha et al., 2017)
Galaxolide	*Danio rerio*	(Fischer et al., 2013)
Isoeugenol	*Danio rerio*	(Cunha et al., 2017)
Musk xylene	*Danio rerio*	(Cunha et al., 2017)
Nerol	*Danio rerio*	(Cunha et al., 2017)
Tonalide	*Danio rerio*	(Fischer et al., 2013)
Pesticides		
Chlorpyrifos	*Poeciliopsis Lucida*	(Zaja et al., 2011)
Diazinon	*Poeciliopsis Lucida*	(Zaja et al., 2011)
Emamectin benzoate	*Oncorhynchus mykiss*	(Kennedy et al., 2014)
Methyl parathion	*Danio rerio*	(Nornberg et al., 2015)
Phosalone	*Poeciliopsis Lucida*	(Zaja et al., 2011)
Hydrophobic Peptide		
Reversin 205	*Poeciliopsis Lucida*	(Zaja et al., 2011)
Immunosuppressants		
Cyclosporin A	*Fundulus heteroclitus, Squalus acanthias*	(Bolten et al., 2022; Masereeuw et al., 2000; Miller et al., 2002a)
Rapamycin	*Fundulus heteroclitus*	(Miller et al., 1997)
Psychoactive Pharmaceuticals		
Bupropion	*Oncorhynchus mykiss*	(Bourgeois et al., 2022)
Citalopram	*Oncorhynchus mykiss*	(Bourgeois et al., 2022)
Clozapine	*Oncorhynchus mykiss*	(Bourgeois et al., 2022)
Fluoxetine	*Danio rerio*	(Cunha et al., 2017)
Venlafaxine	*Oncorhynchus mykiss*	(Bourgeois et al., 2022)
Analgesic Pharmaceuticals		
Colchicine	*Poeciliopsis Lucida*	(Zaja et al., 2011)

Substrate	Species	References
Diclofenac	*Danio rerio*	(Cunha et al., 2017)
Antiarrhythmics		
Acebutolol	*Poeciliopsis Lucida*	(Caminada et al., 2008)
Diltiazem	*Poeciliopsis Lucida*	(Zaja et al., 2011)
Nicardipine	*Poeciliopsis Lucida*	(Zaja et al., 2011)
Prazosin	*Poeciliopsis Lucida*	(Zaja et al., 2011)
Propanolol	*Oncorhynchus mykiss, Poeciliopsis Lucida*	(Stott et al., 2015; Zaja et al., 2011)
Quinidine	*Oncorhynchus mykiss, Poeciliopsis Lucida*	(Johnston and Kennedy, 2023; Zaja et al., 2011)
Verapamil	*Danio rerio, Poeciliopsis Lucida, Fundulus heteroclitus, Squalus acanthias, Oncorhynchus mykiss*	(Fischer et al., 2013; Miller et al., 2002a; Zaja et al., 2008b, 2011)
Antiretroviral		
Saquinavir	*Fundulus heteroclitus*	(Gutmann et al., 1999)
Lipid-Lowering Pharmaceuticals		
Atorvastatin	*Poeciliopsis Lucida*	(Caminada et al., 2008)
Pravastatin	*Poeciliopsis Lucida*	(Zaja et al., 2011)
Simvastatin	*Danio rerio*	(Cunha et al., 2017)
Antiparasitic		
Ivermectin	*Danio rerio, Oncorhynchus mykiss, Fundulus heteroclitus*	(Azevedo and Kennedy, 2022; Bard and Gadbois, 2007; Fricker et al., 1999; Kennedy et al., 2014)
Phosphodiesterase Inhibitor		
Sildenafil	*Poeciliopsis Lucida*	(Zaja et al., 2011)
Anticancer Pharmaceuticals		
AT9283	*Danio rerio*	(Robey et al., 2021)
Bisantrene	*Danio rerio*	(Robey et al., 2021)
Daunomycin (Daunorubicin)	*Fundulus heteroclitus, Pleuronectes americanus*	(Miller, 1995; Sussman-Turner and Renfro, 1995)
Doxorubicin	*Oncorhynchus mykiss, Poeciliopsis Lucida, Danio rerio*	(Caminada et al., 2008; Hildebrand et al., 2009; Robey et al., 2021; Sturm et al., 2001b)
Etoposide	*Danio rerio*	(Robey et al., 2021)

Substrate	Species	References
KW-2478	*Danio rerio*	(Robey et al., 2021)
Mitoxantrone	*Danio rerio*	(Robey et al., 2021)
Paclitaxel	*Danio rerio*	(Robey et al., 2021)
Romidepsin	*Danio rerio*	(Robey et al., 2021)
Sepantronium bromide (YM-155)	*Danio rerio*	(Robey et al., 2021)
Tanespimycin (17-AAG)	*Danio rerio*	(Robey et al., 2021)
Tozasertib (VX-680)	*Danio rerio*	(Robey et al., 2021)
Vinblastine	*Danio rerio, Ictalurus punctatus*	(Doi et al., 2001; Fischer et al., 2013; Robey et al., 2021)
Vincristine	*Danio rerio*	(Fischer et al., 2013)
Vinorelbine	*Oncorhynchus mykiss*	(Johnston and Kennedy, 2024)

Determined by methods including transport inhibition by model chemosensitizers, decreased accumulation in P-gp overexpressing cells, increased cytotoxicity with model chemosensitizers, and increased ATPase activity in combination with inhibition of model substrate accumulation.

Wang et al., 2000; Yano et al., 2019; Zhou, 2008). By managing intracellular levels of these compounds, P-gp plays a pivotal role in hormone signaling, lipid metabolism, and neurotransmission. While research focus has been on xenobiotic efflux and multidrug resistance, understanding the intricate interplay between P-gp and endogenous substrates provides evidence that it has broader regulatory significance in several physiological pathways (Nigam, 2015).

Studies of P-gp expression have provided preliminary evidence that fish P-gp, especially Abcb5, may be involved in processing hormones and growth factors. In zebrafish ovaries, abcb5 is highly expressed in follicles during the early pre-vitellogenic stages of egg development, but is absent during the later vitellogenic and pre-ovulatory stages, while abcb4 is expressed at low levels at all stages of egg development (Robey et al., 2021). This expression pattern suggests a possible role for fish P-gp in transporting hormones that stimulate early oocyte maturation, including follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estradiol (Nagahama and Yamashita, 2008; Patiño and Sullivan, 2002), and that abcb5 may have a higher affinity for these substrates than abcb4 does (Robey et al., 2021). In rainbow trout gills, abcb5 is highly expressed in immature progenitor cells that mature into epithelial pavement (respiratory) cells, but abcb5 is not expressed in mature gill epithelial cells (Goss et al., 1992; Kropf et al., 2020). This suggests that P-gp plays a role in cell maturation in fish gills (Kropf et al., 2020). In mammals, P-gp has been implicated in stem cell differentiation; the ability of stem cells to differentiate into specialized cell types involves the regulation of multiple transport mechanisms including the P-gp transport of endogenous signaling molecules, such as growth factors and hormones, crucial for directing stem cell fate (Frank et al., 2003). Exploring the endogenous substrates of P-gp in fish holds great potential and aligns with similar inquiries into the intrinsic functions of membrane transporters in mammals and other species (Nigam, 2015).

1.7. Regulation of P-glycoprotein expression

Diverse factors including xenobiotic exposure, heat stress, and oxidative stress can increase P-gp expression as part of an adaptive cellular defense response (Silva et al., 2015). In mammals, the induction of P-gp has been extensively studied, especially in the context of drug interactions and multi-drug resistance. Exposure to xenobiotics activates nuclear transcription factors including the pregnane X receptor (PXR),

constitutive androstane receptor (CAR), aryl hydrocarbon receptor (AhR), and nuclear factor erythroid 2-related factor 2 (Nrf2), which bind to specific enhancer sequences in the ABCB1 promoter region, orchestrating the transcriptional upregulation of P-gp (Chan et al., 2013; Mirzaei et al., 2022).

In fish, P-gp induction has gained attention due to its role in environmental detoxification and chemical defense. Exposure to aquatic pollutants, heavy metals, and pharmaceuticals can induce P-gp expression in fish, particularly in the liver, gills, and intestines. The regulation of P-glycoprotein (P-gp) in fish involves molecular mechanisms similar to those in mammals, including interactions between receptors, genetic response elements, and nuclear transcription factors. A key similarity between fish and mammals is the involvement of the nuclear receptors PXR and Nrf2 (Hu et al., 2019; Karmakar et al., 2022). These receptors are activated by oxidative stress (Nrf2), or upon binding to specific ligands (PXR), and induce the subsequent transcription of P-gp and other genes. In fish, PXR has been identified as a central regulator of P-gp expression (Bresolin et al., 2005; Jackson and Kennedy, 2017). Studies have shown that PXR activation by xenobiotics, including pharmaceuticals (e.g., paracetamol, simvastatin) and plant flavonoids (e.g., quercetin, rotenone), leads to the upregulation of P-gp expression in fish tissues (Meinan et al., 2022; Wang et al., 2019, 2020). Similarly, Nrf2 activation has been linked to the regulation of P-gp in both fish and mammals (Karmakar et al., 2022; Wu et al., 2019). For example, in zebrafish, the organophosphate pesticide malathion induced oxidative stress in zebrafish livers, leading to an increase in P-gp expression *via* the Nrf2 pathway (Karmakar et al., 2022).

Despite these similarities, there are notable differences in P-gp regulation between fish and mammals. Although AhR has been shown to regulate the expression of cytochrome P450 in fish, it does not affect P-gp expression (Bard et al., 2002b, 2002a). Fish also lack the constitutive androstane receptor (CAR) that induces P-gp expression in mammals (Reschly et al., 2007). Mammalian research has provided detailed insights into the role of nuclear receptors and transcription factors in P-gp expression, often involving well-defined pathways. In contrast, fish studies are still evolving, and while the key players are identified, the intricate network of interactions remains to be fully elucidated.

1.8. Modulators of P-glycoprotein expression

P-gp expression in fish tissues can be induced or inhibited by many different chemicals, including both xenobiotic and endogenous compounds. Chemicals that modulate P-gp expression include those of anthropogenic origin such as pharmaceuticals (e.g., morphine, paracetamol, simvastatin), pesticides (e.g., malathion, emamectin benzoate), and industrial chemicals (e.g., crude oil, perfluoro octane sulfonate [PFOS]), and those of naturally derived origin such as plant metabolites (e.g., glycyrrhizic acid, quercetin) and bacterial compounds (e.g., microcystin-LR). Endogenous chemical modulators include bile acids and steroid hormones. Compounds that induce P-gp expression in fish are listed in Table 1.4, and those that inhibit P-gp expression are shown in Table 1.5. In addition to the specific modulating substances listed, complex mixtures of toxicants in industrial and urban effluents and receiving waters induce P-gp expression, and contribute to the MXR phenotype (Assef et al., 2019; Bieczynski et al., 2021b; Shúilleabháin et al., 2005). Environmental stressors can also alter P-gp expression. For example, fasted (4 weeks) rainbow trout exhibit induced P-gp expression in intestinal epithelia (Baumgarner et al., 2013).

Alteration in P-gp expression is intricate in terms of timing, effective dosages, and combinations of chemicals. Certain compounds can induce or inhibit P-gp expression at different time intervals, adding to the complexity of this modulation. For example, in zebrafish brain, malathion induced P-gp expression after 3 d, but inhibited its expression after 7 d (Karmakar et al., 2022). Stress that results from acute exposures to toxicants can differ from long-term exposures (Weltje et al., 2013), and may require different gene expression patterns in response. In addition, early induction of coordinately regulated genes (e.g., antioxidant enzymes) may alter the condition of the tissue (e.g., oxidative stress), thereby altering later P-gp expression (Karmakar et al., 2022).

Some substances affect P-gp expression only when present in mixtures. The pesticides cypermethrin and chlorpyrifos significantly decreased P-gp expression in killifish gill and brain tissue in combination, but had no significant effect in these tissues when present individually (Bonansea et al., 2017). Individually, titanium dioxide nanoparticles and the dioxin TCDD did not affect P-gp expression in sea bass liver, but the combination of both compounds caused a significant reduction in P-gp expression

Table 1.4 Inducers of P-glycoprotein (Abcb1, Abcb4, Abcb5, P-glycoprotein not otherwise specified) mRNA or protein expression in fish

Expression Inducer	Species	Tissue	References
Bile Acids and Salts			
5α-cyprinol 27-sulfate	*Danio rerio*	Liver	(Reschly et al., 2007)
Lithocholic acid	*Oncorhynchus mykiss*	Liver	(Wassmur et al., 2010)
Enzyme Cofactor			
Lipoic acid	*Jenynsia multidentate*	Liver	(Monserrat et al., 2014)
Hormone			
Pregnenolone 16α-carbonitrile (Synthetic steroid)	*Oncorhynchus mykiss*	Liver	(Wassmur et al., 2010)
Vitamin			
Vitamin B12	*Danio rerio*	Liver	(Karmakar et al., 2022)
Cyanotoxin			
Microcystin-LR	*Danio rerio*	Embryo	(Lu et al., 2015)
Microcystin-LR	*Jenynsia multidentate*	Liver, gill, brain	(Amé et al., 2009)
Metal Compounds			
Arsenic trioxide (As_2O_3)	*Poeciliopsis Lucida*	Liver	(Della Torre et al., 2012)
Cadmium chloride ($CdCl_2$)	*Trematomus bernacchii*	Liver	(Zucchi et al., 2010)
Cadmium chloride ($CdCl_2$)	*Danio rerio*	Embryo	(Hu et al., 2019)
Silver nitrate ($AgNO_3$)	*Danio rerio*	Embryo	(Hu et al., 2019)
Phytochemicals			
Glycyrrhizic acid	*Ictalurus punctatus*	Kidney	(Wang et al., 2020)
Quercetin	*Ictalurus punctatus*	Kidney	(Wang et al., 2020)
Schisandrin A	*Ictalurus punctatus*	Kidney	(Wang et al., 2020)
Schisandrin B	*Ictalurus punctatus*	Kidney	(Wang et al., 2020)
Schisandrol A	*Ictalurus punctatus*	Kidney	(Wang et al., 2020)
Schisandrol B	*Ictalurus punctatus*	Kidney	(Wang et al., 2020)

Expression Inducer	Species	Tissue	References
Industrial Chemicals			
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	*Dicentrarchus labrax*	Liver	(Della Torre et al., 2014)
Crude oil (Exxon Valdez)	*Anoplarchus purpurescens*	Liver	(Bard et al., 2002b)
Heavy fuel oil (fresh)	*Chelon labrosus*	Liver	(Diaz de Cerio et al., 2012)
Perfluorooctane sulfonate (PFOS)	*Chelon labrosus*	Liver	(Diaz de Cerio et al., 2012)
Pesticides			
Carbendazim	*Jenynsia multidentate*	Gill	(Götte et al., 2020)
Chlorpyrifos	*Jenynsia multidentate*	Liver	(Bonansea et al., 2017)
Emamectin benzoate	*Oncorhynchus mykiss*	Liver	(Cárcamo et al., 2011)
Malathion	*Danio rerio*	Liver, brain	(Karmakar et al., 2022)
Rotenone	*Ictalurus punctaus*	Kidney	(Wang et al., 2020)
Triclosan	*Xiphophorus helleri* (males)	Liver	(Liang et al., 2013)
Zinc pyrithione	*Carassius auratus gibelio*	Liver, kidney	(Ren et al., 2017)
Analgesic Pharmaceuticals			
Diclofenac	*Gambusia affinis*	Liver	(Bao et al., 2018a)
Paracetamol (Acetaminophen)	*Mugilogobius chulae*	Liver	(Meinan et al., 2022)
Antibiotic			
Norfloxacin	*Xiphophorus Helleri*	Liver	(Liang et al., 2015)
Antifungal Pharmaceutical			
Clotrimazole	*Oncorhynchus mykiss*	Intestine, brain	(Love et al., 2021)
Lipid-Lowering Pharmaceutical			
Simvastatin	*Mugilogobius abei*	Liver	(Wang et al., 2019)
Simvastatin	*Gambusia affinis*	Liver	(Bao et al., 2018b)

Table 1.5 Inhibitors of P-glycoprotein (Abcb1, Abcb4, Abcb5, P-glycoprotein not otherwise specified) mRNA or protein expression in fish

Expression Inhibitor	Species	Tissue	References
Hormone			
Pregnenolone 16α-carbonitrile (Synthetic steroid)	*Danio rerio*	Liver	(Jackson and Kennedy, 2017)
Phytochemical			
Genistein	*Ictalurus punctatus*	Kidney	(Wang et al., 2020)
Carbon Allotrope			
Fullerene (nC_{60})	*Cyprinus carpio*	Liver	(Chen et al., 2016)
Industrial Chemicals			
Metal mine tailings (Mn, Cd, As, Cu, Cr)	*Astyanax lacustris*	Liver	(Macêdo et al., 2020)
Pesticides			
Carbendazim	*Jenynsia multidentate*	Liver	(Götte et al., 2020)
Emamectin benzoate	*Oncorhynchus mykiss*	Intestine	(Cárcamo et al., 2011)
Malathion (7-day exposure)	*Danio rerio*	Brain	(Karmakar et al., 2022)
Triclosan	*Xiphophorus helleri* (females)	Liver	(Liang et al., 2013)
Analgesic Pharmaceutical			
Morphine	*Danio rerio*	Embryo	(Mottaz et al., 2017)

(Vannuccini et al., 2015). The pesticide methyl parathion induced P-gp expression in zebrafish liver only in combination with verapamil (Nornberg et al., 2015). These additive or synergistic effects may indicate that each of the compounds in the mixture interacts with different aspects of the mechanisms regulating P-gp expression in fish.

The effects of P-gp expression modulators can be species-specific. For example, the synthetic steroid pregnenolone 16α-carbonitrile (PCN) induced P-gp expression in rainbow trout hepatocytes (Wassmur et al., 2010), inhibited P-gp expression in zebrafish liver (Jackson and Kennedy, 2017), and had no effect on P-gp expression in killifish hepatoma cells (Wassmur et al., 2013). Likewise, the bile salt 5α-cyprinol 27-sulfate induced P-gp expression in zebrafish hepatocytes, but similar induction effects were not observed in sea lampreys (*Petromyzon marinus*) (Reschly et al., 2007). Within a single species, the effects of expression modulators can vary by sex and by tissue. For example, the antimicrobial agent triclosan increased liver P-gp expression in male swordtail fish (*Xiphophorus helleri*), but decreased expression in females (Liang et al., 2013). The fungicide carbendazim induced P-gp expression in killifish gill, but decreased expression in liver (Götte et al., 2020). Emamectin benzoate-treated rainbow trout had increased P-gp expression in liver, but decreased P-gp expression in intestine (Cárcamo et al., 2011). These differences may arise from variations in exposure profiles between tissues, sexes, and species, which would require different chemical defense strategies.

Modulation of P-gp expression is of great importance in both clinical and environmental contexts. In clinical settings, changes in P-gp expression can alter the bioavailability and pharmacokinetics of therapeutic drugs. In aquatic toxicology, P-gp expression confers resistance to environmental toxicants, thus alterations can have major impacts on fitness and survival in contaminated environments. Further research into inducers and inhibitors of P-gp expression in fish will therefore provide valuable information relating to veterinary medicine, aquaculture, and environmental risk assessment.

1.9. Inhibitors of P-glycoprotein transport activity

P-gp activity inhibitors attenuate the efflux function of the transporter, thereby interfering with its role in actively exporting substrates out of cells. Inhibitors of P-gp transport activity are being recognized as an important class of environmental pollutants

that inhibit chemical defense enzymes and transporters (Kurth et al., 2015). They interfere with MXR mechanisms that contribute to chemical defense in contaminated environments (Bieczynski et al., 2021b; Ferreira et al., 2014). P-gp is the most studied MXR protein, but many transporters and enzymes contribute to the MXR phenotype (Kurelec and Pivčević, 1991, 1989; Luckenbach et al., 2014). The inhibition of MXR proteins allow substrates to exert harmful effects at lower concentrations than they would in the absence of these inhibitors (Faria et al., 2016; Kurth et al., 2015; Müller et al., 1998; Smital et al., 2004; Wu et al., 2015). This increases the organism's sensitivity to environmental toxicants; thus, these inhibitors are referred to as chemosensitizers. Chemosensitizers in complex mixtures increase the toxicity of the mixture (Kurth et al., 2015). Inhibitors of MXR transporters including P-gp, MRPs, BCRP, and MATEs interfere with transport activity, which leads to increased intracellular accumulation of their substrates (Ferreira et al., 2014; Kurth et al., 2015). In clinical contexts, compounds that act as chemosensitizers are major contributors to drug-drug and drug-food interactions, since they can alter the pharmacokinetics of co-administered pharmaceuticals (Varma et al., 2015).

Chemosensitizers in fish are usually studied in the context of complex mixtures of environmental pollutants: most identified inhibitors of fish P-gp are anthropogenic in origin (e.g., pesticides, industrial chemicals, pharmaceuticals, personal care products). Only one endogenous P-gp inhibitor is known in fish, the bile acid taurochenodeoxycholate (Zaja et al., 2011). More P-gp inhibitors have been identified in fish than any other type of interacting chemical (inhibitors, substrates, expression modulators). Chemosensitizers are relatively easy to detect using *in vitro* methods, especially dye accumulation and ATP consumption assays (Kurth et al., 2015; Zaja et al., 2011). However, experiments of this nature, especially if they use a high-throughput design, usually provide a simple confirmation of effect rather than a more thorough concentration-response relationship (Kurth et al., 2015). The known inhibitors of fish P-gp activity are shown in Table 1.6.

P-gp inhibitors act by one of two general mechanisms: competitive and non-competitive inhibition. Competitive inhibitors are P-gp substrates that inhibit the transport of other substrates by occupying P-gp transport capacity (Kurth et al., 2015). The most potent competitive P-gp inhibitors have high affinity in the P-gp drug binding pocket, and high membrane permeability (Doppenschmitt et al., 1999; von Richter et al., 2009).

Table 1.6 Inhibitors of fish P-glycoprotein activity

Inhibitor	Species	References
Fluorescent Dye		
Hoechst 3334	*Poeciliopsis lucida*	(Zaja et al., 2011)
Cyanotoxin		
Microcystin-LR	*Oncorhynchus mykiss*	(Bieczynski et al., 2014)
Metal Compounds		
Arsenic trioxide (As_2O_3)	*Poeciliopsis lucida*	(Della Torre et al., 2012)
Cadmium chloride ($CdCl_2$)	*Poeciliopsis lucida*	(Della Torre et al., 2012)
Mercury (II) chloride ($HgCl_2$)	*Poeciliopsis lucida*	(Della Torre et al., 2012)
Orthovanadate (VO_4)	*Poeciliopsis lucida*	(Zaja et al., 2011)
Potassium dichromate ($K_2Cr_2O_7$)	*Poeciliopsis lucida*	(Della Torre et al., 2012)
Hormone		
Octreotide (synthetic somatostatin)	*Fundulus heteroclitus*	(Gutmann et al., 2000)
Bile Acid		
Taurochenodeoxycholate	*Poeciliopsis lucida*	(Zaja et al., 2011)
Fragrance Ingredients		
Galaxolide	*Danio rerio*	(Fischer et al., 2013)
α-hexylcinnamaldehyde	*Danio rerio*	(Cunha et al., 2017)
Isoeugenol	*Danio rerio*	(Cunha et al., 2017)
Musk ketone	*Danio rerio*	(Cunha et al., 2017)
Musk xylene	*Danio rerio*	(Cunha et al., 2017)
Nerol	*Danio rerio*	(Cunha et al., 2017)
Tonalide	*Danio rerio*	(Bieczynski et al., 2021a; Fischer et al., 2013)
Polycyclic Aromatic Hydrocarbon		
Phenanthrene	*Danio rerio*	(Fischer et al., 2013)

Inhibitor	Species	References
Industrial Chemicals		
Oil Sands Process-Affected Water (fresh)	*Oryzias latipes*	(Alharbi et al., 2016)
Polychlorinated Biphenyls (PCBs)	*Thunnus albacares*	(Nicklisch et al., 2021)
Surfactants		
Alcohol polyethoxylates	*Poeciliopsis lucida*	(Zaja et al., 2013)
C-12 linear alkylbenzene sulfonate	*Ictalurus punctatus*	(Tan et al., 2010)
Nonylphenol diethoxylate	*Oncorhynchus mykiss*	(Sturm et al., 2001a)
Perfluorooctane sulfonate (PFOS)	*Danio rerio*	(Keiter et al., 2016)
Polypropylene glycols	*Poeciliopsis lucida*	(Zaja et al., 2013)
Flame Retardants		
Polybrominated diphenyl ethers (BDEs)	*Thunnus albacares*	(Nicklisch et al., 2021)
Pesticides		
Azinphos-methyl	*Danio rerio*	(Bieczynski et al., 2021a)
Diazinon	*Poeciliopsis lucida*	(Zaja et al., 2011)
Dichlorodiphenyldichloroethane (DDD)	*Thunnus albacares*	(Nicklisch et al., 2021)
Dichlorodiphenyldichloroethylene (DDE)	*Thunnus albacares, Poeciliopsis lucida*	(Nicklisch et al., 2021; Zaja et al., 2011)
Dichlorodiphenyltrichloroethane (DDT)	*Thunnus albacares*	(Nicklisch et al., 2021)
Dieldrin	*Thunnus albacares*	(Nicklisch et al., 2021)
2,4-dinitrophenol	*Fundulus heteroclitus*	(Schramm et al., 1995)
Emamectin benzoate	*Oncorhynchus mykiss*	(Kennedy et al., 2014)
Endosulfan	*Poeciliopsis lucida*	(Zaja et al., 2011)
Endrin	*Thunnus albacares*	(Nicklisch et al., 2021)
Metazachlor	*Danio rerio*	(Bieczynski et al., 2021a)
Mirex	*Thunnus albacares*	(Nicklisch et al., 2021)
Phosalone	*Poeciliopsis lucida*	(Zaja et al., 2011)
Prochloraz	*Oncorhynchus mykiss*	(Sturm et al., 2001a)
Terbuthylazine	*Danio rerio*	(Bieczynski et al., 2021a)

Inhibitor	Species	References
Hydrophobic Peptide		
Reversin 205	*Oncorhynchus mykiss, Poeciliopsis lucida*	(Fischer et al., 2011; Kropf et al., 2020; Zaja et al., 2011, 2007)
Anticoagulant		
Dipyridamole	*Danio rerio*	(Bieczynski et al., 2021a)
Antimalarial		
Quinine	*Fundulus heteroclitus*	(Schramm et al., 1995)
Antiparasitic		
Ivermectin	*Danio rerio, Oncorhynchus mykiss, Fundulus heteroclitus*	(Bieczynski et al., 2021a; Fricker et al., 1999; Kennedy et al., 2014)
Leukotriene Antagonist		
MK571	*Danio rerio, Poeciliopsis lucida*	(Bieczynski et al., 2021a; Fischer et al., 2013; Zaja et al., 2011)
Antihistamine		
Cimetidine	*Oncorhynchus mykiss*	(Stott et al., 2015)
Antibiotics		
Erythromycin	*Danio rerio, Poeciliopsis lucida*	(Bolten et al., 2022; Zaja et al., 2011)
Troleandomycin	*Poeciliopsis lucida*	(Wassmur et al., 2013)
Analgesic Pharmaceutical		
Diclofenac	*Danio rerio, Poeciliopsis lucida*	(Cunha et al., 2017; Wassmur et al., 2013)
Antifungal Pharmaceutical		
Clotrimazole	*Oncorhynchus mykiss*	(Kropf et al., 2016)
Anti-Inflammatories		
Indomethacin	*Poeciliopsis lucida*	(Zaja et al., 2007)
Sulfasalazine	*Poeciliopsis lucida*	(Zaja et al., 2011)
Antihypertensives		
Furosemide	*Poeciliopsis lucida*	(Caminada et al., 2008)
Reserpine	*Poeciliopsis lucida*	(Zaja et al., 2011)

Inhibitor	Species	References
Antihyperuricemic		
Probenecid	*Poeciliopsis lucida*	(Zaja et al., 2007)
Phosphodiesterase Inhibitor		
Sildenafil	*Poeciliopsis lucida*	(Caminada et al., 2008; Zaja et al., 2011)
Antiretrovirals		
Ritonavir	*Fundulus heteroclitus*	(Gutmann et al., 1999)
Saquinavir	*Fundulus heteroclitus*	(Gutmann et al., 1999)
Lipid-Lowering Pharmaceuticals		
Atorvastatin	*Poeciliopsis lucida*	(Caminada et al., 2008; Zaja et al., 2011)
Gemfibrozil	*Poeciliopsis lucida*	(Caminada et al., 2008)
Pravastatin	*Poeciliopsis lucida*	(Caminada et al., 2008)
Simvastatin	*Poeciliopsis lucida*	(Caminada et al., 2008)
Psychoactive Pharmaceuticals		
Carbamazepine	*Danio rerio*	(Bieczynski et al., 2021a)
Fluoxetine	*Danio rerio*	(Cunha et al., 2017)
Sertraline	*Danio rerio*	(Cunha et al., 2017)
Trifluoperazine	*Poeciliopsis lucida*	(Zaja et al., 2011)
Immunosuppressants		
Cyclosporin A	*Danio rerio, Oncorhynchus mykiss, Thunnus albacares, Poeciliopsis lucida, Cyprinus carpio, Fundulus heteroclitus, Pleuronectes americanus*	(Bieczynski et al., 2021a; Caminada et al., 2008; Della Torre et al., 2012; Fischer et al., 2013, 2011; Gordon et al., 2019; Gutmann et al., 2000; Johnston and Kennedy, 2023; Kropf et al., 2020; Miller, 1995; Miller et al., 1997; Nicklisch et al., 2021; Smital and Sauerborn, 2002; Stott et al., 2015; Sturm et al., 2001a; Sussman-Turner and Renfro, 1995; Zaja et al., 2013, 2011, 2008a, 2007)
Cyclosporin G	*Fundulus heteroclitus*	(Schramm et al., 1995)
Rapamycin	*Fundulus heteroclitus*	(Miller et al., 1997)
Tacrolimus (FK506)	*Fundulus heteroclitus*	(Miller et al., 1997)

Inhibitor	Species	References
Antiarrhythmics		
Diltiazem	*Poeciliopsis lucida*	(Zaja et al., 2011)
Nicardipine	*Poeciliopsis lucida*	(Zaja et al., 2011)
Prazosin	*Poeciliopsis lucida*	(Zaja et al., 2011)
Propranolol	*Poeciliopsis lucida*	(Zaja et al., 2011)
Quinidine	*Oncorhynchus mykiss, Poeciliopsis lucida*	(Johnston and Kennedy, 2023; Stott et al., 2015; Zaja et al., 2011)
Verapamil	*Danio rerio, Oncorhynchus mykiss, Thunnus albacares, Hatcheria macraei, Salmo trutta, Oncorhynchus tshawytscha, Poeciliopsis lucida, Scophthalmus maximus, Cyprinus carpio, Ictalurus punctatus, Fundulus heteroclitus, Pleuronectes americanus*	(Assef et al., 2019; Bieczynski et al., 2021a; Bolten et al., 2022; Cunha et al., 2017; Doi et al., 2001; Fischer et al., 2013; Fricker et al., 1999; Gutmann et al., 2000; Johnston and Kennedy, 2023; Miller et al., 1997, 1997; Nicklisch et al., 2021; Robey et al., 2021; Schramm et al., 1995; Smital and Sauerborn, 2002; Sturm et al., 2001a, 2001b; Sussman-Turner and Renfro, 1995; Renaud Tutundjian et al., 2002; Zaja et al., 2011, 2008b, 2007)
Anti-Cancer Pharmaceuticals		
Doxorubicin	*Oncorhynchus mykiss*	(Sturm et al., 2001b)
Elacridar	*Danio rerio*	(Robey et al., 2021)
Fluorouracil	*Poeciliopsis lucida*	(Zaja et al., 2011)
Mitoxantrone	*Poeciliopsis lucida*	(Zaja et al., 2011)
Tamoxifen	*Poeciliopsis lucida*	(Zaja et al., 2011)
Tariquidar (XR9576)	*Danio rerio, Oncorhynchus mykiss*	(Hildebrand et al., 2009; Robey et al., 2021)
Valspodar (PSC833)	*Oncorhynchus mykiss, Thunnus albacares, Danio rerio, Poeciliopsis lucida, Fundulus heteroclitus, Squalus acanthias*	(Fischer et al., 2013, 2011; Fricker et al., 1999; Gordon et al., 2019; Gutmann et al., 2000; Johnston and Kennedy, 2023; Miller et al., 2002a, 2002a, 1997; Nicklisch et al., 2021; Robey et al., 2021; Zaja et al., 2011, 2008a)

Inhibitor	Species	References
Vinblastine	*Danio rerio, Oncorhynchus mykiss, Fundulus heteroclitus, Pleuronectes americanus*	(Fischer et al., 2013; Gordon et al., 2019; Schramm et al., 1995; Sturm et al., 2001b, 2001a; Sussman-Turner and Renfro, 1995)

Determined by methods including inhibition of transport of a known P-gp substrate, and inhibition of stimulated ATPase activity.

These properties allow for a futile and energetically costly cycle of outward active transport and inward diffusion (Luckenbach et al., 2014). Non-competitive inhibitors impede some aspect of the P-gp transport mechanism. Vanadate, for example, inhibits P-gp ATPase activity, while tariquidar blocks the movement of transmembrane helices necessary for substrate translocation (Nosol et al., 2020). Non-competitive inhibitors reduce ATP consumption by preventing P-gp from actively transporting substrates (Luckenbach et al., 2014; Zaja et al., 2011). Many studies do not determine the inhibition mechanism of chemosensitizers; however, if a substance has been identified as both a substrate and an inhibitor, it is likely a competitive inhibitor.

In vivo P-gp inhibition can exacerbate the behavioural effects of substrate exposure, as observed with the neurotoxic P-gp substrate ivermectin (IVM). IVM exposure causes behavioural deficits in fish, including altered swimming performance, poor motor coordination, and lethargy (Azevedo and Kennedy, 2022; Bard and Gadbois, 2007; Kennedy et al., 2014). During co-exposure to the P-gp inhibitor cyclosporin A, all of these neurotoxic IVM effects were more severe in killifish, rainbow trout, and zebrafish (Azevedo and Kennedy, 2022; Bard and Gadbois, 2007; Kennedy et al., 2014).

Substantial knowledge gaps remain with respect to the magnitude of chemosensitizer effects as environmental pollutants (Chu et al., 2013; Kurth et al., 2015). More research is also needed to understand the contribution of the inhibition of other MXR transporters in chemical defense, since nearly all studies of chemosensitizers in aquatic animals have focused on P-gp (Kurth et al., 2015). A greater diversity of chemical compounds, and combinations of compounds, should be tested for chemosensitizing effects, especially industrial and agricultural chemicals in current use. More detailed information regarding effect concentration thresholds is needed as well. Studies of chemosensitizer exposure in aquatic media should report measured concentrations wherever possible, rather than nominal concentrations. Many reported IC50 concentrations for chemosensitizers exceed the water solubility of those chemicals, thus the observed chemosensitizing effects will have occurred at much lower aqueous concentrations than those reported (Kurth et al., 2015). Expanding the available information about chemosensitizer effects in these areas will greatly improve our ability to accurately account for chemosensitizers in environmental risk assessments.

1.10. Energetic costs of P-glycoprotein activity

P-gp transport activity is powered by ATP hydrolysis, and may be energetically costly in terms of overall energy budgets. P-gp activity increases cellular energy consumption when fish hepatocytes are challenged *in vitro* with P-gp substrates (Bains and Kennedy, 2005; Hildebrand et al., 2009). During *in vivo* exposure, P-gp induction alone does not increase whole-body respiration rates in rainbow trout (Kennedy, 2021). Increased P-gp activity during substrate exposure likewise does not affect whole-animal energy consumption (Kennedy, 2021). Only when P-gp expression is induced and the organism is simultaneously challenged with substrate exposure does P-gp activity increase the *in vivo* respiration rate of rainbow trout (Kennedy, 2021).

The protective function of P-gp has a high priority in fish energy budgets. P-gp activity is maintained during fasting and starvation in zebrafish and rainbow trout (Azevedo and Kennedy, 2022; Gourley and Kennedy, 2009). Similarly, four weeks of starvation induced P-gp expression in rainbow trout intestinal epithelia (Baumgarner et al., 2013). Thus, the cellular-level activity of P-gp is integrated into protective effects at the organismal level, and this protection is prioritized even during periods of limited resources.

1.11. Conclusions

In fish, P-gp protects sensitive tissues from its potentially harmful substrates, and helps to remove those substrates from the body. Although P-gp has been studied less extensively in fish than in mammals, it seems to share similar functions in both animal lineages. P-gp expression confers resistance to toxic substrates, and this resistance can be diminished by chemosensitizing P-gp inhibitors. Fish P-gp has been studied primarily in the context of resistance to anthropogenic contamination, so its endogenous functions are only beginning to be explored.

P-gp likely has broad regulatory significance in fish, potentially transporting hormones, growth factors, and other signaling molecules, thereby influencing processes including cell maturation, reproductive development, and energy metabolism. P-gp transport helps to maintain homeostasis at all levels of biological organization, from molecules to the whole organism, and contributes to the health of populations and

ecosystems (Nigam, 2015; Nigam et al., 2020). A deeper understanding of the endogenous physiological functions of P-gp will allow for more accurate predictions of off-target effects of drugs, and the identification of sensitive endpoints in ecotoxicology research.

Environmental exposure to P-gp inhibitors and toxic P-gp substrates presents serious risks to the health of aquatic ecosystems. P-gp confers multixenobiotic resistance to fish and invertebrates living in contaminated environments, but chemosensitizing compounds inhibit this resistance. While substantial research efforts have identified a wide range of environmental contaminants that are substrates, inhibitors, and expression modulators of fish P-gp, much remains to be discovered with respect to their effect concentrations, physiological effects, and interactions with other compounds. Continued research into the endogenous functions and xenobiotic protective effects of P-gp in fish will provide valuable information for applications including veterinary medicine, aquaculture, and environmental monitoring.

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Chapter 2. Potency and mechanism of P-glycoprotein chemosensitizers in rainbow trout (*Oncorhynchus mykiss*) hepatocytes

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Christina U. Johnston and Christopher J. Kennedy†

Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia, Canada

Abstract

Elevated levels of the membrane efflux transporter P-glycoprotein (P-gp, [ABCB1, MDR1]) in aquatic organisms can result in the multixenobiotic resistant (MXR) phenotype, enabling survival in contaminated environments. Little information exists on P-gp inhibition by chemosensitizers, compounds which lower toxicity thresholds for harmful P-gp substrates in complex mixtures. The effects of four known mammalian chemosensitizers (cyclosporin A [CsA], quinidine, valspodar [PSC833], and verapamil) on the P-gp-mediated transport of rhodamine 123 (R123) and cortisol in primary cultures of rainbow trout (*Oncorhynchus mykiss*) hepatocytes were examined. Competitive accumulation assays using 25 μM R123 or cortisol and varying concentrations of chemosensitizers (0-500 μM) were used. CsA, quinidine, and verapamil inhibited R123 export (IC50 values ± SE: 132±60, 83.3±27.2, and 43.2±13.6 μM, respectively). CsA and valspodar inhibited cortisol export (IC50 values: 294±106 and 92.2±34.9 μM, respectively). In an ATP depletion assay, hepatocytes incubated with all four chemosensitizers resulted in lower free ATP concentrations, suggesting that they act *via* competitive inhibition. Chemosensitizers that inhibit MXR transporters are an important class of environmental pollutant, and these results show that rainbow trout transporters are inhibited by similar chemosensitizers (and mostly at similar concentrations) as seen in mammals and other fish species.

Keywords: P-glycoprotein, chemosensitizer, rainbow trout, hepatocytes

2.1. Introduction

The multixenobiotic resistance (MXR) phenotype in aquatic animals allows them to survive and reproduce in polluted environments (Bieczynski et al., 2021b; Ferreira et al., 2014) and manifests itself through an increased expression of xenobiotic defense proteins, in particular the membrane efflux transporter permeability-glycoprotein (P-glycoprotein [P-gp]) (Kurelec and Pivčević, 1991, 1989; Luckenbach et al., 2014). P-gp is an ATP-dependent exporter with a broad range of moderately hydrophobic substrates (Silva et al., 2015), and in vertebrates is expressed in absorptive and excretory tissues to avert systemic accumulation, and in barrier tissues to prevent their entry into potentially sensitive cells (Leslie et al., 2005; Lončar et al., 2010; Love et al., 2021).

P-gp inhibitors have been termed chemosensitizers (Smital et al., 2004), and were categorized as a class of pollutants soon after initial investigations into the underlying mechanisms behind MXR (Kurelec, 1995; Kurelec et al., 1995; Smital and Kurelec, 1998). They are particularly relevant to complex mixture exposures, and can result in lower toxicity thresholds for harmful P-gp substrates in their presence (Faria et al., 2016; Kurth et al., 2015; Müller et al., 1998; Smital et al., 2004; Wu et al., 2015). Chemosensitizers can inhibit P-gp transport in either a competitive or non-competitive manner (Kurth et al., 2015). Competitive inhibitors are substrates that alter P-gp transport capacity by reducing the transport rates of other competing substrates (Kurth et al., 2015). Potent competitive inhibitors generally have high membrane permeability, and are high-affinity substrates of P-gp (Doppenschmitt et al., 1999; von Richter et al., 2009). Competitive P-gp inhibition results in increased ATP consumption due to the cycle of inward diffusion and outward active transport (Luckenbach et al., 2014). Conversely, non-competitive P-gp inhibitors prevent P-gp from transporting substrates through several known mechanisms including the inhibition of ATP hydrolysis (e.g. vanadate) and blocking the movement of transmembrane helices during substrate transport (e.g. tariquidar) (Nosol et al., 2020). ATP consumption by P-gp decreases during non-competitive inhibition (Luckenbach et al., 2014; Zaja et al., 2011).

The MXR phenotype in aquatic animals occurs by the same mechanism as the multidrug resistance (MDR) phenotype in mammalian tumors (Luckenbach et al., 2014) and so chemosensitizers, especially those that are pharmaceuticals, have been well-studied for their interaction with mammalian P-gp (Dong et al., 2020). In fish,

chemosensitizer research is more recent and less extensive. The effects of chemosensitizers on P-gp substrate accumulation in teleost cells has been studied in zebrafish embryos (Bieczynski et al., 2021a; Fischer et al., 2013; Keiter et al., 2016) and killifish hepatoma cells (Zaja et al., 2013, 2011). Chemosensitizers identified in these studies include perfluorooctane sulfonate, polypropylene glycols, bile salts, cyclosporin A (CsA), ivermectin, verapamil, and valspodar (PSC833). In rainbow trout hepatocytes, several known chemosensitizers including verapamil, CsA, doxorubicin, and tariquidar, have been shown to inhibit the export of P-gp substrates (Bains and Kennedy, 2005; Hildebrand et al., 2009; Sturm et al., 2001); however these studies involved only a limited investigation of concentration-dependence. Concentration-dependent inhibition of P-gp transport was demonstrated in trout hepatocytes by verapamil, CsA, and reversin 205 in a study examining the role of several ABC transporters in these cells (Zaja et al., 2008b).

Little research has focused on identifying chemosensitizers and characterizing their *in vitro* interactions with active transporters in aquatic animals, and even less attention has been directed toward whole organism effects (Chu et al., 2013; Nigam, 2015). The emerging importance of P-gp inhibitors in ecotoxicology has been recognized, but substantial knowledge gaps limit the understanding of the role of chemosensitization in contaminant exposure (Kurth et al., 2015). Thus, investigations to examine the inhibiting effects of a broader range of chemicals, as well as the concentration-dependence of those effects, is necessary (Kurth et al., 2015). The primary goal of this study was to explore and expand the range of known inhibitors of teleost P-gp, and to investigate the concentration-dependence and mechanism of their effects on substrate efflux. Using primary cultures of rainbow trout hepatocytes, 4 known mammalian chemosensitizers (cyclosporin A [CsA], quinidine, valspodar [PSC833], and verapamil) were examined for their ability to inhibit the export of the exogenous P-gp substrate rhodamine 123 (R123), and the endogenous substrate cortisol. The underlying mechanism of inhibition of the four chemosensitizers was also examined.

2.2. Methods

2.2.1. Fish

Adult mixed-sex rainbow trout (*Oncorhynchus mykiss*) (mass range 500-1200 g) were purchased from LSL Living Seafoods (Langley, BC). Fish were acclimated to holding conditions for a minimum of 14 d before an experiment. Fish were housed in 2500 L fiberglass tanks supplied with continuously flowing dechlorinated municipal tap water (pH 6.6-7.4, 7.5-16.1°C, hardness 6.1 g/mL $CaCO_3$, O_2 saturation 90-95%) under a 12h light:12h dark cycle. Fish were fed *ad libitum* with Pacific floating complete feed for salmonids (EWOS Canada Ltd. Surrey, BC).

2.2.2. Chemicals

Sodium bicarbonate, DL-lactic acid (90%), hydrocortisone (cortisol), dimethyl sulphoxide, trypan blue, 1-butanol, rhodamine 123 (≥85%), cyclosporin A (≥95%), quinidine (≥98%), verapamil hydrochloride (≥99%), valspodar (≥98%), collagenase (Type IV from *Clostridium histolyticum*), bovine serum albumin (fatty acid free, ≥98%), and Hanks' Balanced Salts (powder, modified without calcium chloride, magnesium sulfate, phenol red and sodium bicarbonate) were purchased from Sigma-Aldrich (Oakville, ON). D-glucose was obtained from BDH Inc. (Toronto, ON). MS-222 (tricaine-S) was obtained from Syndel (Ferndale, WA). Calcium carbonate was purchased from Fisher Scientific Company (Fair Lawn, NJ).

2.2.3. Hepatocyte primary cell culture

Fish were anaesthetized using 0.2 g/L buffered tricaine methanesulfonate. Hepatocytes were cultured according to Moon et al. (1985) and Bains and Kennedy (2005). Briefly, the liver was perfused *via* the portal vein with sterile, oxygenated Ca^{2+}-free Hanks balanced salt solution (HSS) to clear blood from the organ. The liver was then perfused with HSS containing 0.3 g/L collagenase until visible signs of disintegration appeared. The liver was then removed from the fish and minced with a razor blade. Hepatocytes were disaggregated by rinsing liver tissue with HSS through two nylon mesh filters (253 μm and 73 μm). Hepatocyte suspensions were concentrated by centrifugation (50 *xg*, 3 min) at 4°C, then re-suspended in HSS containing glucose (3

mM), sodium bicarbonate (6 mM), lactic acid (1 mM), calcium chloride (1.5 mM) and bovine serum albumin (10 g/L) (hereafter called HSSB) and kept on ice until use. Cells were not pooled between fish. Cell viabilities were quantified by trypan blue exclusion (Braunbeck and Storch, 1992) during incubations.

2.2.4. Chemosensitizer inhibition assay

An inhibition assay protocol adapted from Pivčević and Žaja (2006) and Uchea et al. (2015) was used: the intracellular accumulation of a P-gp substrate (R123 or cortisol) was measured alone and in the presence of varying concentrations of a model chemosensitizer (cyclosporin A, quinidine, valspodar, or verapamil). 125 μL aliquots of hepatocyte suspension in HSSB were added to plastic culture tubes (final cell concentration 25 mg/mL, approx. $2.4x10^5$ cells/mL). Samples were pre-incubated in the dark with gentle agitation (60 min, 12°C) to acclimate cells to experimental conditions. A stock solution of chemosensitizer in DMSO was diluted in HSSB to produce experimental chemosensitizer concentrations in appropriate cell media (final concentrations 25, 50, 125, 250, 500 μM). Chemosensitizer in HSSB was added to hepatocyte suspensions and cells were incubated for 15 min with chemosensitizer only. To begin the accumulation phase of the assay, substrate in HSSB was added (final concentration 25 μM), and samples were incubated for a further 60 min with both chemosensitizer and substrate. To end the assay, samples were centrifuged (50 *xg*, 3 min). Supernatant containing extracellular chemosensitizer and substrate was discarded, and pellets containing cells and intracellular substrate were retained for analysis. Pellets were re-suspended in 250 μL HSSB and stored frozen at -20°C until chemical analysis.

2.2.5. Chemical analysis

Cells containing R123 were thawed at room temperature, then sonicated on ice (3x 2s pulses) to lyse the cells. Samples were then extracted with 1-butanol (2x volume) in the dark with vigorous shaking (200 rpm) on an orbital shaker at 20°C for 2 h (Sturm et al., 2001). 100 μL of the butanol layer of each sample was then removed and analyzed in triplicate (ex = 517 nm, em = 532 nm) (Sturm et al., 2001) in a 96-well plate using a SpectraMax M2e Microplate Reader (Molecular Devices, San Jose, CA, USA) (Sturm et al., 2001). Cortisol samples were thawed at room temperature and sonicated

on ice as described above. Cortisol concentrations were quantified using an enzyme-linked immunosorbent assay (ELISA) kit (Catalogue # k003-H5W, Arbor Assays, Ann Arbor, MI, USA) according to manufacturer's protocols.

2.2.6. ATP consumption assay

The mechanism of P-gp inhibition by the four model chemosensitizers was investigated by measuring free ATP in isolated hepatocytes using a luciferase assay (Matsunaga et al., 2006). Cortisol was used as the substrate in ATP consumption assays because R123 fluorescence could interact with luciferase luminescence. ATP consumption was measured in the presence of the chemosensitizer concentration that caused maximum cortisol accumulation in the inhibition assay: 125 μM for verapamil, 250 μM for quinidine and valspodar, and 500 μM for CsA. Depletion of free ATP indicates an increase in P-gp ATPase activity (Matsunaga et al., 2006), which would be observed during competitive inhibition, but not during non-competitive inhibition (Luckenbach et al., 2014). Rainbow trout hepatocytes were isolated as described above, and 125 μL aliquots of hepatocyte suspension in HSSB were added to plastic culture tubes (final concentration 25 mg/mL, approx. $2.4x10^5$ cells/mL). Cell samples were pre-incubated in the dark (60 min, 12°C) with gentle agitation. Experimental media was prepared with vehicle (DMSO), cortisol (25 μM), chemosensitizer, or chemosensitizer + cortisol. The ATP consumption assay was started by adding experimental media to cell cultures, and cell samples were incubated at 12°C for 60 min in the dark with gentle agitation. To end the assay, samples were centrifuged (50 *xg*, 3 min), and the supernatant was discarded. Pellets were retained and stored frozen at -20°C until ATP quantification. Free ATP was quantified using a firefly luciferase luminescence ATP detection assay kit (Catalogue #700410, Cayman Chemical, Ann Arbor, MI, USA) according to manufacturer's instructions.

2.2.7. Calculations and statistics

Statistical analyses were conducted using JMP (Version 15.0.0, SAS Software, Cary, NC, USA) unless otherwise noted. Cell viabilities, free ATP concentrations, and the effect of chemosensitizer concentration on intracellular substrate accumulation were analyzed using a repeated-measures ANOVA followed by Dunnett's test. To determine

the half maximum inhibitory concentrations (IC50 values), intracellular substrate concentrations expressed as percent of maximum effect, and concentration-response curves were produced in Prism 8 (Version 8.4.3, Graphpad Software LLC., San Diego, CA, USA). In cases where the chemosensitizer concentration was found to affect substrate accumulation by the ANOVA described above, a hyperbolic curve was fitted in Prism 8 using the hill equation as a model. Data are reported as mean ± SE. Differences were considered significant at p=0.05.

2.3. Results

2.3.1. Cell viability and cytotoxicity

There were no significant differences in cell viability between control cells and any of the R123 + 500 µM chemosensitizer combinations tested at 75 min (range 93.9±1.3% to 95.6±1.2% viable). There were no significant differences in cell viability between control cells and cortisol + 500 µM CsA, quinidine, or valspodar (range 94.0±1.8% to 97.6±0.9% viable). Cell viability for cortisol + 500 µM verapamil samples was significantly lower than control cells from the same fish (ANOVA, $F_{4,24}$=9.8107, p=0.0003). Cortisol + 500 µM verapamil cell cultures had 88.9±2.1% viability, and were still within the acceptable >80% range (Sturm et al., 2001). In the free ATP assay, there were no significant differences in cell viability between control cells and any of the cortisol + chemosensitizer combinations tested (range 93.0±1.2% to 96.5±1.2%).

2.3.2. Inhibition of R123 efflux

To assess the inhibitory effects of the four chemosensitizers on the P-gp-mediated transport of a xenobiotic substrate, intracellular R123 accumulation in isolated hepatocytes was quantified after a 60 min co-incubation of R123 (25 µM) and a chemosensitizer (0, 25, 50, 125, 250, 500 µM). Increased intracellular accumulation of R123 indicated P-gp efflux inhibition. Significant concentration-dependent increases in R123 accumulation were seen for CsA, quinidine and verapamil (Fig. 2.1A, B, D). CsA caused a significant and maximal 1.5-fold increase in R123 accumulation at 250 µM CsA (ANOVA, $F_{5,29}$=2.9894, p=0.0357) (Fig. 2.1A). The maximal increase in R123 accumulation (2-fold) with quinidine occurred at 250 µM (ANOVA, $F_{5,29}$=6.5249, p=0.0009) (Fig. 2.1B) and the maximal increase in R123 accumulation for Verapamil

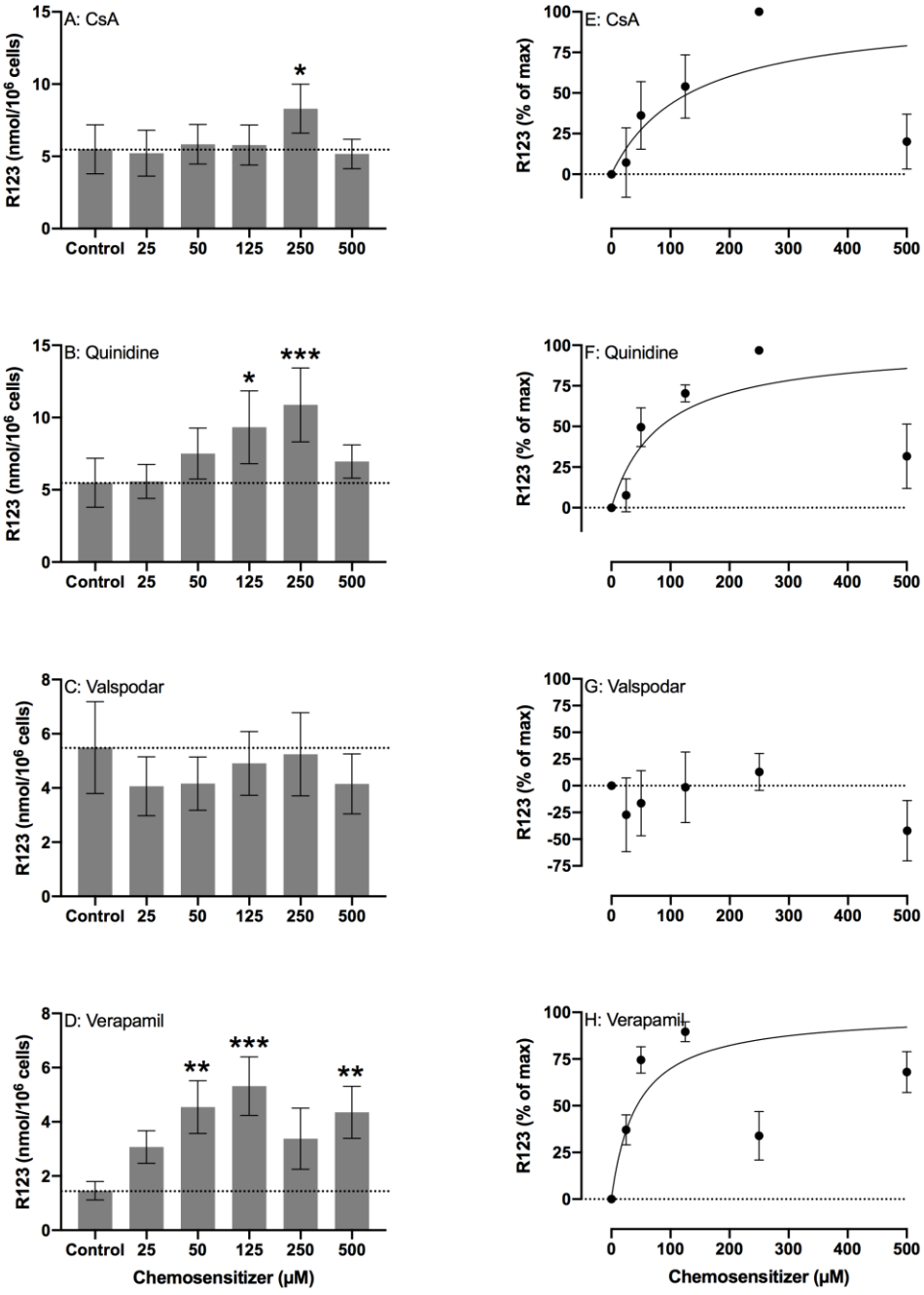

Figure 2.1 Intracellular rhodamine 123 (R123) accumulation incubated with four model chemosensitizers

Intracellular concentrations of the P-gp substrate rhodamine 123 in cultured rainbow trout hepatocytes after 15 min of incubation with chemosensitizer only, then 60 min with chemosensitizer + 25μM rhodamine 123. A – D: Intracellular R123 concentrations. E – H: Normalized intracellular R123 concentrations with fitted Hill Equation curves. Dotted lines show intracellular rhodamine 123 concentrations in control cells incubated in chemosensitizer-free media. Asterisks indicate significant differences compared to chemosensitizer-free control cells from the same fish. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Data are shown as mean ± SEM. N=6 for verapamil, N=5 for CYSA, quinidine, and valspodar.

(3.7-fold) occurred at 125 μM (ANOVA, $F_{5,35}$=6.8638, p=0.0004) (Fig. 2.1D). Valspodar co-incubation had no significant effect on R123 accumulation (Fig. 2.1C). Intracellular R123 concentrations normalized to the percent of maximum response are shown in Figs. 1E-H). For each chemosensitizer that significantly increased R123 accumulation (CsA, quinidine, and verapamil), data were fitted using the Hill equation in order to calculate IC50 values (Table 2.1).

Table 2.1 Inhibition parameters for four model chemosensitizers incubated with a xenobiotic (R123) or endogenous (cortisol) substrate

Substrate:	Rhodamine 123		Cortisol	
Chemosensitizer	**IC50 (μM)**	**Max Concentration (% of control)**	**IC50 (μM)**	**Max Concentration (% of control)**
Cyclosporine A	132±60	151±56	294±106	192±87
Quinidine	83.3±27.2	198±77	-	-
Valspodar	-	-	92.2±34.9	294±120
Verapamil	43.2±13.6	365±113	-	-

2.3.3. Inhibition of cortisol efflux

The P-gp-mediated transport of cortisol (25 μM) was examined by measuring intracellular accumulation after 60 min with a co-incubation with one of the chemosensitizers (at 0, 25, 50, 125, 250, 500 μM). CsA caused a marginally significant 1.9-fold increase in cortisol accumulation at 250 μM CsA (ANOVA, $F_{5,29}$=2.6711, p=0.0525) (Fig. 2.2A). Valspodar caused a significant concentration-dependent increase cortisol accumulation (ANOVA, $F_{5,29}$=4.3409, p=0.0077) (maximum 2.9-fold increase at 250 μM valspodar) (Fig. 2.2C). Quinidine and verapamil did not have a significant effect on cortisol accumulation (Fig. 2.2B, D). Intracellular cortisol concentrations normalized to the percent maximum response are shown in Fig. 2.2E-H). For For each chemosensitizer that significantly increased cortisol accumulation (CsA and valspodar) data were fitted using the Hill equation in order to calculate IC50 values (Table 2.1).

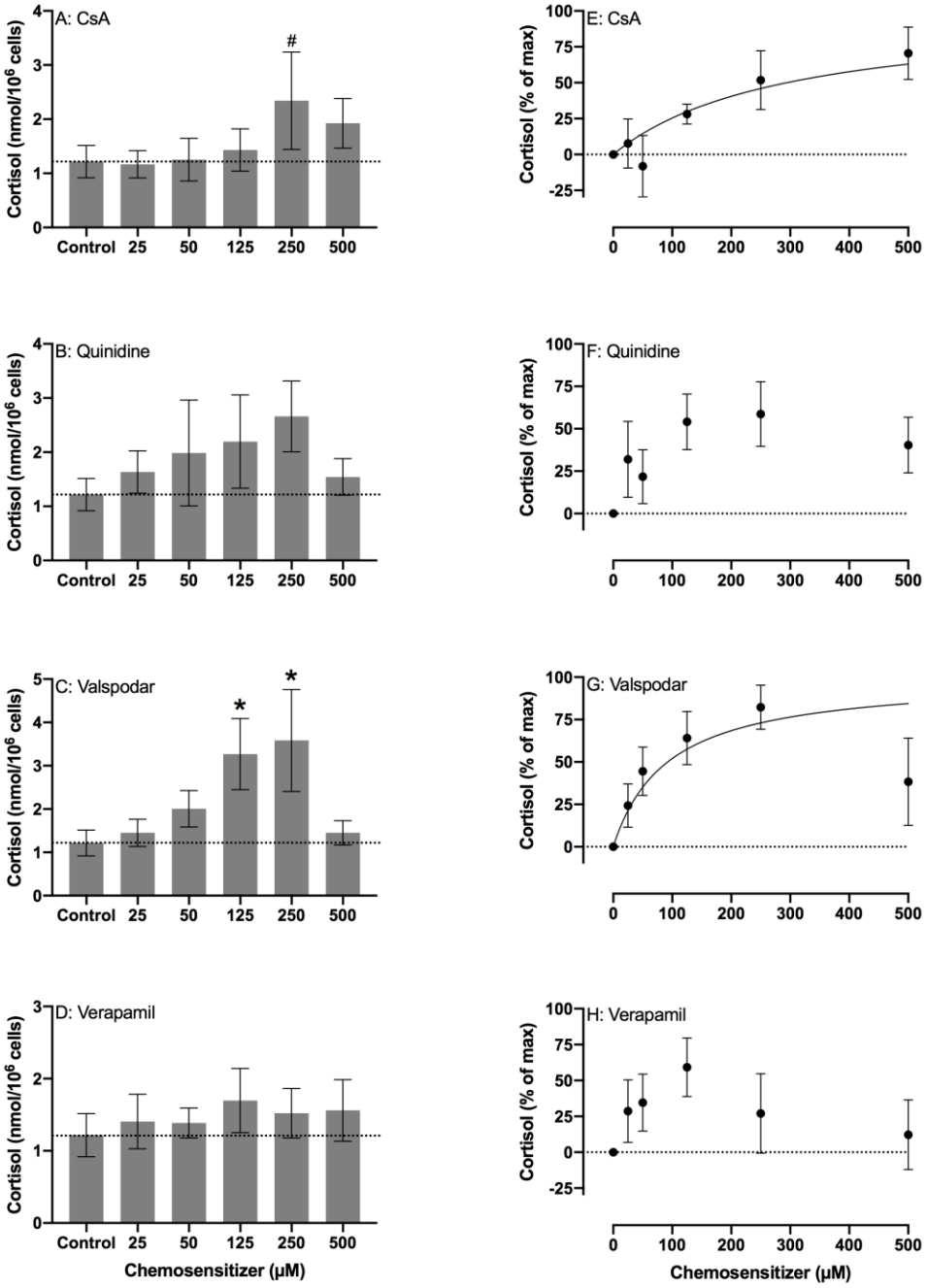

Figure 2.2 Intracellular cortisol accumulation incubated with four model chemosensitizers

Intracellular concentrations of cortisol in cultured rainbow trout hepatocytes after 15 min of incubation with chemosensitizer only, then 60 min with chemosensitizer + 25μM cortisol. A – D: Intracellular cortisol concentrations. E – H: Normalized cortisol concentrations with fitted Hill Equation curves. Dotted lines show intracellular cortisol concentrations in control cells in chemosensitizer-free media. Asterisks and pound signs/hashtags indicate significant differences with respect to chemosensitizer-free control cells from the same fish. # $p=0.0525$, * $p<0.05$. Data are shown as mean ± SEM. N=5 for each chemosensitizer.

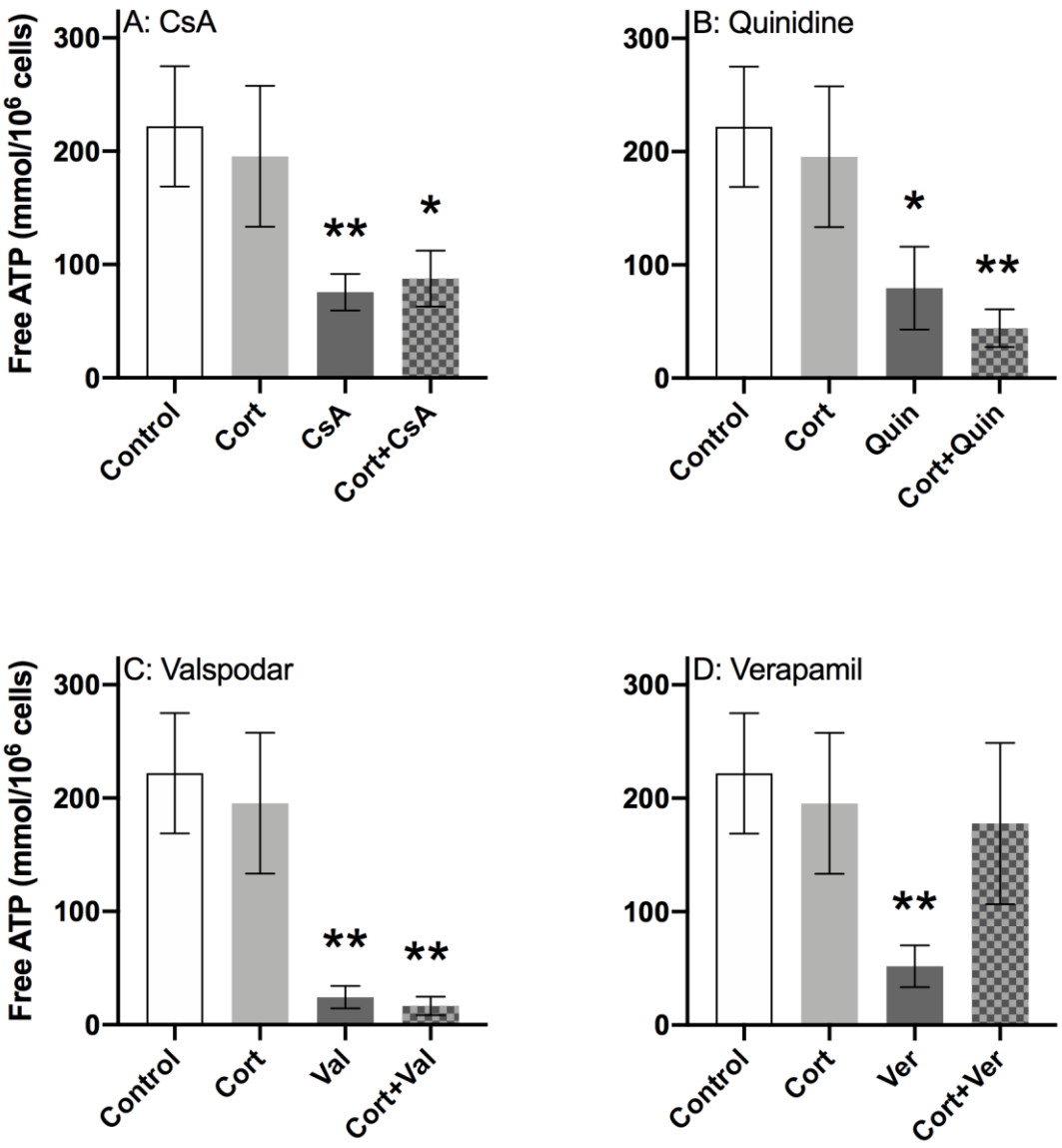

Figure 2.3 ATP consumption with four model chemosensitizers and cortisol
Free ATP in cultured rainbow trout hepatocytes quantified using firefly luciferase. Cells were incubated for 60 min with vehicle, cortisol (25 μM), chemosensitizer, or chemosensitizer + cortisol. Chemosensitizer concentrations were those that showed maximum transport inhibition (125 μM for verapamil, 250 μM for quinidine and valspodar, and 500 μM for CYSA). Asterisks indicate significant differences compared to cortisol- and chemosensitizer-free control cells from the same fish. * $p<0.05$, ** $p<0.01$. Data are shown as mean ± SEM, N=5 fish.

2.3.4. ATP consumption during inhibition

To determine if each chemosensitizer acted by competitive or non-competitive inhibition, intracellular free ATP concentrations were measured in control hepatocytes, and those incubated with cortisol or chemosensitizer alone, or cortisol plus

chemosensitizer in combination. Depletion of free [ATP] indicates an increase in P-gp ATPase activity (Matsunaga et al., 2006), which would be observed during competitive inhibition, but not non-competitive inhibition (Luckenbach et al., 2014). CsA (500 μM) caused a significant reduction of free [ATP] compared to controls, both alone and in combination with cortisol (ANOVA, $F_{3,19}$=7.2425, p=0.0050) (40.8±7.6% of controls for CsA+cortisol, 35.4±2.8% of controls for CsA alone) (Fig. 2.3A). Free [ATP] was significantly lower in cells incubated with 250 μM quinidine, alone and in combination with cortisol (ANOVA, $F_{3,19}$=8.6424, p=0.0025) (23.6±8.9% of controls for quinidine+cortisol, 28.0±9.8% of controls for quinidine) (Fig. 2.3B). Valspodar (250 μM) resulted in a significant reduction of free [ATP] compared to controls, both alone and in combination with cortisol (ANOVA, $F_{3,19}$=10.6212, p=0.0011) (9.0±4.7% of controls for valspodar+cortisol, 12.3±4.3% of controls for valspodar) (Fig. 2.3C). Verapamil (125 μM) significantly reduced free [ATP] compared to controls (ANOVA, $F_{3,19}$=9.2492, p=0.0019) (20.0±5.7% of controls for verapamil), but verapamil in combination with cortisol did not significantly differ from controls (66.5±17% of controls for verapamil+cortisol) (Fig. 2.3D).

2.4. Discussion

Primary cultures of trout hepatocytes were used to determine the concentration-dependent effects and mechanisms of inhibition of four known mammalian chemosensitizers on the P-gp-mediated transport of the xenobiotic and endogenous substrates R123 and cortisol, respectively, in order to begin a comparative assessment of this defense system in mammals and fish.

All of the chemosensitizers tested inhibited the transport of at least one of the P-gp substrates (R123 or cortisol), demonstrating that they are all inhibitors of rainbow trout P-gp. Thus, the range of rainbow trout chemosensitizers has a substantial overlap with the range of mammalian chemosensitizers, consistent with similar data in zebrafish, tuna, and killifish. In a screening of 10 known mammalian chemosensitizers with zebrafish Abcb4, all of the test compounds were able to either stimulate or inhibit P-gp ATPase activity, indicating either competitive or non-competitive P-gp inhibition (Bieczynski et al., 2021a). Similarly, in a screening of 31 compounds comparing tuna and mouse P-gp, 30 chemosensitizers inhibited P-gp from both species at similar concentrations, while one chemosensitizer inhibited tuna P-gp but not mouse P-gp (Nicklisch et al., 2021). Among 28 chemosensitizers tested using killifish and human P-

gp, 16 compounds inhibited P-gp from both species, while 5 compounds inhibited only killifish P-gp and 7 compounds inhibited only human P-gp (Zaja et al., 2011).

The efficacy of the chemosensitizers in this study were substrate-dependent. CsA inhibited the transport of both R123 and cortisol, while the other chemosensitizers inhibited transport of R123 but not cortisol (quinidine and verapamil), or cortisol but not R123 (valspodar). This substrate-dependence occurs as a result of the complex interactions between the substrate, the inhibitor, and the amino acids lining the P-gp drug-binding pocket: even potent competitive inhibitors cannot inhibit the transport of all known P-gp substrates (Robey et al., 2021). The inhibition efficacy of a substrate-chemosensitizer pair depends their binding sites and binding affinities within the P-gp molecule. Competitive inhibition of P-gp transport activity occurs when a high-affinity chemosensitizer displaces a lower-affinity substrate from their shared binding site in the P-gp drug-binding pocket (Kurth et al., 2015). Thus, inhibition will only occur if the chemosensitizer binds to the same binding site as the substrate, and with higher affinity.

P-gp binding affinity was initially measured using radiolabeled substrates binding to P-gp in membrane vesicles (Demmer et al., 1996; Doppenschmitt et al., 1999, 1998; Neuhoff et al., 2000). Since the elucidation of the 3-dimensional structure of mammalian P-gp, the relative affinity of P-gp for its substrates can be determined by a combination of substrate accumulation assays, ATPase assays, and molecular docking models (Chang et al., 2020; Hu et al., 2018; Pang et al., 2017; Qian et al., 2019). Higher-affinity substrates are able to displace lower-affinity substrates from their shared binding site (Doppenschmitt et al., 1999; Kurth et al., 2015). Thus, if the shared P-gp binding site has a higher affinity for the substrate than the chemosensitizer, no inhibition will be observed.

The P-gp drug binding pocket is lined with hydrophobic, aromatic, and polar/charged amino acid side chains (Aller et al., 2009). Different subsets of these amino acids in different regions of the drug binding pocket form multiple substrate binding sites (Martinez et al., 2014; Shapiro and Ling, 1997). When two compounds bind to different sites within the drug-binding pocket, P-gp is able to transport both molecules at the same time (Gutmann et al., 2010; Shapiro and Ling, 1997; Zhang et al., 2021). Indeed, when two substrates bind simultaneously at different binding sites, each substrate has a stimulatory effect on the other's P-gp-mediated transport rate (Shapiro

and Ling, 1997). Thus, if the chemosensitizer has a different binding site than the substrate, P-gp will transport both molecules simultaneously and no inhibition will be observed.

From the concentration-response curves for quinidine and verapamil (R123 and cortisol), it appears likely that all four molecules share a common binding site. R123 accumulation increases during co-exposure with both of the chemosensitizers, suggesting that P-gp has a higher affinity for the chemosensitizers than for R123. During co-exposure to cortisol and verapamil or quinidine, cortisol accumulation has an upward trend similar to the corresponding R123 accumulation, but this increase is smaller and not statistically significant. This trend suggests that cortisol is competing for transport with quinidine and verapamil, but cortisol may have a higher affinity at their shared binding site.

Quinidine and verapamil inhibited R123 transport in a similar concentration range in this study as previously reported for mammalian and fish P-gp. For example, the IC50 value for quinidine in this study (83.3±27 μM) was similar to those previously reported for human P-gp (34.7 to 51.3 μM) (Boer et al., 1994; Jouan et al., 2016). The IC50 value for verapamil (43.2±14 μM), was very close to that reported in zebrafish embryos for the inhibition of the chemically similar substrate rhodamine B (39.81 μM) (Bieczynski et al., 2021). Verapamil had a similar but lower inhibitory potency for R123 transport by rainbow trout P-gp (43.2±14 μM) compared to mammalian P-gp (IC50s 6.8 to 14.3 μM) (Boer et al., 1994; Jouan et al., 2016) and other aquatic animals (IC50s 1.6 to 19.6 μM) (Kovačević et al., 2021; Zaja et al., 2007, 2008b, 2011). This similarity between rainbow trout P-gp and P-gp from other species suggests that the binding site and relative affinities for R123, quinidine, and verapamil within the P-gp drug-binding pocket are shared across these taxonomic groups.

CsA and valspodar, both cyclosporin derivatives, were much less potent inhibitors of R123 transport in trout P-gp than in other species. Studies in mammals, invertebrates, and several other fish species reported IC50 values for CsA ranging from 0.27 to 7.1 μM (Boer et al., 1994; Caminada et al., 2008; Jouan et al., 2016; Kovačević et al., 2021; Smital et al., 2000; Zaja et al., 2007), compared to 132 ± 60 μM in the present study. However, in agreement with our results, 5 and 15 μM CsA had no effect on R123 accumulation in primary cultures of rainbow trout gill cells (Kropf et al., 2020).

Valspodar is a potent inhibitor of R123 transport by human P-gp, with a reported IC50 of 0.33 μM (Boer et al., 1994). In contrast, valspodar had no significant effect on R123 accumulation in this study at concentrations up to 500 μM. The lower inhibition potency of CsA and valspodar observed may arise from differences in affinity for these cyclosporins at their binding site in rainbow trout P-gp compared to P-gp from other species. A lower affinity for cyclosporins at their binding site would reduce the potency of the inhibitory interaction with P-gp substrates. The arrangement of amino acids in the drug-binding pocket differs between human P-gp and zebrafish P-gp, and between zebrafish abcb4 and abcb5 proteins (Robey et al., 2021), thus inter-species differences in binding affinity for some substrates is likely.

The difference in potency of these cyclosporins could also be related to the types of cells in which their inhibitory effects were investigated. The studies that found low potency of cyclosporins (this study and Kropf et al. (2020)) used primary cultures of cells isolated directly from healthy animals. By contrast, all of the vertebrate studies that found high potency of cyclosporins used immortalized cancer cell lines, most of which were multidrug resistant: human MDR leukemia cells (Boer et al., 1994), human MDR breast cancer cells (Jouan et al., 2016), killifish MDR hepatoma cells (Caminada et al., 2008), and killifish non-MDR hepatoma cells (Zaja et al., 2007). MDR cancer cell lines undergo 3 sets of selective process that differentiate them from the somatic cells that gave rise to their progenitors: 1) becoming cancerous by changes leading to abnormal cell growth and proliferation; 2) gaining the ability to reproduce *in vitro* indefinitely; and 3) becoming multidrug resistant by surviving drug concentrations that are cytotoxic to non-resistant cells (Amaral et al., 2019; Mirabelli et al., 2019; van Staveren et al., 2009; Zaja et al., 2008a). Each of these steps involves gene mutations, epigenetic changes, and gene expression alterations that result in cells with very different phenotypes from their predecessors (Amaral et al., 2019; Mirabelli et al., 2019; van Staveren et al., 2009). In P-gp studies using cancer cell lines, the phenotypic change that is desired and quantified is P-gp overexpression (Boer et al., 1994; Jouan et al., 2016; Zaja et al., 2008a). However, the extent of these phenotypic changes are rarely investigated, leading to substantial gaps in applicability of results from cell lines to *in vivo* tumors (Mirabelli et al., 2019; van Staveren et al., 2009). In ecotoxicology studies, we aim to extrapolate another step backwards to non-cancerous cells in healthy tissues (Kurth et al., 2015), adding another layer of uncertainty (Mirabelli et al., 2019; van Staveren et al., 2009). Thus,

further studies are necessary to determine if the different potencies of cyclosporins arise from interspecies differences in P-gp affinity, phenotypes of cancer cell lines compared to primary cultures of wild-type cells, or other factors.

All of the chemosensitizers examined in this study were less potent inhibitors of cortisol transport compared to inhibition in mammalian P-gp. Verapamil had no significant effect on cortisol transport by rainbow trout P-gp at concentrations up to 500 μM, but 16 μM verapamil was sufficient to significantly increase cortisol accumulation in Chinese hamster cells (van Kalken et al., 1993). CsA inhibition of cortisol transport by trout P-gp was relatively weak (IC50 294 $\pm$ 106 μM), in contrast to that in mammals where CsA is an extremely potent inhibitor, although the extent of the concentration-dependence of this inhibition has not been established (van Kalken et al., 1993). Valspodar exhibited low potency in inhibiting cortisol transport in trout (IC50 92.2 $\pm$ 35 μM). In contrast, 8 μM valspodar caused a substantial increase in intracellular accumulation of cortisol in Chinese hamster cells (van Kalken et al., 1993). This pattern of lower potency of inhibition of cortisol transport by trout P-gp suggests a higher affinity for cortisol in fish than in mammals.

This difference in affinity for cortisol between teleost and mammalian P-gp parallels the different physiological roles of corticosteroid hormones between these animal lineages. The mammalian corticosteroid system involves two ligand/receptor pairs: cortisol with the glucocorticoid receptor (GR), and aldosterone with the mineralocorticoid receptor (MR) (Bury and Sturm, 2007; Denver, 2009). Fish lack aldosterone, but rainbow trout and most fish species have 3 corticosteroid receptors that are activated by cortisol binding (MR, GR1, GR2) (Bury, 2017). Thus, in addition to its standard vertebrate regulatory functions, cortisol is also responsible for water and mineral balance in fish, while this is regulated separately by aldosterone in mammals (Kiilerich et al., 2015). These 3 corticosteroid receptors are activated at different cortisol concentrations: MR and GR2 are activated at basal, unstressed plasma cortisol concentrations, while GR1 is activated at elevated stress-induced plasma cortisol concentrations (Bury, 2017; Bury et al., 2003; Sturm et al., 2005). Due to its broader regulatory role and more complex interactions with more receptors in fish than in mammals, control of physiological cortisol concentrations has been under strong selective pressure in fish (Bury, 2017).

All four tested chemosensitizers were able to stimulate ATP consumption in trout hepatocytes, indicating that they are being transported by P-gp (Luckenbach et al., 2014). Inhibitors that are also transported by P-gp act *via* a competitive mechanism, displacing other substrates and increasing ATP consumption due to higher P-gp activity (Luckenbach et al., 2014). CsA, quinidine, and valspodar stimulated ATP consumption both alone and in the presence of the substrate, demonstrating competitive inhibition of rainbow trout P-gp. Similarly, CsA and valspodar are substrates of mammalian P-gp, and are able to competitively inhibit its transport activity (Watanabe et al., 1997). A fluorescent analogue of CsA was transported by killifish P-gp, and this transport was competitively inhibited by valspodar (Miller et al., 2002). Quinidine has previously been shown to competitively inhibit rat and killifish P-gp (Miyazaki et al., 2014; Zaja et al., 2011).

ATP consumption significantly increased in the presence of verapamil, indicating that verapamil is a competitive inhibitor of rainbow trout P-gp. Similarly, verapamil has been shown to be a competitive inhibitor of both mammalian and teleost P-gp (Bieczynski et al., 2021a; Litman et al., 1997; Zaja et al., 2011). However, verapamil was unique in that it stimulated ATP consumption alone, but did not stimulate ATP consumption in combination with cortisol. Verapamil increases ATP consumption due to its high affinity for P-gp and high membrane permeability, leading to a cycle of inward diffusion and outward active transport (Doppenschmitt et al., 1999; Luckenbach et al., 2014; von Richter et al., 2009). The presence of cortisol is able to break this cycle, decreasing verapamil-stimulated ATP consumption. Inhibition of verapamil-stimulated ATPase activity, as observed here with cortisol, is a standard diagnostic criterion for identifying P-gp inhibitors (Bieczynski et al., 2021a; Fischer et al., 2013; Sarkadi et al., 1992). Thus, cortisol is an inhibitor of verapamil transport by rainbow trout P-gp. Since both cortisol and verapamil are P-gp substrates, this suggests that rainbow trout P-gp has a higher affinity for cortisol than for verapamil. This result provides further evidence for the tight regulation of intracellular cortisol concentrations in fish, necessitated by the complex interactions of cortisol with multiple corticosteroid receptors in this animal lineage.

In this study, the concentration- and substrate-dependence of four known mammalian P-gp inhibitors in trout primary hepatocytes were determined. Trout P-gp is inhibited by a set of chemosensitizers that is closely aligned with inhibitors of P-gp in

mammals, invertebrates, and other fish species; these inhibitors also act by the same competitive inhibitory mechanism. Differences between species were evident in the potency of inhibition, with all tested chemosensitizers having a lower potency in trout than in other species. With the xenobiotic substrate R123, verapamil and quinidine had IC50 values that were fairly similar to those reported for mammalian P-gp, while CsA much less potent and valspodar had no inhibitory effect. With the endogenous substrate cortisol, all chemosensitizers either lacked inhibitory effect or had much lower potency than reported in mammals, which may reflect a higher affinity of trout P-gp for cortisol. Intracellular cortisol concentrations are expected to be under tighter control in rainbow trout than in mammals due to the regulation of the salmonid corticosteroid system by different cortisol concentrations, rather than by different hormones. This difference in hormone regulation suggests that, although P-gp and related trans-membrane transporters are most often studied in the context of xenobiotic toxicants, there are likely many endogenous functions that have yet to be investigated (Nigam, 2015). Chemosensitizers are recognized as an important class of environmental pollutants; these new insights into P-gp inhibition in rainbow trout will improve our understanding of the effects of complex mixtures of contaminants in aquatic environments.

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Chapter 3. Effects of the chemosensitizer verapamil on P-glycoprotein substrate efflux in rainbow trout hepatocytes

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Christina U. Johnston and Christopher J. Kennedy†

Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia, Canada

Abstract

The ATP-dependent membrane transporter P-glycoprotein (P-gp) is associated with resistance to a wide variety of chemical substrates, as well as the multi-drug resistance (MDR) phenotype in mammals. Less is known regarding P-gp's function and relevance in teleosts; this study expanded the range of known substrates and the inhibitory effects of a model chemosensitizer verapamil. The P-gp-mediated uptake and efflux dynamics of 5 known mammalian substrates (berberine, cortisol, doxorubicin, rhodamine 123 [R123], and vinorelbine) were examined in isolated rainbow trout (*Oncorhynchus mykiss*) hepatocytes with and without co-exposure to varying doses of verapamil. Initial substrate uptake rates (pmol/10^6 cells/min) varied widely and were in order: berberine (482±94) > R123 (364±67) > doxorubicin (158±41) > cortisol (20.3±5.9) > vinorelbine (15.3±3.5). Initial efflux rates (pmol/10^6 cells/min) were highest in berberine (464±110) > doxorubicin (341±57) > R123 (106±33) > cortisol (26.6±6.1) > vinorelbine (9.0±2.4). Transport of vinorelbine and R123 is verapamil sensitive, but verapamil had no effect on transport of berberine, cortisol, or doxorubicin. Cortisol and doxorubicin showed evidence of high P-gp affinity, thus displacing verapamil from their shared P-gp binding site. Cortisol, doxorubicin, R123, and vinorelbine transport by rainbow trout P-gp was confirmed, while berberine could not be confirmed or excluded as a substrate. Binding sites and affinities were similar between mammalian and trout P-gp for doxorubicin, R123, and vinorelbine, while fish P-gp had a higher affinity for cortisol than mammalian P-gp. This study demonstrated that the range of substrates, as well as binding sites and affinities, of fish P-gp are well-aligned with those in mammals.

Keywords: P-glycoprotein, chemosensitizer, substrates, rainbow trout, hepatocyte, verapamil

3.1. Introduction

Exposure to natural xenobiotics has been an important selective pressure over time, leading to the evolution of highly conserved chemical defense mechanisms (Nebert and Dieter, 2000). Chemical defense is generally categorized into 4 phases: (0) unmodified efflux, (I) functionalization reactions, (II) conjugation reactions, and (III) metabolite elimination. In chemical biotransformation (phases I and II), hydrophobic compounds are usually converted to more hydrophilic metabolites. In phase III, hydrophilic metabolites (mainly conjugates) are actively transported from the cell by trans-membrane proteins including multidrug resistance proteins (MRPs) and breast cancer resistance protein (BCRP) (Iversen et al., 2022). In phase 0, transport proteins export unmodified parent compounds from the cell (Epel et al., 2008; Ferreira et al., 2014; Kroll et al., 2021). Permeability-glycoprotein (p-glycoprotein [P-gp]) is an active trans-membrane transporter involved primarily in phase 0 export in absorptive, secretory, and sanctuary tissues (Leslie et al., 2005; Love et al., 2021). Export of xenobiotic compounds by P-gp makes cells resistant to the harmful effects of its substrates, conferring multi-drug resistance (MDR) to tumors, and multi-xenobiotic resistance (MXR) to aquatic animals inhabiting contaminated environments (Bieczynski et al., 2021; Ferreira et al., 2014).

P-gp is an ATP-binding cassette (ABC) transporter, encoded in mammals by the abcb1, abcb4, and abcb5 genes, with the abcb1 gene product performing most of the xenobiotic transport activity (Fischer et al., 2013). In fish, species including rainbow trout, zebrafish, and catfish have the abcb4 and abcb5 genes; the abcb4 gene product most closely resembles mammalian P-gp in structure and function (Bieczynski et al., 2021; Luckenbach et al., 2014). ATP hydrolysis drives the active export of a broad range of mildly hydrophobic substrates, which can enter the transporter from the hydrophobic region of the plasma membrane or the cytoplasm (Aller et al., 2009; Nosol et al., 2020). Mammalian P-gp (also called multidrug resistance protein 1 [MDR1]) has been extensively studied, leading to the identification of a large substrate base (Silva et al., 2015), three-dimensional structure (Aller et al., 2009; Ling, 1992; Rosenberg et al., 2003), and transport mechanism (Callaghan et al., 2006; Nosol et al., 2020). Research into teleost P-gp is more recent and less is known regarding its structure and functional characteristics, as well as its physiological significance and environmental relevance.

The remarkably broad substrate base of mammalian P-gp arises from the protein's internal drug binding pocket, which is lined with hydrophobic, aromatic, and several polar/charged amino acid side chains (Aller et al., 2009). In the drug binding pocket, each substrate interacts with a subset of these residues, which constitute that substrate's binding site (Aller et al., 2009). Once a substrate has bound, the P-gp molecule shifts its transmembrane helices from an inward-facing conformation to an outward-facing conformation, releases the substrate into the extracellular space, and the hydrolysis of ATP allows it to return to its inward-facing conformation (Nosol et al., 2020).

Fish and mammalian P-gp are expected to share similar substrate specificities due to the closely matching genetic sequences and tissue expression patterns for P-gp (Fischer et al., 2013; Leslie et al., 2005; Love et al., 2021). Substrates of mammalian P-gp tend to be moderately hydrophobic and mildly amphipathic, and often contain a positively charged nitrogen atom and aromatic rings (Silva et al., 2015). Many pharmaceuticals have been identified as substrates of mammalian P-gp, including chemotherapeutic agents, opioids, antibiotics, calcium channel blockers, and antidepressants (Silva et al., 2015). P-gp also transports numerous endogenous substrates, including steroid hormones, peptides, bilirubin, and sphingolipids (Aye et al., 2009; Bellarosa et al., 2009; Silva et al., 2015). In contrast to the hundreds of substrates known for mammalian P-gp, only a few dozen substrates have been identified for fish P-gp, including several pharmaceuticals, pesticides, cyanobacteria toxins, and polycyclic aromatic hydrocarbons (Bieczynski et al., 2021; Luckenbach et al., 2014). Thus, the full range of xenobiotic and endogenous substrates of teleost P-gp has only begun to be explored.

While P-gp and other transmembrane transporters make cells resistant to xenobiotic accumulation, inhibition of these transporters makes cells sensitive to potential toxicants that would otherwise be removed by them (Ferreira et al., 2014; Kurth et al., 2015). Thus, inhibitors of multi-xenobiotic resistance (MXR) transporters, including P-gp, are referred to as chemosensitizers (Kurth et al., 2015). Chemosensitizers were first identified in clinical settings, making tumor cells resistant to chemotherapeutic agents (Silva et al., 2015). Since the identification of the protective role of MXR transporters to aquatic animals in contaminated environments, chemosensitizers have been increasingly recognized as an important class of environmental pollutant,

especially in complex mixtures containing both chemosensitizers and P-gp substrates (Kurth et al., 2015). Many chemosensitizers are high-affinity transporter substrates, competitively inhibiting the export of other substrates (Doppenschmitt et al., 1999b; Kurth et al., 2015; von Richter et al., 2009). Other chemosensitizers can non-competitively interfere with an aspect of the transport mechanism, for example by inhibiting ATP hydrolysis (e.g., vanadate) or by blocking the movement of transmembrane helices necessary for substrate release (e.g., tariquidar) (Nosol et al., 2020).

Inhibition of transport by model chemosensitizers is often used as a diagnostic criterion for recognizing substrates of MXR transporters (Bieczynski et al., 2021; Oude Elferink et al., 1995). Since these model chemosensitizers are specific to only one transporter type (e.g., verapamil [P-gp inhibitor], MK571 [multidrug resistance-associated protein inhibitor]), they are useful tools to identify the transporter responsible for substrate export (Sturm et al., 2001). While transport inhibition by a model chemosensitizer serves as a strong positive identification of efflux by a particular transporter type, this diagnostic criterion is vulnerable to false negatives. The efficacy of chemosensitizers is substrate-dependent; even potent chemosensitizers can have little or no effect on the transport of a subset of substrates (Robey et al., 2021). When substrates are identified by other lines of evidence, these false negatives can provide insight into the binding site and affinity of the substrate in the P-gp protein. The chemosensitizer can have a stimulatory rather than inhibitory effect on substrate transport if they have different binding sites, allowing P-gp to transport both the substrate and chemosensitizer simultaneously (Gutmann et al., 2010; Shapiro and Ling, 1997; Zhang et al., 2021). Similarly, a high affinity substrate can displace a lower affinity chemosensitizer from its binding site, thus no inhibition of substrate transport would be observed (Doppenschmitt et al., 1999b; Kurth et al., 2015).

The primary goal of this study was to begin an assessment of the similarity between the known mammalian P-gp substrate base and those that are also transported by teleost P-gp. The accumulation and efflux kinetics of 5 putative teleost P-gp substrates (berberine, cortisol, doxorubicin, rhodamine 123, and vinorelbine) were measured in primary cultures of rainbow trout hepatocytes. The inhibition of substrate transport by verapamil was also assessed to gain insight into the interactions of these substrates with rainbow trout P-gp.

3.2. Methods

3.2.1. Fish

Adult rainbow trout (*Oncorhynchus mykiss*) (mass range 500-1200 g) were obtained from LSL Living Seafoods (Langley, BC). Fish were kept in 2500 L fiberglass tanks supplied with continuously flowing dechlorinated municipal water (pH 6.6-7.4, 7.5-16.1°C, hardness 6.1 g/mL $CaCO_3$, O_2 saturation 90-95%) under a 12h light/12h dark photoperiod. Fish were fed twice daily *ad libitum* with Pacific Floating Complete Feed for Salmonids (EWOS Canada Ltd. Surrey, BC). Fish were acclimated to housing conditions for a minimum of 14 d before experiments began.

3.2.2. Chemicals

Sodium bicarbonate, DL-lactic acid (90%), rhodamine 123 (≥85%), doxorubicin hydrochloride (≥98%), vinorelbine detartrate salt hydrate (≥98%), hydrocortisone (cortisol), berberine chloride, dimethyl sulphoxide, trypan blue, verapamil hydrochloride (≥99%), 1-butanol, collagenase (Type IV from Clostridium histolyticum), bovine serum albumin (fatty acid free, ≥98%), Hanks' Balanced Salts (powder, modified without calcium chloride, magnesium sulfate, phenol red and sodium bicarbonate) and sodium dodecyl sulphate were purchased from Sigma-Aldrich (Oakville, ON, Canada). D-glucose was obtained from BDH Inc. (Toronto, ON). MS-222 (tricaine-S) was obtained from Syndel (Ferndale, WA, USA). Calcium carbonate was obtained from Fisher Scientific Company (Fair Lawn, NJ, USA).

3.2.3. Hepatocyte primary cell culture

Fish were anaesthetized with 0.2 g/L buffered tricaine methane sulfonate. Hepatocytes were isolated according to Moon et al. (1985), and Bains and Kennedy (2004). Briefly, the liver was perfused *via* the hepatic portal vein with sterile, oxygenated (Ca^{2+})-free Hanks balanced salt solution (HSS) to clear blood from the organ. The liver was then perfused with HSS containing 0.3 g/L collagenase until signs of disintegration became apparent. The liver and gallbladder were removed from the fish, the gallbladder carefully removed, and the liver minced with a razor blade. Liver tissue was rinsed with HSS through two nylon mesh filters (253 μm and 73 μm) to disaggregate hepatocytes.

Hepatocyte suspensions were concentrated by centrifugation (50 *xg*, 3 min, 4°C) and re-suspended in Hanks' Balanced Salts solution containing glucose (3 mM), sodium bicarbonate (6 mM), lactic acid (1 mM), calcium chloride (1.5 mM) and bovine serum albumin (10 g/L) (hereafter called HSSB) and kept on ice until use. Cells were not pooled between fish. Cell viabilities were assessed by the trypan blue exclusion test (Braunbeck and Storch, 1992) during experiments.

3.2.4. Substrate accumulation and efflux assays

Substrate accumulation and efflux protocols (Bains and Kennedy, 2005; Sturm et al., 2001) were used in isolated hepatocyte primary cultures with each substrate tested individually. Cell yields from a single fish were sufficient for accumulation and efflux assays for all time points for a single substrate. Sample sizes (n) refer to the number of fish used. Briefly, 1 mL aliquots of the hepatocyte suspension were added to 15 mL culture tubes (final cell concentration 25 mg/mL, approx. $2.4x10^5$ cells/mL) and were pre-incubated in the dark for 60 min at 12°C with gentle agitation to acclimate cells to experimental conditions. A stock solution of substrate in DMSO (10 mM) was diluted in HSSB to produce substrate culture media. Substrate culture media was added to cell suspensions to achieve a final concentration of 25 μM of substrate to begin the accumulation assay. At various time points, cell suspensions were removed and centrifuged (50 xg) for 3 min. The supernatant was removed, and the cell pellet was re-suspended in 1 mL HSSB and stored frozen at -20°C until chemical analysis of substrate concentrations.

In experiments to determine efflux rates, cells were first incubated with a substrate for the full 90 min to 'load' the cells and then centrifuged (50 xg) for 3 min. The supernatant containing substrate that was not taken up by cells was discarded. Cell pellets were re-suspended in fresh substrate-free HSSB, and then incubated for a further 90 min to allow for elimination of substrate. At various time points, cell suspensions were removed and centrifuged at 50 xg for 3 min to separate the cells and supernatant, which were both retained. The pellet was re-suspended in 1 mL fresh HSSB, and both supernatant and pellet were stored at -20°C until chemical analysis of substrate concentrations.

3.2.5. Inhibition assay

In order to determine if the co-incubation of the substrates with verapamil resulted in increased accumulation, the described accumulation assay was performed with the following modification: after the 60 min pre-incubation period, verapamil in HSSB (final concentrations 25, 50, 125, 250, 500 μM) was added, and samples were incubated for a further 15 min before the addition of a test substrate in HSSB (final concentration 25 μM). Cells were incubated for a further 60 min and then isolated from the medium as described above. The cell pellet was re-suspended in 1 mL HSSB and frozen at -20°C until chemical analysis of substrate concentrations.

3.2.6. Chemical analysis

Doxorubicin, R123, and vinorelbine samples were defrosted and then sonicated on ice (3x 2s pulses) and extracted with 2 volumes of 1-butanol. Samples containing 1-butanol were incubated in the dark for 2 h at 20ºC with shaking (200 rpm) on an orbital shaker (Robieux et al., 1996; Sturm et al., 2001). 100 µL of the butanol layer of each sample was removed and spectrophotometrically analyzed (in triplicate) on a 96-well plate using a SpectraMax M2e Microplate Reader (Molecular Devices, San Jose, CA). The wavelength settings used for the analyses were: doxorubicin (excitation [ex] = 470 nm, emission [em] = 585 nm) (Hildebrand et al., 2009), R123 (ex = 517 nm, em = 532 nm) (Sturm et al., 2001), vinorelbine (ex = 280 nm, em = 360 nm) (Robieux et al., 1996). Samples containing berberine were defrosted for 45 min and sonicated on ice (3 x 2s pulses). Sodium dodecyl sulphate (SDS) in RO water was added to a final concentration of 10 mM (Nies et al., 2008). 100 µL of the sample was removed and analyzed (in triplicate) using fluorescence spectroscopy as described above (ex = 355 nm, em = 538 nm) (Nies et al., 2008). Samples from the cortisol assays were defrosted and sonicated as described above. Cortisol concentrations in samples were quantified using an enzyme-linked immunosorbent assay (ELISA) kit (Lot #0148, Neogen, Lansing, MI, USA) according to manufacturer's instructions.

3.2.7. Calculations and statistics

Statistical analyses were conducted using JMP (Version 15.0.0, SAS Software, Cary, NC, USA). Cell viability, maximum intracellular concentrations, and fraction of

initial intracellular concentration exported from the cells at steady state were compared using a one-way ANOVA and Tukey's HSD test using a randomized incomplete block design. Initial accumulation and efflux rates were determined by linear regression using the first 3 time points on the accumulation curve (0, 5, 15 min). Rates were compared with ANCOVA using a randomized incomplete block design. The categorical treatment variable in the ANCOVA analysis was substrate identity, the continuous variable was time (0 to 15 min), and the measured dependent variable was intracellular substrate concentration. The effect of verapamil on intracellular substrate accumulation was compared with a repeated-measures ANOVA followed by Dunnett's test using a randomized incomplete block design. Results are expressed as mean±SE. Differences were considered significant at p=0.05.

3.3. Results

3.3.1. Cell viability

Cell viability was high in all experiments, with no significant differences detected between control cells and those in substrate treatment groups after 180 min of incubation (range 86.3±2.6% to 97.0±0.6%). In verapamil inhibition assays, there were no significant differences in cell viability between controls and verapamil/substrate combination treatments (range 83.1±2.6% to 96.0±1.0%).

Table 3.1 Molecular properties of tested substrates

Substrate	Molar Mass (g/mol)	Log K_{OW}
Berberine	336.4	-0.99
Cortisol	362.5	1.61
Doxorubicin	543.5	1.27
Rhodamine 123	380.8	0.25
Vinorelbine	778.9	4.84

Log K_{OW} values from PubChem, except rhodamine 123 from Bhattarai et al., (2020).

3.3.2. Substrate accumulation and efflux

The kinetics of accumulation and efflux for each substrate were determined in isolated rainbow trout hepatocytes. The molecular properties of the 5 tested substrates are shown in Table 3.1. The initial accumulation of all substrates was linear, however,

the time of linear accumulation to saturation was different between compounds: 15 min for cortisol and vinorelbine, 30 min for doxorubicin, and 60 min for berberine and R123 (Fig. 3.1). Initial rates of accumulation (0 to 15 min) calculated for berberine, R123, doxorubicin, cortisol, and vinorelbine were 482±94, 364±67, 158±41, 20.3±5.9, and 15.3±3.5 pmol/10^6 cells/min, respectively, and were significantly different from each other (ANCOVA, $F_{4,83}$=14.6847, p<0.0001) (Fig. 3.2). At steady state (plateau phase) (Altenberg et al., 1994), intracellular concentrations were constant and were significantly different between substrates (ANOVA, $F_{4,27}$=9.7525, p< 0.0001) (Fig. 3.3).

In efflux assays using cells placed in substrate free media, intracellular concentrations decreased as extracellular concentrations increased over time (Fig. 3.4). Accumulation in the extracellular compartment was initially linear for 10 to 15 min, depending on the substrate. Initial rates of efflux ranged from 9 to 464 pmol/10^6 cells/min and in rank order (highest to lowest) were: berberine, doxorubicin, R123, cortisol, and vinorelbine. Initial efflux rates were significantly different from each other (ANCOVA, $F_{4,93}$=11.2199, p<0.0001) (Fig. 3.5). For most of the substrates tested, steady state was achieved between 15 and 30 min. The percent of the initial intracellular concentration exported from the cells at steady state was significantly different between substrates (ANOVA, $F_{4,27}$=14.5988, p<0.0001) and were ranked cortisol, doxorubicin, berberine, vinorelbine, and R123, highest to lowest (Fig. 3.6).

3.3.3. Verapamil inhibition

To test the inhibitory effect of verapamil on the transport of each substrate, total intracellular substrate accumulation was measured after 60 min of co-incubation with 25 μM substrate and 0, 25, 50, 125, 250, or 500 μM verapamil. Verapamil treatment had no significant effect on the accumulation of cortisol, doxorubicin and berberine (Fig. 3.7). In verapamil treated cells, the total intracellular accumulation of vinorelbine was significantly increased in a concentration-dependent manner over controls (ANOVA, $F_{5,47}$=4.3060, p=0.0037). Total intracellular vinorelbine accumulation was 5.6-fold higher at the highest concentration of verapamil compared to control cells.

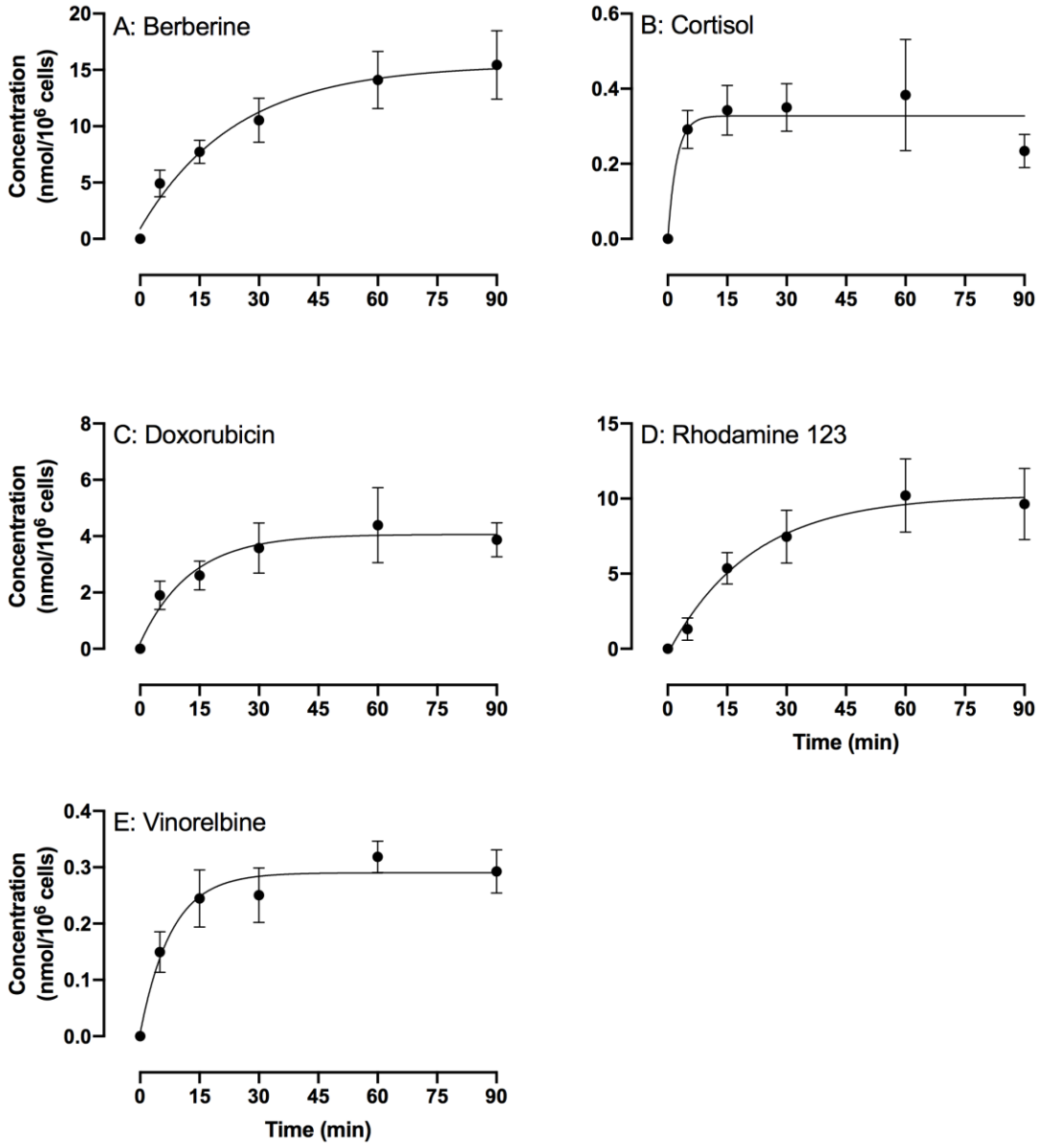

Figure 3.1 Intracellular substrate accumulation
Intracellular concentrations of P-gp substrates in trout hepatocytes during 90 min of incubation with 25 μM substrate in media. Data are shown as mean ± SEM. N=7 for doxorubicin and rhodamine 123, N=5 for berberine and vinorelbine, N=4 for cortisol.

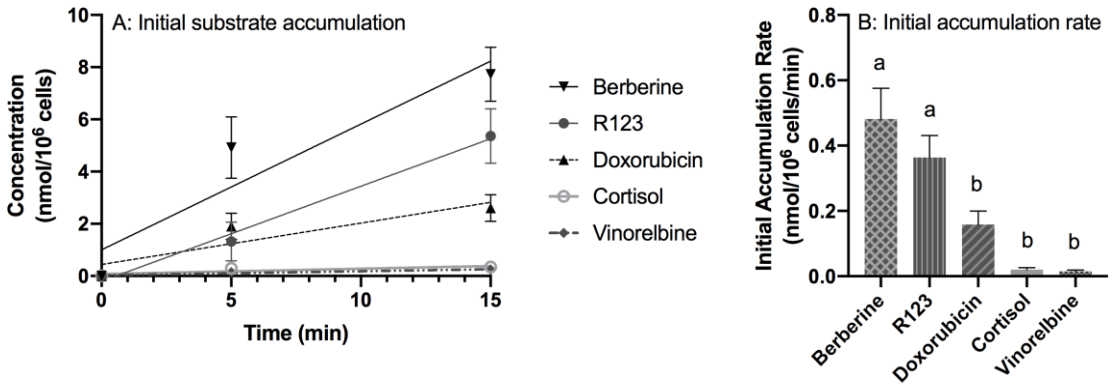

Figure 3.2 Initial uptake rates of P-gp substrates into trout hepatocytes
A: Intracellular substrate accumulation-time curves for the first 15 min of incubation in 25 μM substrate in media for substrates berberine (—▼—), rhodamine 123 (—●—), doxorubicin (--▲--), cortisol (—○—), and vinorelbine (--◆--). B: The initial rate of substrate accumulation for five P-gp substrates. Bars with the same letter are not significantly different from each other. Bars that do not share a letter are significantly different from each other ($p<0.05$). Data are shown as mean ± SEM. N=7 for doxorubicin and rhodamine 123, N=5 for berberine and vinorelbine, N=4 for cortisol.

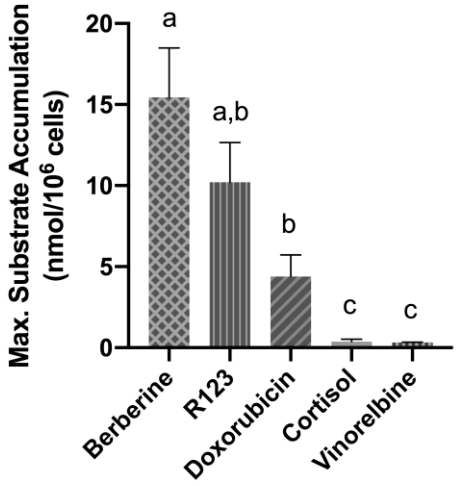

Figure 3.3 Maximum substrate accumulation
Maximum accumulation of P-gp substrates into rainbow trout hepatocytes after 90 min of incubation in 25 μM substrate in media. Bars with the same letter are not significantly different from each other. Bars that do not share a letter are significantly different from each other ($p<0.05$). Data are shown as mean ± SEM. N=7 for doxorubicin and rhodamine 123, N=5 for berberine and vinorelbine, N=4 for cortisol.

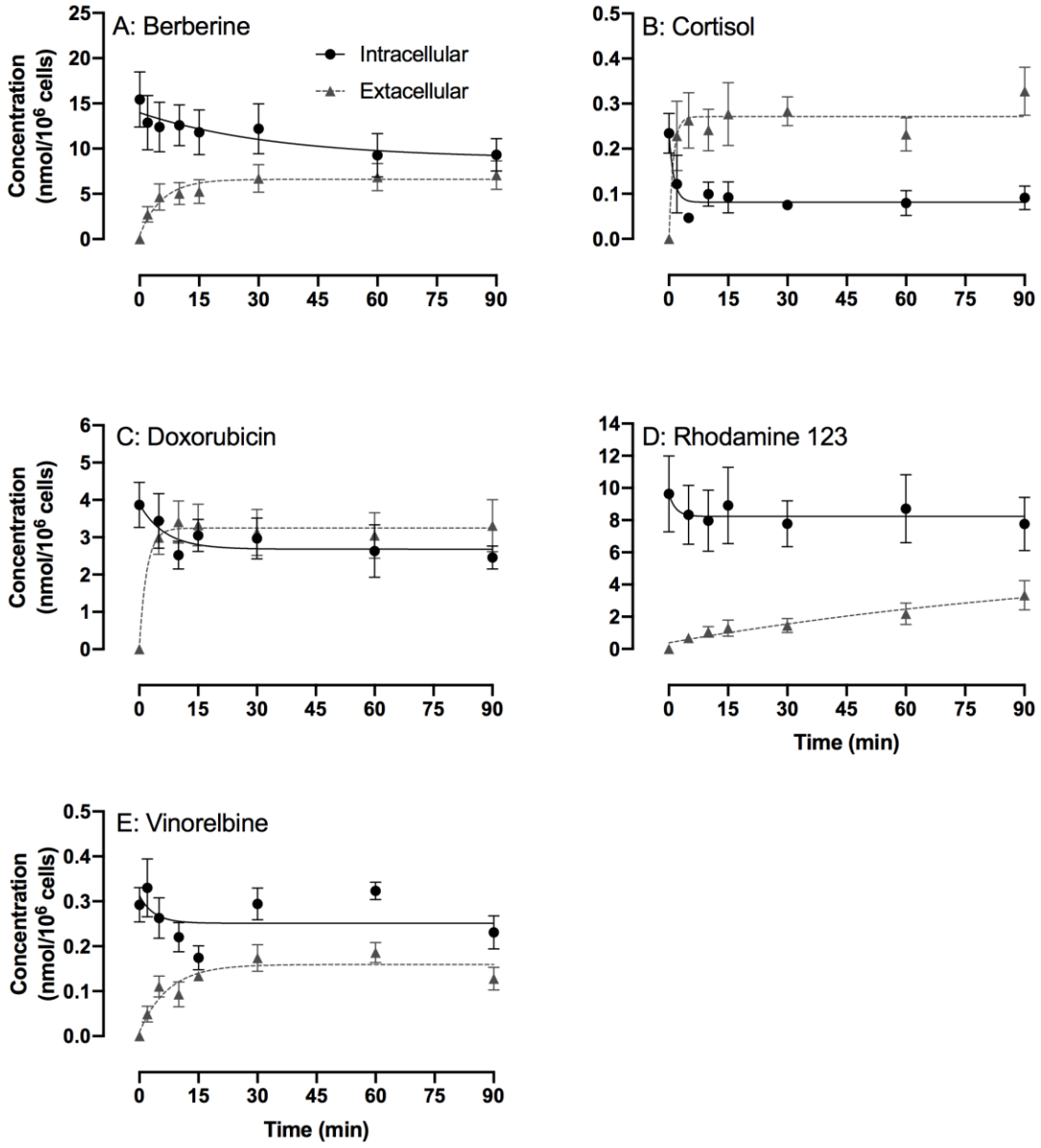

Figure 3.4 Substrate efflux

After 90 min of incubation in 25 μM substrate in media, extracellular substrate was removed, then cells were washed once and re-suspended in fresh substrate-free media. Intracellular substrate concentrations are shown (—●—), along with extracellular concentrations of substrate that has been transported out of the same cells (--▼--). Data are shown as mean ± SEM. N=7 for doxorubicin and rhodamine 123, N=5 for berberine and vinorelbine, N=4 for cortisol.

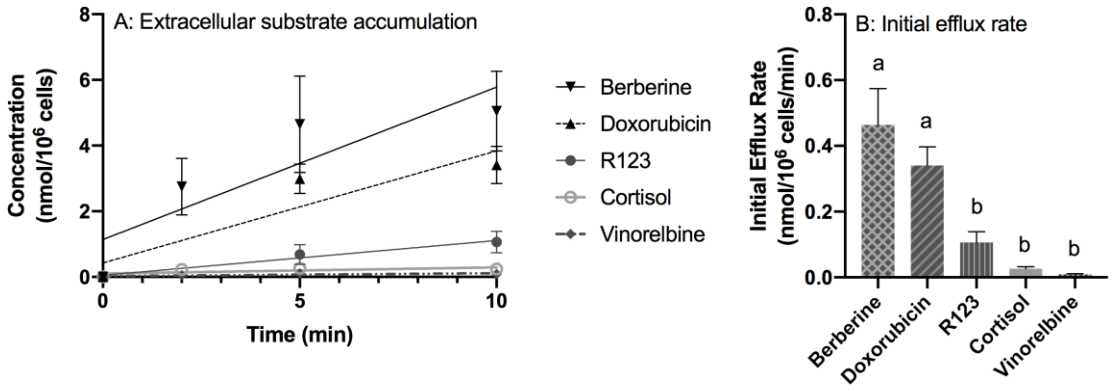

Figure 3.5 Extracellular accumulation of P-gp substrates exported from rainbow trout hepatocytes pre-loaded with a substrate

A: Extracellular substrate accumulation-time over the first 10 min after cells that were re-suspended in substrate-free media for substrates berberine (—▼—), rhodamine 123 (—●—), doxorubicin (- -▲- -), cortisol (—○—), and vinorelbine (- -◆—). B: Initial rate of efflux for 5 P-gp substrates. Bars with the same letter are not significantly different from each other. Bars that do not share a letter are significantly different from each other ($p<0.05$). Data are shown as mean ± SEM. N=7 for doxorubicin and rhodamine 123, N=5 for berberine and vinorelbine, N=4 for cortisol.

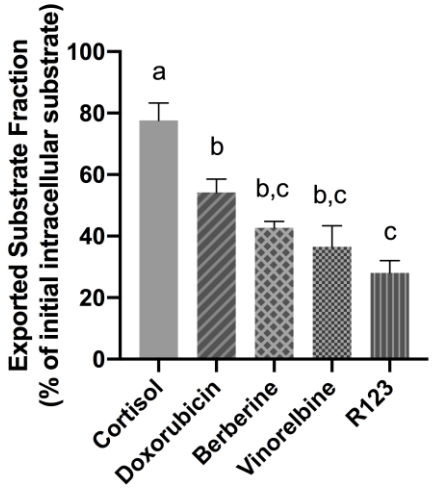

Figure 3.6 Exported fraction of accumulated substrate

Percentage of total substrate that has been exported from rainbow trout hepatocytes 90 min after re-suspension in substrate-free media. Bars with the same letter are not significantly different from each other. Bars that do not share a letter are significantly different from each other ($p<0.05$). Data are shown as mean±SEM. N=7 for doxorubicin and rhodamine 123, N=5 for berberine and vinorelbine, N=4 for cortisol.

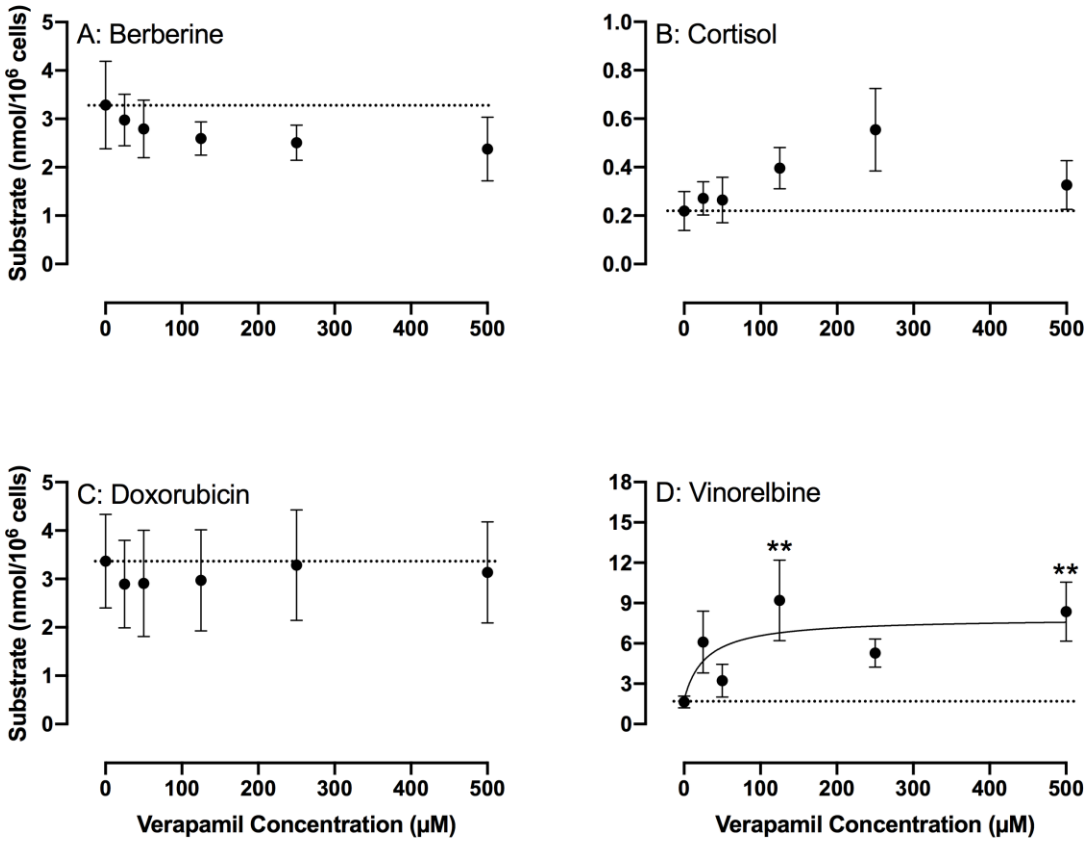

Figure 3.7 Verapamil inhibition of substrate transport
Intracellular substrate concentrations in cultured rainbow trout hepatocytes after 15 min of incubation with verapamil, followed by 60 min of incubation with verapamil + 25μM substrate. Dotted lines indicate intracellular concentration in control cells in verapamil-free media. An increase in intracellular concentration shows inhibition of efflux processes. Asterisks indicate significant differences compared to verapamil-free control cell cultures from the same fish. * $p<0.05$, ** $p<0.01$. Data are shown as mean±SEM. N=8 for vinorelbine, N=5 for doxorubicin, N=4 for berberine.

3.4. Discussion

Chemical defense mechanisms, including P-gp-mediated efflux, preserve chemical homeostasis by reducing the intracellular accumulation of both endogenous and exogenous compounds. Although the range of P-gp substrates has been well-characterized for mammalian P-gp (Silva et al., 2015), the full range of teleost P-gp substrates has only begun to be explored. To examine potential substrate overlap between the mammalian and teleost substrate base, the transport activity of P-gp toward five known mammalian P-gp substrates was measured in isolated rainbow trout hepatocytes. The model chemosensitizer verapamil was used to gain insight into the interactions of these substrate molecules with rainbow trout P-gp, particularly with

respect to binding affinity (Doppenschmitt et al., 1999a; Kurth et al., 2015) and binding site (Gutmann et al., 2010; Shapiro and Ling, 1997; Zhang et al., 2021).

P-gp genetic sequences and tissue expression patterns are similar between fish and mammals, suggesting that they likely share a common set of substrates (Fischer et al., 2013; Leslie et al., 2005; Love et al., 2021). The amino acids lining the drug-binding pocket of human and zebrafish P-gp are mostly the same or very similar, but there are a few amino acids in this region that have substantially different properties between the two species (Robey et al., 2021). Thus, some interspecies diversity of substrate binding is expected. In a study screening P-gp-interacting chemicals in human and killifish cells, 9 substrates were found to be common to both species, 2 were substrates of only killifish P-gp, and 3 were substrates of only human P-gp (Zaja et al., 2011). Similarly, two organochlorine pesticides (DDT and DDD) were found to be weak substrates of yellowfin tuna P-gp but were not transported by mouse P-gp (Nicklisch et al., 2021). In a cytotoxicity assay testing 90 P-gp substrates, zebrafish Abcb4 and human P-gp conferred nearly identical resistance to all tested compounds, while zebrafish Abcb5 conferred less resistance (Robey et al., 2021). Thus, we expect rainbow trout P-gp to transport a similar, but perhaps not identical, set of substrates as mammalian P-gp and P-gp from other fish species.

The rainbow trout hepatocyte model system has been used extensively to characterize substrate transport by fish P-gp (Hildebrand et al., 2009; Sturm et al., 2001; Zaja et al., 2008). Hepatocytes express P-gp on their apical surface, exporting substrates into the bile canaliculi, thus efflux of substrates from these cells corresponds to biliary excretion from the intact liver (Sturm et al., 2001; Zaja et al., 2008). The accumulation and efflux kinetics of all five substrates tested in this study are consistent with previous reports showing P-gp-mediated transport in this model system (Bains and Kennedy, 2005; Hildebrand et al., 2009; Sturm et al., 2001).

P-gp substrates tend to enter cells by passive diffusion across the plasma membrane, and diffusion rates are generally higher for compounds with increasing lipophilicity and limited by increasing molecular weight (Camenisch et al., 1998; Rowland and Tozer, 2011). Based on their molecular properties, the order of membrane permeabilities of the substrates in this study is cortisol > R123 > berberine > doxorubicin > vinorelbine (Camenisch et al., 1998). Despite having the highest membrane

permeability, cortisol had among the lowest initial accumulation rates. Berberine had the highest initial accumulation rate and maximum intracellular accumulation, but only moderate membrane permeability. Doxorubicin had a low membrane permeability, but a moderate accumulation rate. This lack of correlation between membrane permeability and substrate accumulation rates demonstrates that active transport plays a large role in controlling the intracellular concentrations of these substrates (Camenisch et al., 1998). R123 and vinorelbine had the closest alignment between their membrane permeability and accumulation rates, being second and fifth, respectively, in both; this suggests that active transport may play a more limited role for these substrates (Camenisch et al., 1998).

Verapamil is a chemosensitizer with a high P-gp affinity and high membrane permeability that competitively inhibits substrate transport by displacing lower-affinity substrates from their shared binding site (Doppenschmitt et al., 1999b; Kurth et al., 2015). If a substrate's transport is inhibited by this model chemosensitizer (binding to a shared binding site), P-gp-mediated transport is confirmed (Bieczynski et al., 2021; Oude Elferink et al., 1995). If no inhibition is observed, the substrate may not be transported by P-gp, or may be transported by P-gp, but there is a higher affinity for the substrate compared to verapamil (Doppenschmitt et al., 1998; Kurth et al., 2015). The substrate may also bind to a different region of the P-gp drug-binding pocket than verapamil, allowing both to be transported simultaneously, with each stimulating the other's efflux rate (Gutmann et al., 2010; Shapiro and Ling, 1997; Zhang et al., 2021).

Verapamil co-exposure significantly increased vinorelbine and R123 accumulation in trout hepatocytes. In mammals, verapamil treatment restored sensitivity to vinorelbine cytotoxicity in vinorelbine-resistant mouse and human cell lines (Etiévant et al., 1993). P-gp-overexpressing mouse and human cell lines resistant to vinorelbine also showed cross-resistance to other P-gp substrates including doxorubicin, vincristine, and vinblastine; this resistance was similarly reversed by verapamil treatment (Adams and Knick, 1995). Verapamil had a similar effect in P-gp-overexpressing human cells, increasing R123 accumulation in a concentration-dependent manner (Boer et al., 1994; Jouan et al., 2016). This transport inhibition by verapamil demonstrates that both R123 and vinorelbine are substrates of rainbow trout P-gp, with a lower binding affinity than verapamil, and at the same binding site as the model chemosensitizer. This binding site and relative affinity are also shared between teleost and mammalian P-gp.

Verapamil had no effect on doxorubicin accumulation after 60 min of co-exposure in this study. In a previous study using rainbow trout hepatocytes, verapamil inhibited doxorubicin efflux after 90 min of co-exposure, but not at earlier time points (Sturm et al., 2001). This apparent delayed onset of verapamil inhibition may indicate that rainbow trout P-gp has similar affinities for doxorubicin and verapamil. In human ovarian and kidney cell lines, intracellular doxorubicin accumulation increased 2- to 3-fold after 2 h of co-exposure to doxorubicin and verapamil (Chu et al., 2015), suggesting a similar binding site and relative affinity for doxorubicin between mammalian and rainbow trout P-gp.

There was no significant effect of verapamil on cortisol accumulation in this study. In another study, the P-gp inhibitors cyclosporin A and valspodar (PSC833) caused a significant increase in cortisol accumulation in trout hepatocytes (Johnston and Kennedy, 2023), confirming that cortisol is a substrate of rainbow trout P-gp. The absence of verapamil inhibition for cortisol transport suggests that rainbow trout P-gp has a higher affinity for cortisol than for verapamil. This high affinity for cortisol is also reflected in the exported fraction of cortisol from pre-loaded cells placed in substrate-free media: nearly 80% of intracellular cortisol was exported, more than all other tested substrates. These results are consistent with the previous observation that cortisol inhibits verapamil-stimulated ATP consumption in this cell type (Johnston and Kennedy, 2023). In contrast, verapamil can inhibit cortisol transport by mammalian P-gp. In human intestinal and lymphocyte cell lines, verapamil significantly reduced cortisol efflux within 1 to 2 h of co-exposure (Farrell et al., 2002). Chronic stress caused an increase in circulating cortisol concentrations and significantly increased the volume of distribution of verapamil in rat brains (de Klerk et al., 2010). This suggests that P-gp has a higher affinity for cortisol in fish it does in mammals.

This higher affinity could be related to the broader physiological roles of the glucocorticoid cortisol in fish, which lack the mineralocorticoid hormone aldosterone (Denver, 2009). In mammals, cortisol binds to a single glucocorticoid receptor (GR), and aldosterone binds to a single mineralocorticoid receptor (MR) (Bury and Sturm, 2007; Denver, 2009). In rainbow trout and related fish species, cortisol binds to 3 receptors (MR, GR1, GR2), which are activated at different cortisol concentrations (Bury, 2017; Bury and Sturm, 2007; McCormick et al., 2008). As a result, cortisol is responsible for water and mineral balance in fish in addition to its standard vertebrate regulatory

functions (Kiilerich et al., 2015). Thus, P-gp-mediated cortisol efflux likely plays an important role in the tight control of intracellular cortisol necessary to maintain homeostasis in fish.

There was no significant change in berberine accumulation with co-exposure to verapamil, thus berberine cannot be confirmed as a substrate of rainbow trout P-gp. Berberine's accumulation and efflux kinetics were consistent with active transport across the plasma membrane (Camenisch et al., 1998; Sturm et al., 2001; Tang et al., 2014; Vergote et al., 1998), but further studies are necessary to determine which transporter(s) it interacts with in fish cells. A small concentration-dependent decrease in berberine accumulation was observed with increasing verapamil concentrations, which may be consistent with stimulation of P-gp-mediated berberine export if berberine and verapamil have different P-gp binding sites (Shapiro and Ling, 1997). Berberine is a substrate of human, rat, and chicken P-gp (Chen et al., 2008; Maeng et al., 2002; Nies et al., 2008; Zhang et al., 2019), but this initial study examining berberine transport in fish is inconclusive as to its transport by rainbow trout P-gp.

This study conducted a functional characterization of the transport of five known mammalian P-gp substrates in rainbow trout hepatocytes and provided information that aids in elucidating the interactions of these substrates with teleost P-gp. Four of the five tested mammalian substrates (cortisol, doxorubicin, rhodamine 123, and vinorelbine) were transported by rainbow trout P-gp, which binds them at the same site as verapamil. Rainbow trout P-gp had similar binding affinities for these substrates as mammalian P-gp, except for a higher affinity for the steroid hormone cortisol, which has a broader physiological role in teleosts than in mammals. Berberine could not be confirmed or excluded as a substrate of rainbow trout P-gp, thus its active transport in fish cells calls for further study. The substrate range of teleost P-gp is only beginning to be investigated in detail, but thus far it closely mirrors that of mammalian P-gp.

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Consent to participate: All authors consent to participate.

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Chapter 4. Ivermectin toxicokinetics in rainbow trout (*Oncorhynchus mykiss*) following P-glycoprotein inhibition

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Christina U. Johnston, Vinicius Cavicchioli Azevedo and Christopher J. Kennedy†

Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia, Canada

Abstract

Changes to ivermectin (IVM [22,23-dihydro avermectin B1a + 22,23-dihydro avermectin B1b]) toxicokinetics (TK) with and without P-glycoprotein (P-gp) inhibition by cyclosporin A (CsA) were examined in rainbow trout (*Oncorhynchus mykiss*). Rainbow trout were injected with 175 μg/kg ^{3}H-IVM (8.6 μCi/mg IVM) with or without co-administration of 480 μg/kg CsA into the caudal vasculature. Fish were sacrificed at various time points (0.25, 0.5, 1, 3, 24, 48, 96, and 168 h) for organ and tissue sampling (blood, liver, kidney, gill, intestines, brain [5 regions], eye, gonad, and fat) which were analyzed for IVM-derived radioactivity. IVM concentration decreased over time in blood, liver, kidney, and gill, while concentrations in other tissues remained constant. The highest maximum IVM concentration (C_{max}) was found in kidney, followed by liver; the lowest C_{max} was found in eye, followed by brain and adipose tissue. The highest % of the administered dose was found in the blood 15 min post-IVM administration, followed by the intestine at 60 min post-IVM administration. P-gp inhibition by CsA did not significantly affect calculated TK parameters (AUC [7.33±0.73 – 11.5±2.5 mg·h/kg], mean residence time [84.7±21 – 125±55 h], $T_{1/2}$ [58.7±15 – 86.8±38 h], clearance rate [0.0152±0.0033 – 0.0239±0.0024 L/kg·h], or volume of distribution [1.91±0.47 – 2.02±0.33 L/kg]), but resulted in small but significant changes in the % administered dose found in blood and medulla. These results suggest that P-gp plays a limited role in overall IVM TK, and that its role in xenobiotic protection may be much less robust in fish than it is in mammals.

Keywords: P-glycoprotein, toxicokinetics, inhibition, ivermectin, cyclosporin A, rainbow trout

4.1. Introduction

Aquatic animals expressing the multixenobiotic resistance (MXR) phenotype in contaminated environments exhibit higher activities of xenobiotic defense proteins, including the transmembrane exporter permeability-glycoprotein (P-glycoprotein [P-gp]) (Kurelec and Pivčević, 1991; Luckenbach et al., 2014). P-gp is an ATP-dependent efflux protein with a broad range of xenobiotic and endogenous substrates that tend to be hydrophobic and amphipathic, and often include aromatic rings or positively charged nitrogen atoms in their structure (Silva et al., 2015). A wide variety of known P-gp substrates are environmental contaminants (e.g. cyanobacteria toxins, pesticides [e.g., diazinon, chlorpyrifos]) and pharmaceuticals (e.g., anthelminthics, chemotherapeutics, antibiotics) (Bieczynski et al., 2021; Zaja et al., 2011). Substances that can inhibit P-gp and reduce its protective potential are termed chemosensitizers (Kurelec, 1992; Smital and Kurelec, 1998), and include many environmental pollutants (Kurth et al., 2015; Smital et al., 2004).

In rainbow trout and related fish species, P-gp is encoded by two genes: abcb4 and abcb5 (Bieczynski et al., 2021; Luckenbach et al., 2014). The abcb4 protein has more known substrates than abcb5, and more closely resembles mammalian P-gp in structure and function (Fischer et al., 2013). P-gp is expressed in a wide variety of tissue types, particularly absorptive tissues (e.g. intestine), excretory tissues (e.g., liver, kidney), and barrier tissues around sensitive organs (e.g., brain, gonad) (Leslie et al., 2005; Love et al., 2021). In rainbow trout, abcb4 and abcb5 have different tissue expression patterns; most tissues express more abcb4 than abcb5, but a few (kidney, gill, cerebrum, olfactory bulb) express similar amounts of both proteins. The liver expresses only abcb4, with no abcb5 detected (Love et al., 2021).

P-gp is generally located on the apical surface of epithelial cells, directed outwards from the organ or tissue expressing it (Bieczynski et al., 2021). In absorptive and excretory tissues, P-gp exports substrates into an excretory fluid (e.g., bile, feces, urine) (Bieczynski et al., 2021). The gill is capable of acting as both an absorptive and excretory tissue, but P-gp (mostly abcb5) is expressed at very low levels in this tissue, and only in interlamellar cells that are not in direct contact with either the blood or the water (Kropf et al., 2020). To protect sensitive organs, P-gp is expressed in barrier tissues around the organ, with vectorial transport from the sensitive tissue into the blood.

P-gp is an integral part of the blood-brain barrier in fish (Miller et al., 2002) and is expressed in the mammalian and fish eye as part of the blood-retina barrier (Chen, 2020; Kajikawa et al., 1999). The mammalian blood-testis barrier includes P-gp (Su et al., 2009), and P-gp is expressed in mammalian ovaries (Guerreiro et al., 2018), but its role in the ovarian blood-follicle barrier is not well-studied. P-gp is expressed in rainbow trout gonads (Lončar et al., 2010; Love et al., 2021), but its localization to a blood-gonad barrier has not been explored.

The expression, protein distribution, and activity pattern of P-gp suggests an important role in the absorption, distribution, and elimination of its substrates. The significance of P-gp in the pharmacokinetics of its substrates has been well-studied in mammals, but much less so in fish (Leopoldo et al., 2019; Shaikh et al., 2007). Previous studies in rainbow trout and zebrafish have demonstrated behavioural deficits due to the neurotoxic P-gp substrate ivermectin, exacerbated by co-administration of the P-gp inhibitor cyclosporin A ((Kennedy et al. 2014; Azevedo and Kennedy 2022), suggesting altered distribution of ivermectin in the brain. The goal of this study was to investigate the role of P-gp in altering xenobiotic toxicokinetics in fish, as well as the toxicokinetic significance of P-gp inhibition at the whole-animal level. The effects of the competitive P-gp inhibitor cyclosporin A (CsA) on the toxicokinetics of the anthelminthic neurotoxin and model P-gp substrate ivermectin (IVM) in rainbow trout were examined by measuring the concentrations of IVM in the organs and tissues of trout for 7 d following an intravenous administration of IVM, alone or in combination with CsA.

4.2. Methods

4.2.1. Chemicals

Ivermectin (IVM) (>96%), cyclosporin A (CsA) (>95%), NaCl, KCl, $CaCl_2$, $MgSO_4$, $NaHCO_3$, and NaH_2PO_4 were obtained from Sigma-Aldrich (Oakville, ON, Canada). MS-222 (tricaine-S) was obtained from Syndel (Ferndale, WA). Tritiated IVM (^{3}H-IVM) (ivermectin-B1A[22,23-3H], 40-60 Ci/mmol, 1 mCi/mL in ethanol) was purchased from American Radiolabeled Chemicals Inc (St. Louis, MO). Solvable alkaline hydrolysis solubilizer and Ultima Gold liquid scintillation cocktail were purchased from PerkinElmer (Woodbridge, ON).

4.2.2. Fish

Young adult female rainbow trout (*Oncorhynchus mykiss*) (average weight 453 ± 8 g, range 360-616 g) were obtained from Miracle Springs (Mission, BC, Can). Fish were allowed to acclimate for a minimum of 14 d after transport. Fish were housed indoors with a 12h light/12h dark photoperiod. Fish were kept in 2500 L fibreglass tanks containing continuously flowing dechlorinated municipal tap water (pH 7.0, 11 ± 1 °C, hardness 6.1 g/mL $CaCO_3$, O_2 saturation > 90%). Fish were fed twice daily *ad libitum* with Pacific Floating Complete Feed for Salmonids (EWOS Canada Ltd., Surrey, BC, Can), until 2 d before the beginning of the experiment. Fish were not fed for the duration of the experiment. Fasting (7 d) has no effect on P-gp activity in fish, nor on general health or swimming activity (Azevedo and Kennedy 2022).

4.2.3. Solution preparation

Stock solutions of unlabeled IVM (cold-IVM) and CsA in DMSO were prepared and stored frozen at -80 °C. An IVM-only injection solution was prepared by diluting cold-IVM stock solution in fish saline (110 mM NaCl, 2.0 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 1.0 mM $NaHCO_3$, 0.5 mM NaH_2PO_4) (Hoar and Hickman, 1975) to a final concentration of 200 μM (175 μg/L). An IVM + CsA injection solution was prepared by diluting the respective stock solutions in fish saline to a final concentration of 200 μM and 400 μM respectively. This ratio of CsA to IVM inhibits P-gp transport of IVM without detrimental effects to fish (Azevedo and Kennedy, 2022). ^{3}H-IVM was added to both solutions by diluting the stock solution to a final concentration of 1.5 μCi/mL (8.6 μCi/mg IVM). All injection solutions had a final DMSO concentration of 0.02% v/v.

4.2.4. P-gp inhibition

Fish were lightly anesthetized with 80 mg/L buffered MS-222 until gross body movement ceased, but fish continued regular opercular movements. Fish were weighed, and anesthesia maintained by passing water containing 60 mg/L buffered MS-222 over the gills while chemicals were administered. An ^{3}H-IVM solution (0.2 μmol/kg [0.18 mg/kg]) was administered intravascularly (injection volume 1 mL/kg) into the caudal vasculature. The ^{3}H-IVM solution was injected alone (control fish) or in combination with

0.4 μmol/kg (0.48 mg/kg) CsA in the same injection solution to inhibit P-gp in a second treatment group of fish. Following injection, fish were allowed to recover from anesthesia and injections in individual 150L recovery tanks.

Fish were euthanized at various times (0.25, 0.5, 1, 3, 24, 48, 96, 168 h) following injections using a 250 mg/L buffered MS-222 solution. A blood sample (~3 mL) was collected from the caudal vein in a heparinized vacuum tube (BD Vacutainer, Franklin Lakes, NJ) and fish tissues were dissected, weighed, and frozen at -20°C for further tissue analysis (maximum 7 d). The following organs and tissues were collected (whole, unless otherwise specified): liver, kidney, intestine (pyloric sphincter to the cloaca, lumen contents removed), eye (one eye with as much connective tissue removed as possible), gonad (one entire ovary), fat (two strips of visceral fat from the outer surface of the intestine), gill (gill filaments of all 4 gill arches [both sides], removed from the cartilage), and brain (medulla, cerebrum, cerebellum, optic lobe, and olfactory bulb).

4.2.5. Tissue preparation and analysis

Sample preparation methods were adapted from the LSC Sample Preparation by Solubilization methods outlined in PerkinElmer (2008). Samples were defrosted at room temperature immediately prior to preparation. Blood (250 μL), brain and gonads were placed in a 20 mL glass scintillation vial, and 1.0 mL Solvable™ was added to each sample. For blood, 100 μL of 0.1M EDTA was also added to prevent foaming. Hydrogen peroxide (30%, 200-300 μL) was added 100 μL at a time, with gentle agitation after each addition. Samples were incubated at 60 °C for 60 min, then allowed to cool at room temperature overnight. For other tissues, a pre-determined volume (5-15 mL) of fish saline was added to samples and homogenized. A subsample of the tissue homogenate (containing 100 mg liver, 80 mg eye, 60 mg intestine, 40 mg kidney, 30 mg fat, and 20 mg gill) was added to a 20 mL glass scintillation vial and treated as above. 15 mL of liquid scintillation cocktail Ultima Gold™ was added to each prepared tissue sample. Samples were light and temperature adapted for 6 h by storage in the dark at room temperature. Total ^{3}H disintegrations per minute (DPM) in the prepared samples were counted using a Beckman Coulter LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter Inc., Fullerton, CA). The background ^{3}H signal was determined for

each tissue using 3 rainbow trout that had not been injected with ^{3}H-IVM, processed and quantified as described above. The background ^{3}H signal for the corresponding tissue was subtracted from the ^{3}H signal in each experimental tissue sample to determine the specific ^{3}H signal from the injected ^{3}H-IVM.

4.2.6. Toxicokinetic parameters

Toxicokinetic parameters were calculated from tissue concentration-time data using a non-compartmental method assuming a serial sampling design (Wolfsegger and Jaki, 2009). After removing outliers using Dixon's Q test, toxicokinetic parameters were calculated in R (version 4.0.2) using the PK package (version 1.3-5) (Jaki and Wolfsegger, 2020). Maximal concentration (C_{max}) and area under the curve to the last point ($AUC_{0\rightarrow last}$) were calculated for all sampled tissues. Area under the curve to infinity ($AUC_{0\rightarrow\infty}$), mean residence time (MRT), and half-life ($T_{1/2}$) were estimated for tissues that had a decreasing concentration trend for the last 4 time points in their concentration-time curve. Clearance (Cl) and volume of distribution at steady state (VD_{SS}) were estimated using the blood concentration-time data.

4.2.7. Body burden

The percent administered dose was calculated for each tissue using the measured amount in each tissue divided by the total administered amount for each fish. This calculation requires the total mass of the tissue; for most organs/tissues sampled, the sample contained either the entire organ (e.g. liver, kidney, intestine) or one of a pair (e.g., eye, gonad). For blood and fat, only a small portion of the total tissue was sampled, therefore total tissue weight was estimated using published literature values for rainbow trout of similar size. Total blood volume was approximately 30 mL/kg body weight (Conklin et al., 1997), and assuming a density of 1 g/mL, the total weight of blood for each fish was estimated using the conversion factor 0.03 g blood/g fish. Adipose tissue represents approximately 2.6% of total body weight of adult rainbow trout (Dumas et al., 2007), therefore a conversion factor of 0.026 g fat/g fish was used.

4.2.8. Statistical analysis

All statistical analyses were performed using Prism 8 (Version 8.4.3, Graphpad Software LLC., San Diego, CA) unless otherwise noted. For toxicokinetic parameters calculated using the non-compartmental model, the model's mean and standard error output were used to perform further statistical tests. A two-factor ANOVA followed by a Tukey-Kramer test were conducted on C_{max} and $AUC_{0\rightarrow last}$ to detect differences between treatments (control and CsA-inhibited) and tissues (liver, kidney, gill, intestine, eye, gonad, fat, whole brain), and between treatments and brain regions (medulla, cerebrum, cerebellum, optic lobe, olfactory bulb). A two-factor ANOVA followed by a Tukey-Kramer test were conducted on $AUC_{0\rightarrow\infty}$, MRT, and $T_{1/2}$ to detect differences between treatments (control and CsA-inhibited) and tissues (liver, kidney, gill; tissue with declining IVM concentrations in the elimination phase). If no interaction between treatments and tissues was detected, the mean value of the factors was used in the Tukey-Kramer post-hoc test. For blood, a Welch's t test was used to detect differences between treatments (control and CsA-inhibited) in the calculated toxicokinetic parameters ($AUC_{0\rightarrow\infty}$, MRT, $T_{1/2}$, Cl, and VD_{SS}).

The statistical analysis for % administered dose was conducted using JMP (Version 15.0.0, SAS Software, Cary, NC, USA). The whole-body IVM concentration was the total mass of IVM in all sampled tissues divided by the total mass of those tissues. A two-way ANOVA (treatment *x* time) was performed to compare whole-body IVM concentrations, to determine if CsA treatment affected IVM distribution over time. A two-way ANOVA (treatment *x* time) was performed with the fish as a block to compare % administered dose in different tissues (blood, liver, kidney, gill, intestine, eye, gonad, fat, whole brain) and brain regions (medulla, cerebrum, cerebellum, optic lobe, olfactory bulb), to determine whether CsA treatment affected the internal IVM dose over time in these tissues. If a significant effect of time was detected in a tissue, a Dunnett's test was used to compare % administered dose at later time points with the first time point (0.25 h). The mean value of the factor was used in the post-hoc test if no interaction between time and treatment was detected. If a significant effect of treatment was detected in a tissue, a one-way ANOVA was used to compare treatments at each time point to determine the time (s) after IVM administration when CsA treatment affected IVM

distribution. Data are reported as mean ± SE. Differences were considered significant at p=0.05.

4.3. Results

There were no apparent toxic effects observed in fish treated with ivermectin or cyclosporin A in the experiment.

4.3.1. Concentration-time course of IVM

IVM concentration-time curves for each of the sampled organs and tissues (blood, liver, kidney, gill, intestine, fat, gonad, eye, whole brain) are shown in Fig. 4.1A-I. No detectable ^{3}H-IVM was found in the eye in either control or P-gp-inhibited fish. Curves for the other tissues followed one of two general trends: (1) a high initial concentration followed by an exponential decay, and clearance of IVM from the tissue (blood, liver, kidney, gill), or (2) a lower initial concentration with a generally constant IVM concentration through the 7-d sampling period (intestine, fat, gonad, brain). In both control and CsA-treated fish, the liver IVM concentration exhibited a secondary peak at the 3-h sampling point (Fig. 4.1B), suggesting possible enterohepatic re-circulation. The whole-body IVM concentration declined significantly with time (ANOVA, $F_{1,47}$=28.1284, p<0.0001), but was not affected by CsA treatment, nor was there an interaction between time and treatment. For the 5 brain regions (medulla, cerebrum, cerebellum, optic lobe, olfactory bulb), IVM concentration-time curves are shown in Fig. 4.2A-E. Concentration over time in all 5 brain regions followed the second trend, with concentration remaining constant or declining slowly over the 7-d sampling period.

4.3.2. Toxicokinetic parameters

The maximum tissue concentration (C_{max}) achieved was significantly different between body tissues (ANOVA, $F_{7,32}$=15.02, p<0.0001) (Fig. 4.3A). The highest measured C_{max} values for both treatments were found in kidney (2.2±0.6 mg/kg control, 2.1±0.7 mg/kg CsA-treated), followed by the liver (1.5±0.4 mg/kg control, 1.2±0.1 mg/kg CsA-treated). There was no effect of CsA treatment on C_{max}, nor was there an interaction between tissue and treatment. C_{max} was significantly different between brain regions (ANOVA, $F_{4,20}$=8.158, p=0.0005) (Fig. 4.4A). The highest C_{max} value measured

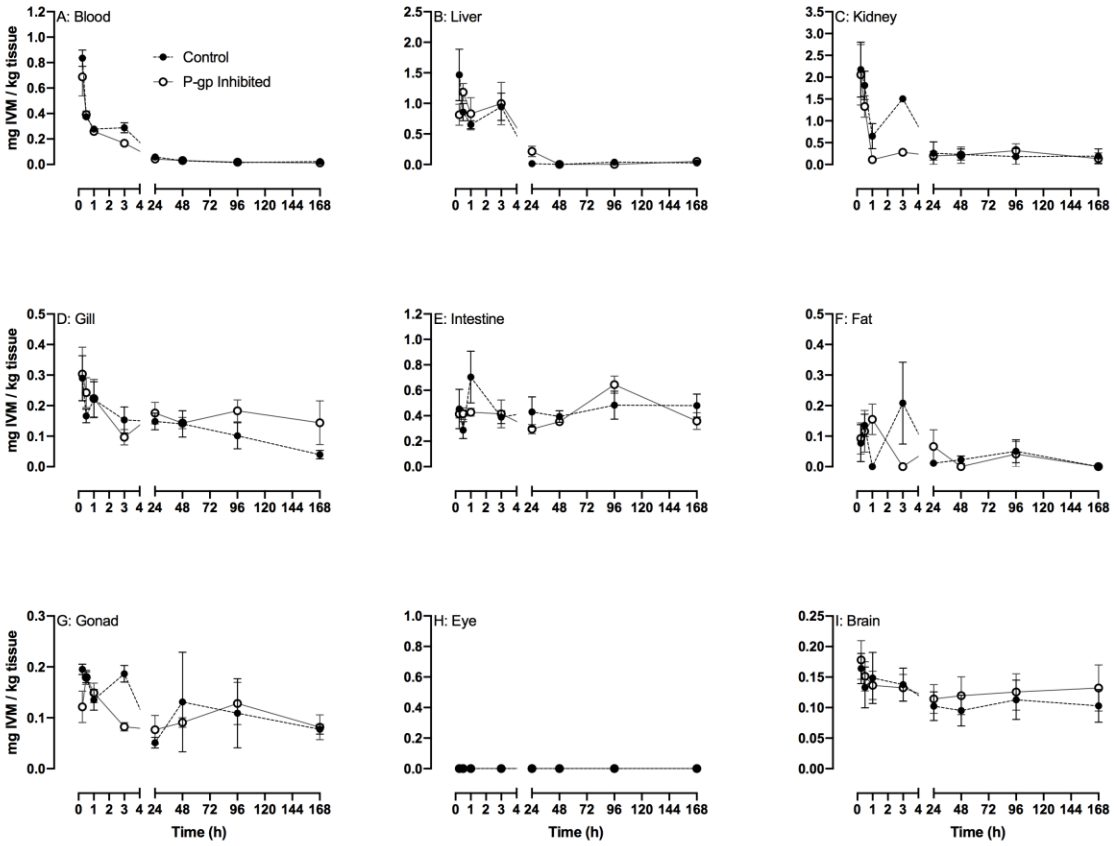

Figure 4.1 Ivermectin concentration vs. time in tissues

Ivermectin concentration vs. time after iv injection of 0.175 mg/kg ivermectin, with (–O–) or without (-●-) the P-glycoprotein inhibitor cyclosporin A (CsA). Data are shown as mean ± SEM. N=3 fish per treatment at each time point.

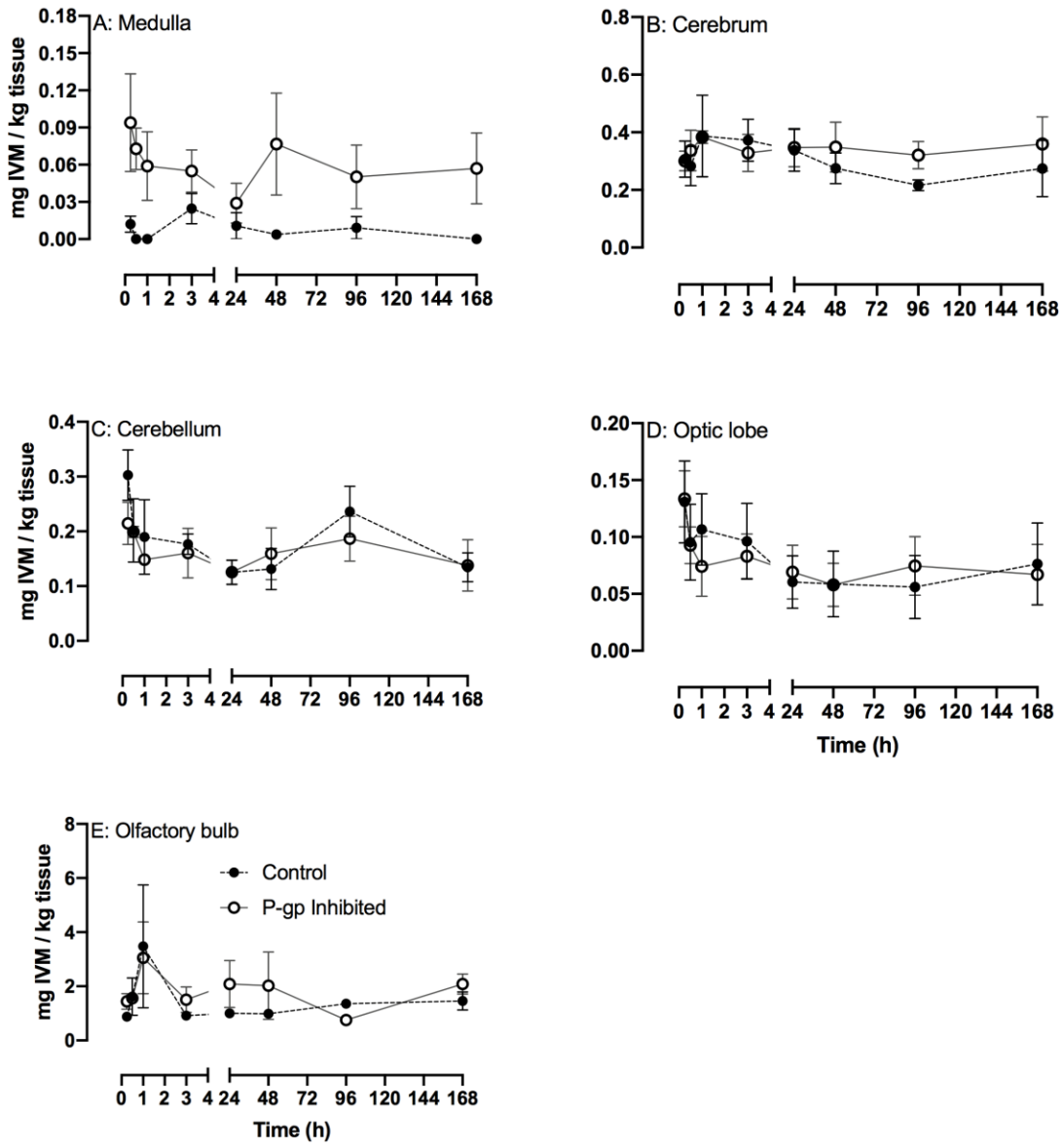

Figure 4.2 Ivermectin concentration vs. time in brain regions
Ivermectin concentration vs. time after iv injection of 0.175 mg/kg ivermectin, with (-O-) or without (-●-) the P-glycoprotein inhibitor cyclosporin A (CsA). Data are shown as mean ± SEM. N=3 fish per treatment at each time point.

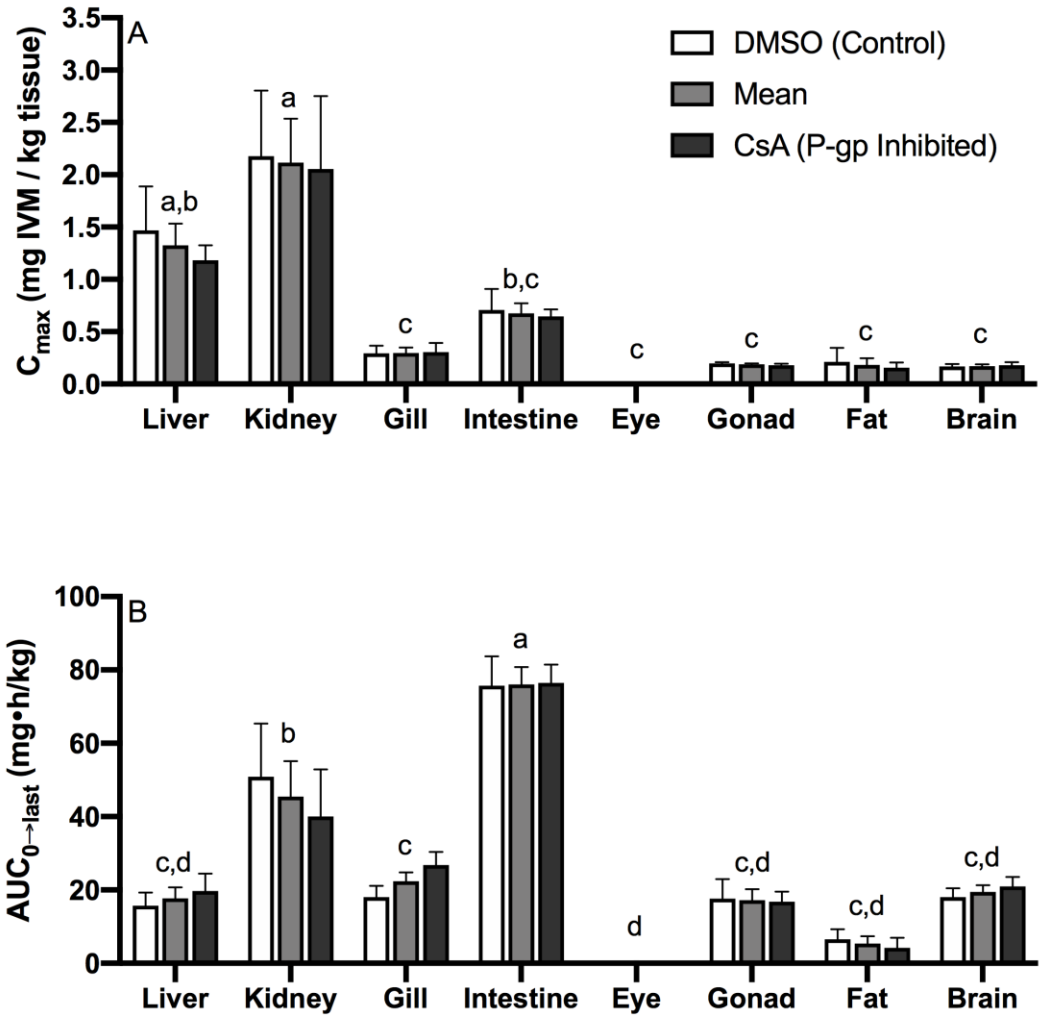

Figure 4.3 Toxicokinetic parameters for tissues

Toxicokinetic parameters for rainbow trout tissues for 7 days of observation after iv injection of 0.175 mg/kg ivermectin, with or without the P-glycoprotein inhibitor cyclosporin A (CsA). There was no effect of CsA treatment on these TK parameters, so tissue comparisons are based on the mean value of control and CsA-treated samples of that tissue. Tissues with the same letter are not significantly different from each other. Tissues that do not share a letter are significantly different from each other ($p<0.05$). Data are shown as mean ± SEM. N=3 fish per treatment at each time point.

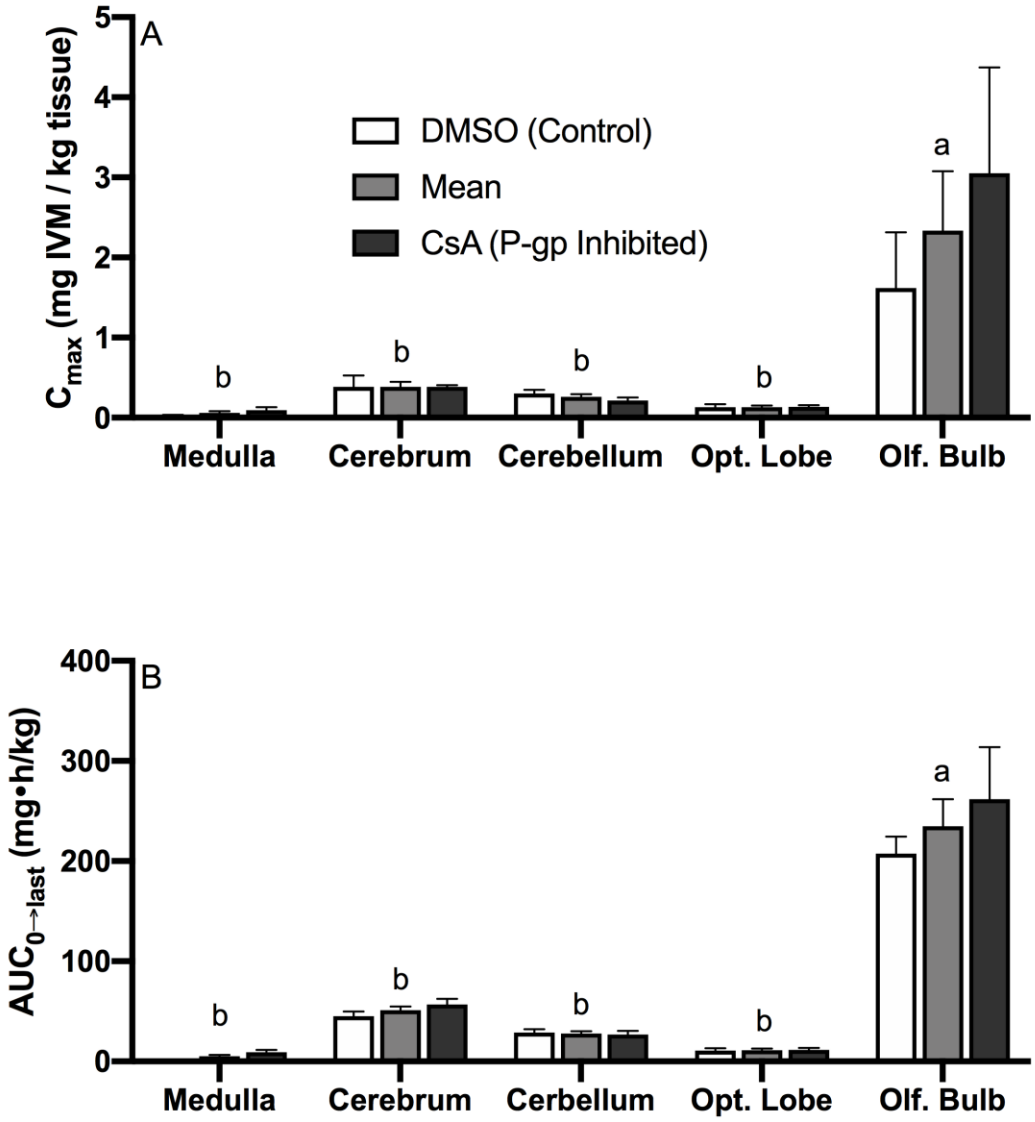

Figure 4.4 Toxicokinetic parameters for brain regions

Toxicokinetic parameters for rainbow trout brain regions for 7 days of observation after iv injection of 0.175 mg/kg ivermectin, with or without the P-glycoprotein inhibitor cyclosporin A (CsA). There was no effect of CsA treatment on these TK parameters, so tissue comparisons are based on the mean value of control and CsA-treated samples of that tissue. Tissues with the same letter are not significantly different from each other. Tissues that do not share a letter are significantly different from each other ($p<0.05$). Data are shown as mean ± SEM. N=3 fish per treatment at each time point.

in the brain was in the olfactory bulb (1.6±0.7 mg/kg control, 3.1±1.3 mg/kg CsA-treated), and the lowest brain C_{max} was in the medulla (0.025±0.012 mg/kg control, 0.094±0.036 mg/kg CsA-treated). There was no effect of treatment on C_{max} in the brain, and there was no interaction between tissue and treatment.

The area under the curve to the last time point ($AUC_{0\rightarrow last}$) (total chemical exposure across time) was significantly different between body tissues (ANOVA, $F_{7,32}=32.62$, $p<0.0001$) (Fig. 4.3B). The largest calculated $AUC_{0\rightarrow last}$ value was in the intestine (75.7±8.0 mg·h/kg control, 76.4±5.0 mg·h/kg CsA-treated), and the lowest $AUC_{0\rightarrow last}$ value was in the eye (0±0 mg·h/kg control and CsA-treated). There was no effect of CsA treatment on $AUC_{0\rightarrow last}$, nor was there an interaction between tissue and treatment. $AUC_{0\rightarrow last}$ was significantly different between brain regions (ANOVA, $F_{4,20}=60.34$, $p<0.0001$) (Fig. 4.4B). The largest $AUC_{0\rightarrow last}$ value in the brain was in the olfactory bulb (208±17 mg·h/kg control, 262±52 mg·h/kg CsA-treated), and the smallest $AUC_{0\rightarrow last}$ was in the medulla (1.2±0.6 mg·h/kg control, 9.2±2.4 mg·h/kg CsA-treated). There was no effect of CsA treatment on $AUC_{0\rightarrow last}$, and there was no interaction between tissue and treatment.

The excretory tissues (liver, kidney, gill) showed a decrease in IVM concentration over the last 4 sampling time points, thus area under the curve to infinity ($AUC_{0\rightarrow\infty}$), half-life ($T_{1/2}$), and mean residence time (MRT) could be estimated (Fig. 4.5A-C). There was no significant effect of tissue or treatment on any of these parameters, and no interaction between tissue and treatment. The mean $AUC_{0\rightarrow\infty}$ ranged from 17.7±3.0 mg·h/kg for liver to 55.9±67 mg·h/kg for gill. Half-life ranged from 17.2±6.8 h in liver to 191±248 h in gill. MRT ranged from 24.9±9.8 h in liver to 276±357 h in gill. In the blood, $AUC_{0\rightarrow\infty}$, $T_{1/2}$, and MRT could be estimated, as well as the clearance (Cl) and the volume of distribution at steady state (VD_{SS}). These parameters are shown in Table 4.1 for both control and CsA-treated fish. There was no significant effect of CsA treatment on any of these parameters in the blood.

Table 4.1 Toxicokinetic parameters in blood

Toxicokinetic Parameter	DMSO (Control)	CsA (P-gp inhibited)	Welch's Test P value
$AUC_{0\rightarrow\infty}$ (mg·h/kg)	11.5±2.5	7.33±0.73	0.2329
Mean Residence Time (h)	125±55	84.7±21	0.5501
Half Life (h)	86.8±38	58.7±15	0.5501
Clearance (L/kg·h)	0.0239±0.0024	0.0152±0.0033	0.1085
Volume of Distribution at Steady State (L/kg)	1.91±0.47	2.02±0.33	0.8541

Calculated toxicokinetic parameters in blood during 7 days of observation after iv injection of 0.175 mg/kg ivermectin. Data are shown as mean ± SEM. N=3 fish per treatment at each time point.

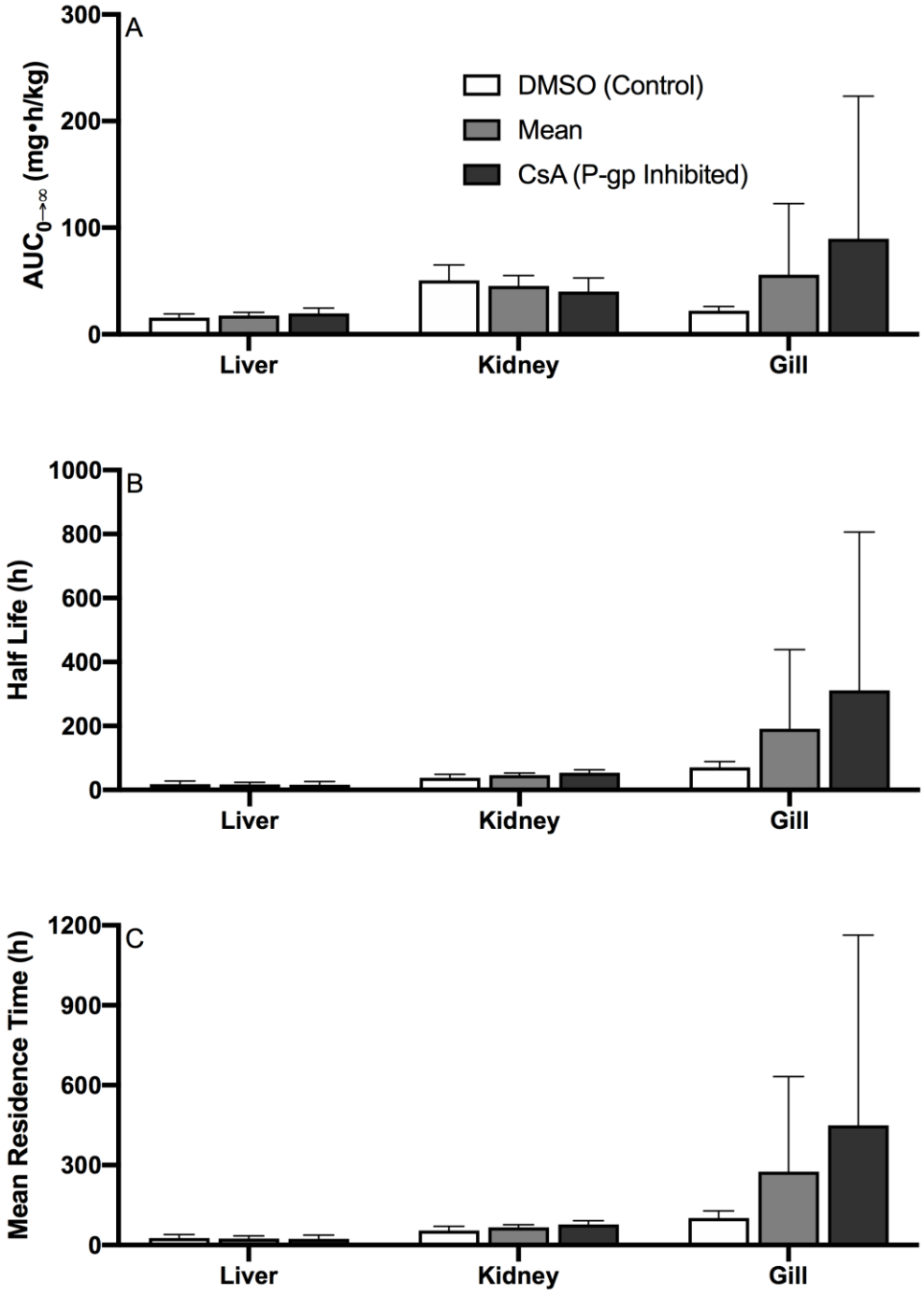

Figure 4.5 Toxicokinetic parameters for excretory tissues
Toxicokinetic parameters for rainbow trout excretory tissues for 7 days of observation after iv injection of 0.175 mg/kg ivermectin, with or without the P-glycoprotein inhibitor cyclosporin A (CsA). There was no effect of CsA treatment on these TK parameters, so tissue comparisons are based on the mean value of control and CsA-treated samples of that tissue. TK parameters did not differ between tissues ($p>0.05$). Data are shown as mean ± SEM. N=3 fish per treatment at each time point.

4.3.3. Percent administered dose

The % administered dose in each tissue and organ is the mass of IVM in the tissue as a percentage of the total mass of administered IVM. The total % administered dose in the sampled tissues declined significantly with time (ANOVA, $F_{7,32}$=29.1683, p<0.0001) (37.4±3.0% to 7.1±1.7% in controls, 33.7±6.8% to 7.1±1.5% in CsA-treated fish), but was not affected by CsA treatment. The % administered dose of IVM in the sampled organs and tissues over the 7-d observation period are shown in Fig. 4.6A-I.

The % administered dose in the blood declined (14.3±1.4% to 0.2±0.05% controls, 11.8±2.6% to 0.2±0.05% CsA-treated) significantly with time (ANOVA, $F_{7,32}$=71.3183, p<0.0001), and was significantly lower in CsA-treated fish compared to controls (ANOVA, $F_{1,32}$=5.6995, p=0.0230). In the liver, kidney, gill, and gonad, the % administered dose declined significantly with time, but was not affected by CsA treatment. Among these tissues, % administered dose was highest in the liver (7.7±2.0% to 0.0±0.0% control, 7.6±0.3% to 0.0±0.0% CsA-treated) and lowest in the gonad (0.11±0.011% to 0.03±0.013% control, 0.11±0.022% to 0.03±0.009% CsA-treated). In the intestine, the % administered dose was consistent over time (3.1±0.6% to 5.9±1.1% control, 3.9±0.7% to 6.7±0.5% CsA-treated), with a significant peak (11.8±4.2% control, 7.2±0.4% CsA-treated) at the 1-hour time point (ANOVA, $F_{7,32}$=2.6104, p=0.0299), but was not affected by CsA treatment. In the adipose tissue and whole brain, there was no effect of time or CsA treatment. The % administered dose was higher in the adipose tissue (0.0±0.0% to 3.1±2.0% control, 0.0±0.0% to 2.3±0.74% CsA-treated) than in the whole brain (0.070±0.015% to 0.042±0.012% control, 0.086±0.018% to 0.051±0.013% CsA-treated). IVM did not enter the eye tissue in any of the fish, so there was no effect of time or CsA treatment on IVM % administered dose in the eye. There was no interaction between treatment and time in any of the sampled tissues.

When the 5 brain regions were analyzed separately, different trends in IVM distribution were evident. The % administered dose of IVM in the brain regions are shown in Fig. 4.7A-E. In the medulla, the % administered dose was not affected by time, but was significantly increased by CsA treatment (ANOVA, $F_{1,32}$=11.4398, p=0.0019). In the other 4 brain regions, the % administered dose was not affected by time or CsA treatment. The % administered dose in the brain was highest in the optic lobe

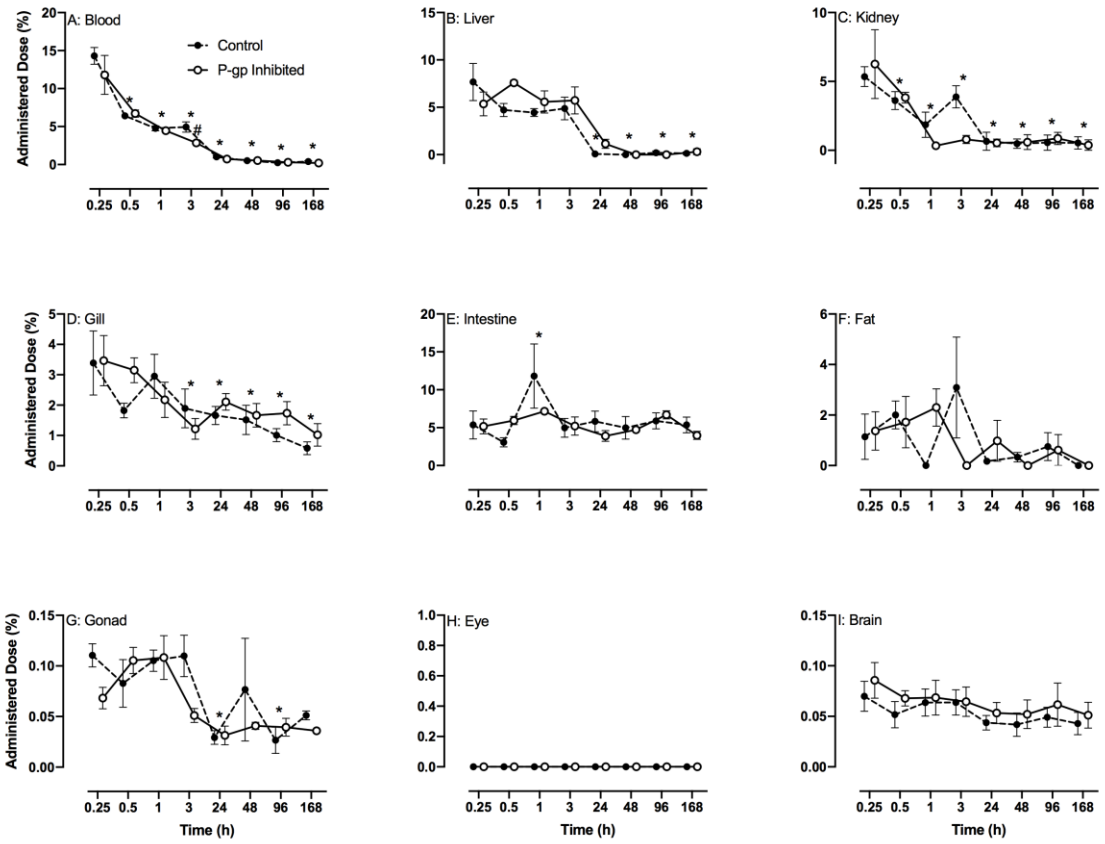

Figure 4.6 Percent administered IVM dose in tissues

Percent administered dose of IVM in rainbow trout tissues for 7 days of observation after iv injection of 0.175 mg/kg IVM, with (-O-) or without (-●-) the P-glycoprotein inhibitor cyclosporin A (CsA). A significant effect of time on % administered dose with respect to the first time point (0.25 h) is shown with asterisks (*): * $p < 0.05$. A significant effect of CsA treatment (P-gp inhibition) on % administered dose is shown with hashtags/pound signs (#): # $p < 0.05$. Data are shown as mean ± SEM. N=3 fish per treatment at each time point.

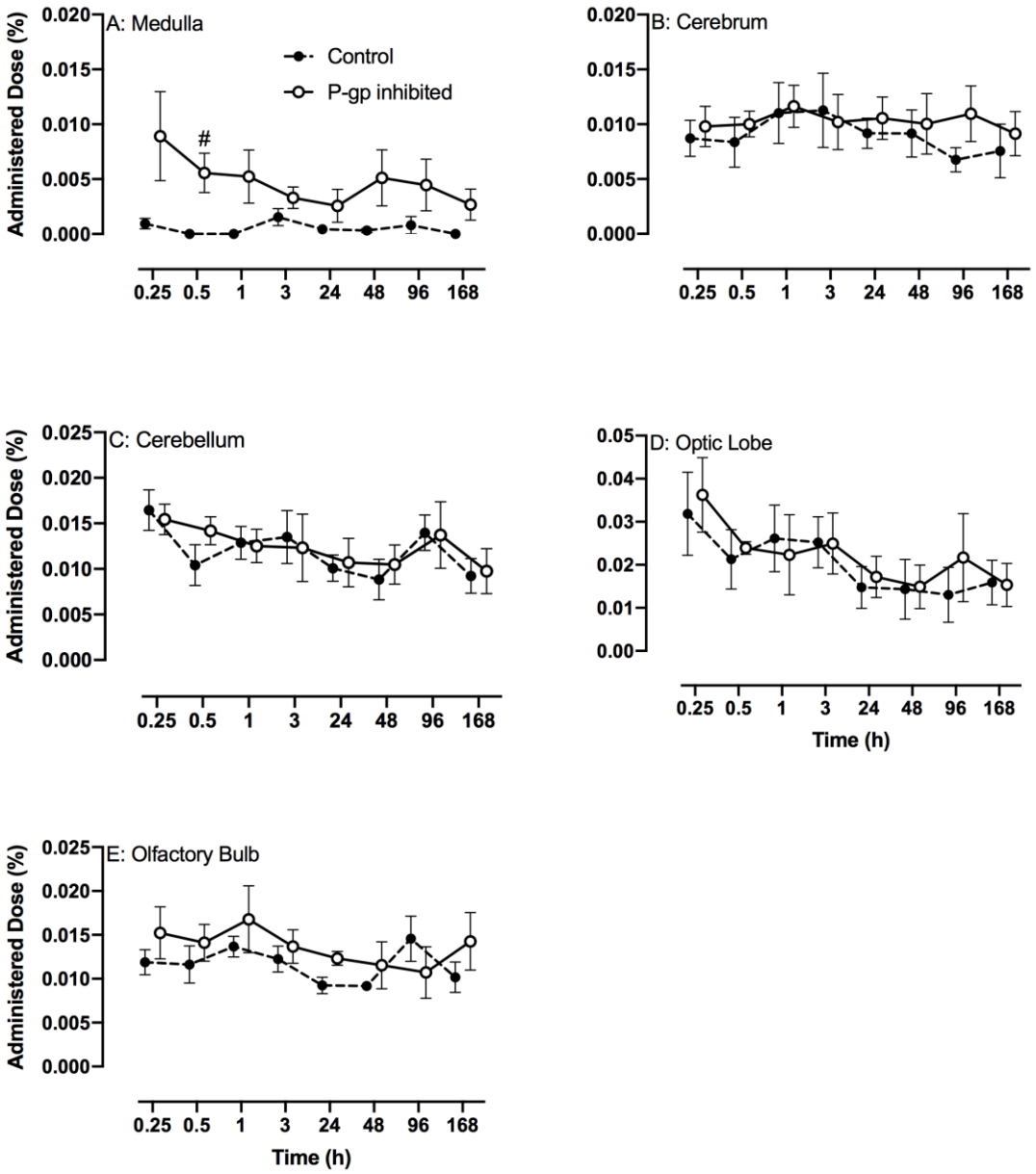

Figure 4.7 Percent administered IVM dose in brain regions

Percent administered dose of IVM in rainbow trout brain regions for 7 days of observation after iv injection of 0.175 mg/kg ivermectin with (–O–) or without (-●-) the P-glycoprotein inhibitor cyclosporin A (CsA). There was no significant effect of time on % administered dose in any of the brain regions. A significant effect of CsA treatment (P-gp inhibition) on % administered dose is shown with hashtags/pound signs (#): # $p < 0.05$. Data are shown as mean ± SEM. N=3 fish per treatment at each time point.

(0.032±0.01% to 0.013±0.006% control, 0.036±0.009% to 0.015±0.005% CsA-treated), and lowest in the medulla (0.0015±0.0008% to 0.0±0.0% control, 0.0089±0.004% to 0.0026±0.001% CsA-treated). There was no interaction between treatment and time in any of the brain regions.

4.4. Discussion

In this study, we measured the concentrations of the P-gp substrate ivermectin (IVM) in tissues known or suspected to express P-gp in rainbow trout, with or without co-administration of the P-gp inhibitor cyclosporin A (CsA). IVM concentration-time data over a 7-d period were analyzed using a non-compartmental method, which, in conjunction with calculated tissue body burden over time, allowed for the determination of the significance of P-gp activity in the distribution and elimination of this P-gp substrate in rainbow trout.

CsA has demonstrated a significant ability to inhibit P-gp transport activity in both mammalian and fish systems (Caminada et al., 2008; Jouan et al., 2016; Zaja et al., 2007). In mammals, CsA has exhibited its inhibitory effect on P-gp through in whole animal studies and cell line investigations (Bauer et al., 2005; Boer et al., 1994; Jouan et al., 2016). For example, in a study conducted with rodents, CsA administration led to a significant increase in the oral bioavailability of P-gp substrates, suggesting a reduction in P-gp activity (Bardelmeijer et al., 2004). Additionally, *in vitro* studies using mammalian cell lines, including Caco-2 cells, have consistently shown that CsA treatment results in decreased efflux of P-gp substrates across cell monolayers (Bleasby et al., 2000; Wu et al., 2000). Similar evidence from studies involving fish species also show CsA to be a potent inhibitor. For example, zebrafish embryos treated with CsA displayed enhanced accumulation of the P-gp substrates rhodamine B and calcein-AM within their tissues (Fischer et al., 2013). Another investigation using rainbow trout hepatocytes demonstrated that CsA treatment led to decreased efflux of P-gp substrates, indicating a substantial inhibitory effect (Johnston and Kennedy, 2023).

The IVM dose used in this study (0.2 μmol/kg) was chosen to be high enough to track its movement through the organism, but not so high that it would cause behavioural deficits. In zebrafish dosed intraperitoneally, 1 μmol/kg IVM was sufficient to decrease swimming speeds, but did not increase time spent immobile or reduce food attraction, as

observed with higher IVM doses (Azevedo and Kennedy 2022). Thus, 0.2 μmol/kg was expected to be below the threshold for behavioural neurotoxic effects in fish. In the same zebrafish study, a 2:1 IVM:CsA molar ratio was sufficient to decrease swimming speeds and increase time spent immobile compared to IVM alone, while higher 2:3 and 2:5 IVM:CsA ratios also decreased food attraction compared to IVM alone (Azevedo and Kennedy 2022). This exacerbation of neurotoxic IMV effects demonstrates that the CsA concentration in the brain was high enough to functionally inhibit IVM transport *in vivo*. Thus, the 2:4 IVM:CsA molar ratio used in this study is sufficient to cause significant P-gp inhibition in target tissues.

In the present study, the total tritium signal from ^{3}H-IVM, which includes both parent IVM and its metabolites was measured; however, in rainbow trout, IVM is metabolized to a limited extent, and excreted mostly as parent compound. For example, in trout dosed orally with IVM, 94% of the IVM in muscle tissue was still the parent compound after 24 h, with 6% of the IVM present as metabolites (Shaikh et al., 2007). This declined to 81% parent IVM after 3 d, and 66% after 7 d (Shaikh et al., 2007). Thus, most of the IVM in the fish was likely present as parent IVM for the entire 7-d exposure period of this experiment. IVM in the bile of Atlantic salmon was 77% parent compound 1 d after administration, then declined to 44% after 3 d, and remained consistent until 14 d post-administration (Høy et al., 1990). Thus, parent IVM is readily eliminated from salmonids, even after being processed through the liver. P-gp transports parent IVM, but generally does not transport its metabolites. All significant effects of P-gp inhibition in this study occurred within 3 h of IVM administration, when nearly all of the IVM in the fish was parent IVM.

CsA is also known to inhibit other efflux transporters, including multidrug resistance protein-1 (MRP1, Abcc1) and breast cancer resistance protein (BCRP, Abcg2), in addition to P-gp (Qadir et al. 2005). These transporters are often expressed in the same cells, and function along with biotransformation enzymes as part of the chemical defense system (Lončar et al. 2010). P-gp transports primarily unmodified substrates, while BCRP and MRPs transport mostly biotransformed substrates as part of phase III of chemical defense (Luckenbach et al. 2014). Considering the limited extent to which IVM is biotransformed in rainbow trout, especially during the first 24 h when all significant effects of CsA treatment were observed in this study, the effects of CsA treatment likely occurred primarily through

P-gp inhibition, rather than through inhibition of MRPs, BCRP, or other phase III transporters.

The toxicokinetic parameters determined for IVM are consistent with those of previous studies in mammals and other fish species. The calculated blood elimination half-life ($T_{1/2}$) in rainbow trout (3.6 d control, 2.4 d CsA-inhibited) was similar to values for various mammal species (e.g. sheep [1.8 d - 7.4 d], cattle [2.8 d - 7.8 d], rabbits [2.0 d], humans [1.5 d]) (Antonić et al., 2011; Elazab and Hsu, 2021; González Canga et al., 2009, 2008; Lifschitz et al., 2010b, 2010a). IVM was eliminated faster in sea bream (0.6 d - 0.9 d) (Katharios et al., 2002) than in the present study for trout. Although a half-life was not calculated, the IVM blood concentration in Atlantic salmon declined slowly, decreasing by less than half from 2 d post-administration to 28 d (Høy et al., 1990).

In this experiment, IVM was injected directly into the caudal vasculature, bypassing the absorption phase of toxicokinetics. When administered orally, IVM is readily absorbed through the intestines of vertebrate animals, although this absorption phase can be slow, and varies with factors such as co-administered food and digestive system type (e.g., ruminant *v*. monogastric) (Chiu et al., 1990; González Canga et al., 2009; Høy et al., 1990; Shaikh et al., 2007). Once IVM has entered the systemic circulation, its distribution to tissues is determined by those tissues' blood perfusion, lipid content, and active transport at blood-tissue interfaces (Rowland and Tozer, 2011).

IVM distribution to excretory and sanctuary tissues depends largely on the blood supply to those tissues. Here, IVM was measured in all sampled tissues except the eye at the first time point (15 min post-injection), demonstrating a rapid and efficient distribution. A similar pattern of rapid distribution to diverse tissues after IVM injection has previously been observed in cattle, sheep, and rats (Chiu et al., 1990). The IVM concentration in the blood showed an exponential decay over time, consistent with distinct distribution and elimination phases (Rowland and Tozer, 2011). In highly-perfused tissues (liver, kidney, gill), IVM accumulation and clearance trends, as well as toxicokinetic parameters ($AUC_{0\rightarrow\infty}$, MRT, and $T_{1/2}$), closely matched those measured in the blood, demonstrating an equilibrium with blood IVM concentrations. In the remaining tissues, IVM distribution was slower, lacking the initial peak seen in liver, kidney, gill, and blood. IVM concentrations remained constant or declined slowly in these tissues over

the exposure period, consistent with expectations for more slowly-perfused tissues (Rowland and Tozer, 2011).

The kidney and liver exhibited the highest maximum IVM concentrations, but the kidney was cleared of IVM more quickly than the liver. The rapid clearance of IVM from the kidney is most likely due to its higher blood flow (Barron et al., 1987). The liver also received prolonged IVM exposure compared to the kidney due to possible enterohepatic re-circulation. This phenomenon is apparent as a secondary peak in IVM concentration in the liver at the 3 h time point. As IVM is removed from the blood by the liver, it is introduced into the intestinal lumen *via* the bile, where it can be re-absorbed and delivered back to the liver through the hepatic portal vein (Okour and Brundage, 2017; Rowland and Tozer, 2011). Enterohepatic re-circulation of IVM has previously been observed in humans and Atlantic salmon (Baraka et al., 1996; Høy et al., 1990).

When IVM is delivered to tissues by perfusion, its partitioning is determined mainly by lipid content. IVM is hydrophobic (log Kow = 3.2), and will preferentially partition into lipid-rich tissues from more aqueous compartments like blood (Liebig et al., 2010). Adipose tissue has the highest lipid content of all tissues (80-95% lipid), followed by the eye (48% lipid) (Manor et al., 2012; Stoknes et al., 2004). The ovary and brain have relatively low lipid content (11-14% and 7%, respectively) (Manor et al., 2012; Stoknes et al., 2004). IVM distribution back into the blood from lipid-rich tissues can occur rapidly by active transport (ABC and SLC transporters at blood-tissue barriers), and more gradually by diffusion as the chemical concentration in the blood decreases during the elimination phase (Rowland and Tozer, 2011). Low IVM concentrations in lipid-rich sanctuary tissues suggests robust protection of that tissue by an active transport barrier, or low perfusion thus minimizing delivery of the lipophilic drug.

The lowest IVM accumulation occurred in the eye, from which IVM was completely excluded. These results differ from Azevedo et al. (2023), in which low IVM concentrations were detected in the eyes of trout using a similar IVM exposure regimen. These results also differ from Høy et al. (1990), who detected IVM in the retina of Atlantic salmon smolts dosed orally with IVM. The eyes of rainbow trout have a high lipid content (Stoknes et al., 2004), making them a likely reservoir for the accumulation of hydrophobic chemicals like IVM. The eyes of trout are also well-supplied with blood directly from the gills *via* the ophthalmic artery (Waser and Heisler, 2004). Fish, like

mammals, possess a blood-retina barrier (Chen, 2020), but it is poorly understood in salmonids. The present results suggest that the blood-retina barrier may provide a robust protection to the eyes of rainbow trout.

In adipose tissue, the IVM concentration was low and remained so throughout the 7-d exposure period. Nearly 50% of fish in both treatment groups had no detectable IVM in their visceral fat. In contrast, pigs that received IVM through subcutaneous injection had higher IVM concentrations in their back fat than in their blood for 21 d following IVM administration (Craven et al., 2002). Likewise, fat had a higher IVM concentration than plasma for 28 d in cattle and sheep, and for 4 d in rats (Chiu et al., 1990). The high lipid content of adipose tissue would make it a favorable reservoir for IVM accumulation with adequate perfusion (Bayen et al., 2005; Manor et al., 2012). Adipose tissue receives relatively little blood flow in rainbow trout due to its low metabolic activity (Edhlund and Lee, 2019; Nichols et al., 1994). The absence or low concentration of IVM in rainbow trout adipose tissue therefore suggests that IVM delivery to adipose tissue is limited by a low perfusion rate in this tissue.

IVM concentration in the trout brain was low, and remained fairly constant post-injection. The lipid content of trout brain tissue is low at 7 g/100 g tissue: this is similar to that of the liver, and lower than found in muscle and ovary (Gélineau et al., 2001; Manor et al., 2012). The brain is highly perfused, and is separated from the systemic circulation by the blood-brain barrier (BBB). The olfactory bulb had the highest cumulative IVM exposure in the brain, having a significantly higher maximum IVM concentration and larger $AUC_{0\rightarrow last}$ than the other brain regions. Any possible differences within the rainbow trout brain with respect to blood flow, lipid content, or BBB protection are not well-studied, thus the reasons for any particular region to accumulate significantly more IVM than other regions are unknown.

In rainbow trout ovaries, IVM concentration was low, and the % administered dose declined toward the end of the sampling period. Rainbow trout ovaries have a moderate lipid content of 11 to 14 g/100 g tissue: higher than brain and liver, and with similar values to muscle (Gélineau et al., 2001; Manor et al., 2012). Mammalian gonads are protected by blood-testis and blood-follicle barriers (Guerreiro et al., 2018; Su et al., 2009), but the existence of such barriers has not been investigated in fish gonads. Rainbow trout ovaries have approximately double the lipid content of the rainbow trout

brain, but IVM cumulative exposures for both tissues were not significantly different. This pattern suggests that the ovary may be better protected from hydrophobic toxicants than the brain in rainbow trout.

The highest cumulative IVM exposure of all sampled tissues was in the intestine, which had the largest $AUC_{0\rightarrow last}$ value. The intestine is an absorptive tissue, exposed to IVM in the blood, as well as in the lumen contents *via* biliary excretion from the liver. During the elimination phase of the experiment (1 – 7 d), most of the IVM that remained in sampled tissues was found in the intestinal tissue. This is possibly due to IVM being introduced into the intestinal lumen through enterohepatic re-circulation (Okour and Brundage, 2017). IVM is excreted mainly *via* the hepatobiliary route; this is the primary (>90%) excretion route for IVM in all mammal species studied to date, as well as in Atlantic salmon (Chiu et al., 1990; González Canga et al., 2009; Høy et al., 1990; Kiki-Mvouaka et al., 2010; Scott and McKellar, 1992). In rainbow trout dosed orally with IVM, the IVM concentration in the bile was the highest of all sampled fluids and tissues from the first sampling point (1 d), reached a maximum at 3 d, and remained high until 7 d post-administration (Shaikh et al., 2007). In the current experiment, the concentration profile of IVM in the intestinal tissue showed evidence of possible enterohepatic re-circulation: there was a significant spike in IVM % administered dose at 1 h post-IVM injection, which was followed by a secondary peak of IVM concentration in the liver (3 h). These local maxima are consistent with intestinal reabsorption followed by biliary excretion of IVM in a complete enterohepatic cycle (Okour and Brundage, 2017).

P-gp inhibition by CsA treatment had no effect on any toxicokinetic parameter for IVM in rainbow trout. This is in sharp contrast to previous work in mammals, in which P-gp inhibition decreased circulating IVM concentrations and prolonged IVM retention in the body. After co-administration of IVM and the P-gp inhibitor loperamide, calves had a higher maximum plasma IVM concentration and larger plasma $AUC_{0\rightarrow last}$, while lambs had a longer elimination half-life and larger plasma $AUC_{0\rightarrow last}$ (Lifschitz et al., 2010b, 2010a). Rabbits co-administered IVM and the P-gp inhibitor verapamil had higher plasma IVM concentrations and higher plasma $AUC_{0\rightarrow last}$, with a greater effect after oral IVM administration than subcutaneous IVM injection (Elazab and Hsu, 2021). P-gp knockout mice administered IVM orally had a larger plasma AUC and slower intestinal

IVM clearance than wild-type mice (Kiki-Mvouaka et al., 2010). Thus, it appears that P-gp plays a much larger role in IVM kinetics in mammals compared to fish.

Despite the lack of effect on IVM toxicokinetic parameters, P-gp inhibition in rainbow trout had small but significant effects on tissue distribution in several tissues. The blood is the central reservoir into which P-gp exports substrates from sanctuary tissues. In P-gp inhibited fish, there was a decrease in % administered dose in the blood compared to controls 3 h after IVM injection. This suggests that more IVM is accumulating in sanctuary tissues in P-gp-inhibited fish than in control fish. This protective effect in rainbow trout is only evident at one time point, during the distribution phase. This small and time-limited effect in fish contrasts with large-scale effects of P-gp inhibition on blood IMV concentrations and elimination kinetics in ruminants, rabbits, and mice (Elazab and Hsu, 2021; Kiki-Mvouaka et al., 2010; Lifschitz et al., 2010b, 2010a). Thus, P-gp is involved in protecting tissues from IVM exposure in trout, but its significance appears to be limited compared to its role in mammals.

In the medulla, the % administered dose was higher in P-gp inhibited compared to control fish. Although this effect was minimal, it may play a protective role against IVM neurotoxicity. IVM can impair swimming performance in rainbow trout, particularly in P-gp inhibited fish (Kennedy et al., 2014). The authors proposed that this exacerbation by CsA treatment resulted from increased IVM penetration into the fish central nervous system (Kennedy et al., 2014), and the present data suggests that the medulla may be the site for this accumulation. However, any protective effects of P-gp in the fish central nervous system are small in comparison to those observed in the mammalian brain. In P-gp knockout mice, for example, the IVM concentration in the brain was 14-fold higher than in wild-type mice 2 h after IVM administration, increasing to 27-fold higher than in wild-type mice after 24 h (Kiki-Mvouaka et al., 2010).

P-gp is expressed in the same tissue types in fish and mammals: high expression in kidney, liver, and intestine to facilitate elimination of substrates from the body, and lower expression in barriers to protect sanctuary tissues (e.g., brain, gonad, eye) from substrate accumulation (Leslie et al., 2005; Lončar et al., 2010; Love et al., 2021). Although P-gp has a smaller effect on IVM kinetics in fish than in mammals, it is still able to protect fish from organismal level toxic effects of IVM. IVM exposure impairs behavioural functions including swimming and motor control in rainbow trout, killifish,

and zebrafish: neurotoxic effects which are exacerbated during P-gp inhibition (Azevedo and Kennedy, 2022; Bard and Gadbois, 2007; Kennedy et al., 2014). Thus, the small alterations to IVM toxicokinetics by P-gp in these fish are able to integrate into protective effects at the whole-animal level during toxicant exposure. This protective function of P-gp is also prioritized during periods of limited energy resources. P-gp activity is maintained during fasting in zebrafish and rainbow trout (Azevedo and Kennedy, 2022; Gourley and Kennedy, 2009), and 4 weeks of starvation induces increased P-gp expression in rainbow trout intestinal epithelia (Baumgarner et al., 2013). Thus, although P-gp protection is less robust in fish than it is in mammals, this protection is still effective and prioritized.

4.4.1. Conclusions

The toxicokinetic profile of IVM is in many aspects similar in fish and mammals. Both animal lineages have a similar range of IVM elimination half-lives, similar toxicokinetic parameters, and evidence of possible enterohepatic re-circulation. IVM in rainbow trout tends to partition into lipid-rich tissues, however several of these tissues (eye, gonad) appear to be well-protected, leading to low or no IVM accumulation: the blood-eye barrier and ovarian blood-follicle barrier may be more robust in rainbow trout than previously thought. P-gp inhibition had a limited effect on IVM tissue distribution, suggesting a smaller but still effective protective role for P-gp in teleosts compared to mammals.

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Consent to participate: all authors consent to participate

Consent for publication: all authors consent to publication

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Chapter 5. Conclusions and future directions

5.1. Summary of results

P-glycoprotein exports a remarkably broad range of xenobiotic and endogenous molecules from cells in a manner that has been compared to a bouncer removing undesirable patrons from a nightclub (Epel et al., 2008). Compounds that are potentially toxic, or that have served their useful purpose and need to be removed, enter P-gp's drug-binding pocket, and are removed from the cell at the expense of ATP hydrolysis. This aspect of chemical defense is common to all of life (Pierman et al., 2018; Rice et al., 2014), but the mechanism is particularly similar within vertebrate lineages of animals (Bieczynski et al., 2021; Luckenbach et al., 2014; Silva et al., 2015).

Mammalian P-gp has been extensively studied, revealing many aspects of its substrate specificity, drug-binding sites, and role in cellular and organismal chemical defenses (Gottesman and Ling, 2006). Investigations into fish P-gp have shown that it functions in a very similar manner to its mammalian counterparts in many respects (Bieczynski et al., 2021; Luckenbach et al., 2014). Differences tend to arise due to divergent evolutionary histories between these animal lineages, including variations in toxicant exposure profiles, and lineage-specific gene duplication events (Luckenbach et al., 2014). Yet, much remains to be discovered about the function and physiological role of P-gp in fish.

5.1.1. Inhibitors of Rainbow Trout P-glycoprotein

Rainbow trout P-gp is inhibited by similar chemosensitizers, mostly at similar concentrations, as mammalian P-gp. All four chemosensitizers tested in this research (cyclosporin A, quinidine, valspodar, and verapamil) operate with a competitive inhibition mechanism, indicating that they are also substrates of rainbow trout P-gp. This suggests that fish and mammalian P-gp have closely matched substrate bases, which reflects the similar protein structures and physiological functions of P-gp in these two animal lineages.

Despite these broad similarities, some P-gp substrates have different physiological roles in fish compared to mammals. Cortisol, for example, interacts with

three different nuclear receptors in fish, as opposed to one receptor in mammals (Bury, 2017; Bury and Sturm, 2007; Denver, 2009). These receptors are activated at different intracellular cortisol concentrations, thus necessitating tighter control of cortisol accumulation and efflux in fish than in mammals (Bury, 2017; Bury et al., 2003; Sturm et al., 2005). Part of this cortisol regulation is performed by P-gp, which has a higher binding affinity for cortisol in fish than it does in mammals.

5.1.2. Substrates of Rainbow Trout P-glycoprotein

Fish and mammalian P-gp have similar binding sites and affinities for doxorubicin, R123, and vinorelbine, further echoing the similarities in structure and function of P-gp in different classes of vertebrate animals. Rainbow trout P-gp has a higher binding affinity for cortisol than mammalian P-gp does, reflecting that P-gp structure has evolved to serve different and specific physiological roles for some substrates, where these differences exist between animal lineages. The interaction(s) of berberine with membrane transporters in rainbow trout hepatocytes could not be determined in this project. This exposes the complexity of the interconnected system of importers and exporters expressed together in animal cells, and the difficulties in determining specific transporter functions experimentally.

5.1.3. P-glycoprotein Inhibition in Ivermectin Toxicokinetics

P-gp inhibition caused small measurable changes in the tissue distribution of the substrate ivermectin in the blood and medulla of rainbow trout. These results suggest that P-gp provides effective protection of sensitive tissues, especially the brain, from accumulation of toxic substrates. These protective effects have been shown at the whole organismal level by preventing behavioural deficits during neurotoxicant exposure (Azevedo and Kennedy, 2022; Bard and Gadbois, 2007; Kennedy et al., 2014), and are prioritized even during times of limited energy resources (Baumgarner et al., 2013; Gourley and Kennedy, 2009). However, the protective effects of P-gp in the central nervous system (CNS) of fish is much less robust than it is in the CNS of mammals (Elazab and Hsu, 2021; Kiki-Mvouaka et al., 2010). This difference may be a consequence of differing life histories between fish and mammals, where longer-lived animals may prioritize learning and memory with strong neuroprotection, while shorter-

lived animals expend more detoxification resources on reproduction (Costantini et al., 2010).

5.2. Future Directions

5.2.1. SLC transporters in cultured cells

Epithelial cells, including hepatocyte primary cultures, express a set of ABC exporters and SLC importers. Transporters from the Abcb, Abcc, Abcg, SLC21, and SLC22 families are commonly expressed on opposite sides of the same epithelial cells (Bolten et al., 2022; Muzzio et al., 2014; Romersi and Nicklisch, 2022). These co-expressed import and export proteins share a very similar set of substrates and inhibitors, as is expected for an integrated system that controls the movement of compounds across barriers (Mihaljević et al., 2017; Nigam, 2015; Popovic et al., 2014; Willi and Fent, 2018). Studies using cultured animal cells usually use intracellular substrate accumulation as the measurable endpoint to examine trans-membrane efflux. These experiments often account for specific transporter types by using nominally specific transport inhibitors like verapamil (P-gp [Abcb] inhibitor), MK571 (MRP [Abcc] inhibitor), and tetraethylammonium (SLC22 inhibitor) (Bieczynski et al., 2014; Sturm et al., 2001). Inhibition of an exporter results in increased intracellular substrate accumulation, while inhibition of an importer causes decreased intracellular accumulation.

Despite their co-expression in the same cells and broad overlap of substrates and inhibitors, ABC and SLC transporters are often studied in complete isolation from each other, by separate research groups (Nigam, 2015). This narrow focus on either importers or exporters can lead to potential misinterpretation of experimental results: inhibition of an importer and stimulation of an exporter will produce the same change in intracellular substrate accumulation, and *vice versa*. For example, in a study of MPP+ transport in human embryonic kidney cells, a set of inhibitors including quinidine, cimetidine, and cyclosporin A caused increases in intracellular substrate accumulation (Floerl et al., 2020). The researchers interpreted these results as stimulation of the SLC22 importer OCT1 by an as-yet undetermined mechanism (Floerl et al., 2020). However, MPP+ is a substrate of ABC transporters including P-gp (Bleasby et al., 2000),

and quinidine, cimetidine, and cyclosporin A are known P-gp inhibitors (Stott et al., 2015). Thus, inhibition of P-gp would also produce the observed effects.

When examining the transport of P-gp substrates in rainbow trout hepatocytes, I encountered a similar type of unexpected result (Chapter 3) (Johnston and Kennedy, 2023a). The inhibitor verapamil caused a concentration-dependent decrease in intracellular berberine accumulation. This result was interpreted as a stimulation of P-gp-mediated berberine export, but it could also be attributed to the inhibition of an SLC import protein. Berberine is a substrate of SLC22 transporters including OCT1 (Mihaljević et al., 2017; Nies et al., 2008), verapamil is a known inhibitor of OCT1 (Tzvetkov et al., 2013; Zhou et al., 2021), and fish express high levels OCT1 in their hepatocytes (Mihaljevic et al., 2016). Since the goal of my study was to gain insight into the function of an ABC transporter, I proposed a mechanism that only included ABC transporters to the exclusion of other transporter types, even though the involvement of other transporters was a possible explanation.

There are several experimental techniques that allow researchers to distinguish between import and export of the same substrate in the same cells. Vectorial transport assays involve harvesting intact epithelial tissues (eg., intestinal wall, renal tubules, brain capillaries) or culturing isolated epithelial cells on 3-dimensional culture inserts (Bieczynski et al., 2014; Miller, 1987; Miller et al., 2002; Nies et al., 2008). In simple suspension or adhesion cultures, the polarity of epithelial cells is disrupted, and there are only two compartments in which the substrate concentration can be measured: intracellular and extracellular. In vectorial cultures, cell membrane polarity is preserved, and there are three compartments: apical (extracellular), intracellular, and basolateral (extracellular). This type of cell or tissue culture allows for the measurement of directional fluxes: apical-to-basolateral and basolateral-to-apical, which will be affected differently by inhibition of importer and exporter proteins. In combination with specific inhibitors for different transporters, this technique provides much more detailed information about the role of transporters in fluxes across epithelial barriers.

5.2.2. Endogenous P-gp substrates and inhibitors

Most studies of P-gp in fish have focused on xenobiotic substrates and inhibitors, and the multixenobiotic resistance (MXR) phenotype that allows them to survive in

contaminated environments. Very few endogenous compounds are known to interact with fish P-gp: cortisol and testosterone are P-gp substrates, and the bile acid taurochenodeoxycholate is a P-gp inhibitor (Johnston and Kennedy, 2023b; Zaja et al., 2011). Mammalian P-gp has a broad range of known endogenous substrates including peptides (eg., enkephalins), lipids (eg., cholesterol, phospholipids), and steroid hormones (eg., aldosterone, cortisol, testosterone) (Abulrob and Gumbleton, 1999; Dagenais et al., 2001; Wang et al., 2000; Yano et al., 2019; Zhou, 2008). P-gp's involvement in controlling intracellular concentrations of these substrates gives this transporter an important role in neurotransmission, lipid metabolism, and hormone signalling. P-gp likely has a similar set of endogenous functions in fish that have yet to be identified.

Steroid hormones appear to be a promising area for discovery of endogenous substrates of fish P-gp. Two have been identified to date: cortisol and testosterone (Johnston and Kennedy, 2023b; Zaja et al., 2011), and P-gp expression patterns provide evidence that there may be more. Zebrafish ovaries express high levels of abcb5 during the early pre-vitellogenic stages of egg development, but not at later stages (Robey et al., 2021). This suggests that fish P-gp, especially abcb5, transports hormones that stimulate early oocyte maturation. Possible candidate hormones are the steroid estradiol, and the associated peptides follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Nagahama and Yamashita, 2008; Patiño and Sullivan, 2002).

P-gp is likely involved in cell differentiation in fish as well. In rainbow trout gills, immature progenitor cells that mature into respiratory cells express high levels of abcb5, but mature gill epithelial cells do not express P-gp (Goss et al., 1992; Kropf et al., 2020). Abcb5 is also associated with cell differentiation in mammals (Frank et al., 2003). This expression pattern suggests that fish P-gp is involved in the transport of endogenous signalling molecules, including growth factors and hormones, that control cell differentiation. In particular, cytokines, growth hormone, and insulin-like growth factors are possible substrates of fish P-gp (Hanington et al., 2009; Koganti et al., 2021; Schulz et al., 2010).

Compounds involved with digestion represent further possible endogenous P-gp substrates in fish. P-gp is highly expressed in bile canaliculi in the liver, as well as in the intestine, especially the distal intestine (Lončar et al., 2010; Love et al., 2021).

Mammalian P-gp is known to transport a range of bile acids (Wang et al., 2009), but only a single bile acid is known to interact with fish P-gp (Zaja et al., 2011). P-gp expression in the rainbow trout intestine increases during starvation (Baumgarner et al., 2013), suggesting that P-gp is involved with regulating intestinal lumen contents even in the absence of food. Bile acids are possible target substrates of this P-gp induction, since they are important inter-organ signalling molecules, regulating metabolic processes in the liver including gluconeogenesis and lipogenesis (Nigam et al., 2020). Starvation causes significant changes in gut microbiome composition in fish, and can increase the risk of infection by opportunistic pathogens present in the intestine (Xia et al., 2014). These simultaneous changes (P-gp induction and microbiome alterations) suggest that P-gp may be involved in the interface between intestinal microflora and their fish hosts, which is another known function of bile acids (Nigam et al., 2020).

P-gp and other chemical defense proteins are conventionally studied in the context of the absorption, distribution, metabolism, and excretion (ADME) of xenobiotic compounds. However, the ubiquity of these proteins in all branches of life suggests that their evolutionary importance lies primarily in endogenous functions (Nigam, 2015). An emerging understanding of the primary functions of chemical defense proteins, termed the Remote Sensing and Signalling Network (RSSN), frames these proteins as a small molecule communication system, linking all levels of biological organization: molecules, organelles, cells, tissues, organs, and the whole body, to maintain homeostasis, and return to homeostasis after disturbances (Nigam et al., 2020). It also incorporates inter-individual (eg., mother/nursing infant) and inter species (eg., plant/pollinator, gut flora/host) interactions that are known to be facilitated by trans-membrane transporters (Nigam, 2015; Nigam et al., 2020). The interactions of drugs and other xenobiotic molecules with this system can help to predict off-target effects of drugs, and may aid in identifying the physiological effects of toxicant exposure (Nigam et al., 2020).

5.2.3. Chemosensitizers in Environmental Risk Assessment

While the research reported in this thesis was primarily conducted from a basic research perspective (i.e. not strictly applied), one of the main practical applications of the resulting information is in assessing the risks of toxicant exposure to fish living in contaminated environments. Valid risk assessments require a broad range of data, including physiological effects, effect concentrations, and exposure routes of toxicants.

Inhibitors of P-gp and other MXR proteins are a particularly under-studied class of environmental contaminants. These compounds decrease the effectiveness of the chemical defense system, allowing substrate toxicants to have harmful effects at lower concentrations than during single exposures, a process referred to as chemosensitization (Faria et al., 2016; Kurth et al., 2015; Müller et al., 1998; Smital et al., 2004; Wu et al., 2015). Chemosensitizers increase the toxicity of toxicant mixtures, therefore their effects should be accounted for in environmental risk assessments (Kurth et al., 2015).

While the chemosensitizing effects of environmental contaminants have been increasingly recognized as a risk to aquatic organisms, relatively little data is available regarding the effects of chemosensitizer exposure. Many studies of chemosensitizers include only a simple confirmation of effect without investigating concentration-response relationships (Kurth et al., 2015). Even in studies that report concentration-response data, effect concentrations are regularly overestimated. Many reported IC50 concentrations for chemosensitizers exceed the water solubility of those chemicals, often by several orders of magnitude (Kurth et al., 2015). Thus, the chemosensitizing effects observed in those studies occurred at much lower aqueous concentrations than reported, and harmful concentrations of those chemosensitizers would be considered safe in risk assessments relying on flawed data.

To improve our understanding of chemosensitizers as contaminants in aquatic environments, exposure studies must measure aqueous dissolved chemosensitizer concentrations, rather than relying on nominal concentrations. Chemosensitizing effects should be investigated in animals exposed to chemosensitizers through realistic exposure routes, including through water, food, and sediment. Combinations of chemicals that often occur together in the environment (eg., agricultural chemicals used in close proximity, industrial contaminants in the same effluent) should be investigated for chemosensitizing effects during co-exposure. To facilitate the identification of sub-lethal toxic effects, measurable endpoints should represent systemic manifestations of known *in vitro* effects of P-gp substrates and inhibitors. More accurate predictions of systemic effects will be possible as more information becomes available about the endogenous functions of P-gp.

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