REGULATION OF P-GLYCOPROTEIN ACTIVITY BY OXIDATIVE STRESS IN ISOLATED RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) HEPATOCYTES

by

Timothy Kimmerly Gray
B.Sc. (Biology), University of Ottawa, 2005

PROJECT SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF ENVIRONMENTAL TOXICOLOGY

In the
Department of Biological Sciences
Faculty of Science

SIMON FRASER UNIVERSITY

Fall 2011

APPROVAL

Name: **Timothy Gray**

Degree: **MASTER OF ENVIRONMENTAL TOXICOLOGY**

Title of Thesis: **REGULATION OF P-GLYCOPROTEIN ACTIVITY BY OXIDATIVE STRESS IN ISOLATED RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) HEPATOCYTES**

Chair: **Dr. G.J. Gries,** Professor, Department of Biological Sciences, S.F.U.

Dr. C.J. Kennedy
Senior Supervisor
Professor, Department of Biological Sciences, S.F.U.

Dr. R.A. Nicholson
Supervisor
Associate Professor, Department of Biological Sciences, S.F.U.

Dr. G.L. Rintoul
Supervisor
Associate Professor, Department of Biological Sciences, S.F.U.

Dr. A. Vieira
External Examiner
Associate Professor, Department of Biomedical Physiology and Kinesiology, S.F.U.

Date Defended/Approved: 16 December 2011

Declaration of
Partial Copyright Licence

SFU SIMON FRASER UNIVERSITY
THINKING OF THE WORLD

STATEMENT OF ETHICS APPROVAL

The author, whose name appears on the title page of this work, has obtained, for the research described in this work, either:

(a) Human research ethics approval from the Simon Fraser University Office of Research Ethics,

or

(b) Advance approval of the animal care protocol from the University Animal Care Committee of Simon Fraser University;

or has conducted the research

(c) as a co-investigator, collaborator or research assistant in a research project approved in advance,

or

(d) as a member of a course approved in advance for minimal risk human research, by the Office of Research Ethics.

A copy of the approval letter has been filed at the Theses Office of the University Library at the time of submission of this thesis or project.

The original application for approval and letter of approval are filed with the relevant offices. Inquiries may be directed to those authorities.

Simon Fraser University Library
Simon Fraser University
Burnaby, BC, Canada

Last update: Spring 2010

ABSTRACT

To investigate the hypothesis that oxidative stress (OS) can regulate P-glycoprotein (P-gp) function in isolated rainbow trout hepatocytes (*Oncorhynchus mykiss*), cell suspensions were exposed *in vitro* to the pro-oxidant treatments diethyl maleate (DEM) or hydrogen peroxide (H_2O_2). Cellular glutathione (GSH) concentrations were depleted following treatment with 2.5 mM DEM and increased by treatment with 2.0 mM GSH. P-gp activity was assessed by measuring the accumulation of the P-gp substrate rhodamine 123 following treatment with either H_2O_2, DEM or GSH. Initial rates of R123 accumulation by hepatocytes treated with 0.025 to 2.5 mM DEM, 0.02 to 2.0 mM GSH, or 3 to 1200 µM H_2O_2 were not significantly different from their respective controls. The findings of this study suggest that P-gp activity in rainbow trout hepatocytes is not acutely modulated by increases in reactive oxygen species or changes in GSH content *in vitro*.

Keywords: P-glycoprotein; oxidative stress; hydrogen peroxide; glutathione; diethyl maleate; rainbow trout

DEDICATION

This thesis is dedicated entirely to my mother, and my father.

ACKNOWLEDGEMENTS

This work was supported by an NSERC discovery grant to Dr. Christopher J. Kennedy.

I would like to acknowledge my colleagues in the Kennedy lab, the MET program and the Biology Department at Simon Fraser University. Reza Babajani is thanked for his generous assistance and instruction on the use of his spectrofluorometer. I would especially like to thank Dr. C. J. Kennedy for giving me the opportunity to work in his lab and for being a mentor throughout my years at Simon Fraser University.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABC	ATP binding cassette
ABCA1	ATP binding cassette A
ABCB1	ATP binding cassette B, also refers to MDR1
ABCC	ATP binding cassette C, also refers to MDR1, also refers to the multidrug resistance associated protein (MRP)
ABCG1	ATP binding cassette G, also refers to the breast cancer drug resistance protein (BCRP)
AhR	Aryl hydrocarbon receptor
ATP	Adenosine triphosphate
BCRP	Breast cancer drug resistance protein, coded for by the ABCG1 gene
β-NADH	β-nicotinamide adenine dinucleotide
DEM	Diethyl maleate
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HSS	Hank's salt solution
LDH	Lactate dehydrogenase
MDR	Multidrug resistance
MRP	Multidrug resistance-associated protein
MS 222	3-aminobenzoic acid ethyl ester

MXR	Multiple xenobiotic resistance
nF-κB	Nuclear factor-κ B
P-gp	P-glycoprotein
PXR	Prenane-X-receptor
R123	Rhodamine 123
ROS	Reactive oxygen species
RXR	Retinoid-X-receptor
OS	Oxidative stress
TTX	Triton X-100

1: INTRODUCTION

1.1 Chemical defense mechanisms

The potential for xenobiotics and endogenous metabolites to cause cellular toxicity is affected by multiple toxicokinetic and toxicodynamic factors. Toxicodynamics refers to the interactions of the toxicant within the organism, especially at sensitive sites, while toxicokinetics refers to those factors that contribute to the disposition and accumulation of these compounds. Within this context, biotransformation reactions that reduce chemical accumulation can be classified as either Phase I functionalization or Phase II conjugation reactions (Williams, 1971). Phase I enzymes are primarily microsomal and include the cytochrome P450 family of enzymes which catalyses the NADPH-dependant oxidation of a wide variety of xenobiotics and endogenous molecules. In general, Phase 1 biotransformation reactions add or expose a functional group onto the chemical, producing a more hydrophilic metabolite. Further increases in the hydrophilicity of metabolites results from the actions of cytosolic Phase II conjugating enzymes such as glutathione transferases (GST) which covalently link an endogenous molecule, specifically glutathione (GSH) with the endogenous cellular metabolite or xenobiotic substrate. GSH conjugation followed by mercapturic acid biosynthesis renders the Phase II metabolites even more hydrophilic. These conjugates are transported from the cytosol to the outside of the cell by several different classes of transport proteins including the

multidrug resistance associated protein (MRP) family of transmembrane efflux proteins (Rebbeor, 2000) and the recently described RLIP76 (Ral-interacting protein) (Singhal, 2007).

Another important transmembrane transport protein that mediates the cellular efflux of a wide variety of xenobtiotics is P-glycoprotein (P-gp). However, unlike the MRP family of proteins, P-gp removes moderately hydrophobic xenobiotics from the plasma membrane without the requirement of prior biotransformation. Transmembrane efflux proteins including the MRP family and P-gp are often referred to as the Phase III line of cellular defence (Xu, 2005). By transporting unmetabolized xenobiotics, P-gp provides an important 'first line' or 'front line' of chemical defence against foreign compounds (Epel, 2008; Callaghan, 2008; Bard, 2000).

1.2 The evolution of xenobiotic efflux transporters

Cellular mechanisms of resistance to cytotoxic chemicals have been described and studied in both bacterial and eukaryotic cells (Saves, 1998). The production of compounds that are deleterious to the health of competitors, predators and prey is well documented in multiple taxa; these toxic compounds include plant secondary metabolites, animal venoms, fungal and bacterial cyclic peptides. Some compounds (venoms) may be directly injected into prey organisms while other compounds may be released into the surrounding environment as a means of deterring competitors. The latter example of toxin dispersal and chemical defence has been reviewed in the context of benthic, sessile marine invertebrates that must compete with a constant flow of pelagic

propagules for surfaces to colonize (Krug, 2006). Allelopathy in fresh water cyanobacteria and terrestrial plants provides another example of environmentally dispersed secondary metabolites adversely affecting the growth and survival of competitors (Leão, 2009; Whittaker and Feeny, 1971).

The recent development of multidrug resistance in pathogenic bacterial strains, pesticide resistant insects and drug resistant human tumours can be conceptualized in terms of a co-evolutionary chemical arms race between humans and our non-human competitors. Through technological advances, humans have continued this chemical arms race with the design, mass production and environmental dispersal of chemicals intended to kill organisms that might eat our food or cause disease. Research into the mechanisms by which organisms develop and maintain chemical resistant phenotypes is an increasingly important field because the ever increasing human population has become reliant on a small number of compounds to protect agricultural crops, livestock and ourselves from phylogenetically diverse competitors and pathogenic organisms (Taylor and Feyereisen, 1996).

Other anthropogenic inputs into the natural environment include industrial and domestic wastewater which may contain a wide array of anthropogenic chemicals designed to be effective at very low concentrations.

Within a population of organisms that are continuously exposed to foreign compounds, individuals that are able to most effectively excrete accumulated xenobiotics may hold a selective advantage over conspecifics having a lesser ability to prevent xenobiotic accumulation. Fitness consequences at various

levels of biological organization have been linked to xenobiotic exposure; for example mummichogs (*Fundulus heteroclitus*) inhabiting contaminated environments have been found to exhibit altered feeding habits (Goto, 2011); flounder (*Platichthys flesus*) populations from polluted environments have been found to have a lower average fecundity, growth rate and condition factors when compared with flounder from less polluted habitats (Marchand, 2004). There are frequent reports that organisms inhabiting polluted environments have an increased ability to prevent the accumulation of xenobiotics (Smital, 1998). There is considerable evidence that points towards increased cellular efflux capabilities in these organisms as the mechanism; this efflux capacity is mediated by transmembrane efflux transport proteins that provide a protective mechanism against the accumulation of natural cytotoxic and membrane disrupting compounds (Smital, 1998; Higgins, 2007).

1.3 The multidrug resistance, multixenobiotic resistance phenotype

The multidrug resistance, multixenobiotic resistance phenotype (MDR/MXR) phenotype has been defined as 'the simultaneous acquisition of resistance to many chemically unrelated compounds to which the cell has never been exposed' and is regarded as one of the most challenging questions in oncology (Higgins, 2007; Shtil, 2005). P-gp is a transmembrane efflux protein that mediates the cellular efflux of many structurally diverse, moderately hydrophobic compounds including many clinically important drugs (Ford and Hait, 1990; Ambudkar, 2003). Increased expression of P-gp at the cell surface is considered to be one of the most important contributors to MDR in human cancer

cells (Callaghan, 2008). The existence of this protein was first described by Ling and Juliano (1976) who noted the increased expression of a 170 kDa protein on the surface of Chinese hamster ovary cells that had developed a resistant phenotype to the cytotoxic effects of a wide variety of structurally unrelated compounds.

Consideration of the cellular and organism level expression pattern of P-gp has lead researchers to compare P-gp to a 'first line' and 'front line' of chemical defence against ingested compounds (Epel, 2008; Callaghan, 2008; Bard, 2000). P-gp is highly expressed on the apical surface of epithelial cells of the gastro-intestinal tract and the apical surface of endothelial cells lining brain capillaries that contribute to the blood-brain barrier in humans (Ambudkar, 1999). It has been suggested that the physiological role of P-gp is to prevent the cellular accumulation of naturally occurring toxins (Klaassen, 2005). This P-gp mediated ability to remove potentially deleterious chemicals from the cell membrane, prior to accessing the cytosol, is adaptive, as it prevents the accumulation of endogenous compounds and xenobiotics which may interfere with cellular processes if allowed to accumulate beyond threshold concentrations.

It has been suggested that the cellular mechanisms responsible for the MDR phenotype in mammalian cancer cells also operate in aquatic organisms inhabiting chronically polluted environments (Kurelec, 1992). The function of P-gp in aquatic organisms is believed to be the removal of both natural and anthropogenic chemicals from the cells of exposed animals (Kurelec 1992). Data from immunohistochemical, genomic and protein function investigations suggest

that the P-gp and MRP-like transporters found in fish perform a similar protective role when compared to their better-studied mammalian orthologs (Zaja, 2007). Annilo (2006) reported that nearly 80% of all human ABC transporters have an ortholog in zebrafish (*Rerio danio*).

In a clinical context, the P-gp mediated MDR phenotype decreases the therapeutic efficacy of drugs which must remain within a target cell in order to achieve their desired pharmacological effect (Juliano and Ling, 1976). Reversal of the MDR / MXR phenotype in human tumours is a challenge that clinicians and researchers have attempted to overcome through various pharmacological interventions. By interfering with the mechanism of P-gp mediated drug efflux, P-gp substrates may remain within target cells for a longer period, increasing clearance time and therapeutic efficacy. These chemicals that increase the clearance time of pharmaceuticals have been termed 'chemosensitizers'. It has been suggested that chemosensitizers ought to be highly prioritized among environmentally hazardous chemicals due to their potential to interfere with P-gp function and disrupt elimination of endogenous substrates (Kurelec, 1992). Using the PHLC cell line, Caminada (2008) reported that many environmentally relevant pharmaceuticals and personal care products had the capacity to significantly inhibit the MXR mechanism, with IC_{50} values in the mM to µM range.

1.4 Lipid membranes, transmembrane efflux transporters and liver function

By transporting compounds from the blood to the bile, the liver removes many endogenous and foreign chemicals from the circulatory system, thus

protecting other organs by preventing exposure. This function is commonly known as the first pass effect (Klaassen, 1988). Due to its anatomical connection with the digestive tract and high metabolic capacity, the liver is vulnerable to the toxic effects of drugs, xenobiotics and oxidative stress (Jaeschke, 2002). Reactive metabolite formation, depletion of anti-oxidants and protein alkylation are among the general mechanisms of hepatotoxicity (Jaeschke, 2002).

The clearance of endogenous compounds and xenobiotics from liver cells is performed by hepatic transport proteins located on the canalicular membrane (Jaeschke, 2002). Regulation of the transport of cellular waste products and nutrients is, in part, made possible by transmembrane transport proteins. Minimizing the risk of interaction between potentially deleterious chemicals and critical biological macromolecules is achieved by the efficient subcellular compartmentalization that is made possible by biological membranes and transmembrane transport proteins, including P-gp (Shtil, 2005).

The ABC (ATP Binding Cassette) family of transport proteins and the solute carrier (SLC) family are the major hepatic transport proteins (Hewitt, 2007). The multi-drug resistance associated proteins (MRPs), multi drug resistance proteins (MDRs), bile salt export pump (BSEP) and breast cancer resistance protein (BCRP) transport proteins belong to this family. The Na^+-taurocholate co-transporting polypeptides (NTCP), organic anion-transporting polypeptides (OATPs), organic anion transporters (OATs) and organic cation transporters (OCTs) belong to the solute carrier SLC family (Hewitt, 2007).

At the cellular level, the uptake of xenobiotics from the blood across the sinusoidal (basolateral) hepatocyte membrane is performed largely by the organic anion transporting polypeptide family (Oatps), the organic cation transporter 1 (Oct1) and the organic anion transporter 2 (Oat2) (Klaassen, 2005). Once inside the hepatocyte, chemicals may be acted upon by cytosolic or microsomal enzymes, conjugating enzymes, or exported across the canicular (apical) membrane into the bile by transport proteins. P-gp and MRP are two such efflux transport proteins. Hepatically-eliminated compounds become biliary components, and are cleared from the body with the feces.

1.5 ABC transporters: phylogenetic distribution, anatomical distribution, mechanisms and substrates

The wide phylogenetic distribution of P-gp genes suggest that these MDR / MXR proteins may be a shared feature common to all organisms (Bard, 2000). In humans, P-gp is expressed in epithelial cells of the gastrointestinal tract, liver, kidneys and capillaries of the brain and gonads (Ambudkar, 2003). The existence of P-gp like proteins in numerous fish species has been reported (Zaja, 2007). The polyclonal murine antibody C-219 has been used to show that P-gp is expressed in numerous tissues from many teleost species, including the bile canaliculi from the guppy (*Poecilia reticulata*) (Hemmer, 1995), rainbow trout (*Oncorhynchus mykiss*) (Sturm, 2001) and channel catfish (*Ictalurus punctatus*), (Kleinow, 2000). P-gp expression has also reported in the proximal tubules of killifish kidneys (Miller, 1995). In fish, two isoforms of P-gp have been reported,

P-gpA and P-gpB (Bard, 2000). For the purposes of this report, only P-gpA will be discussed further, and will simply be referred to as P-gp.

The activity of ABC transport proteins significantly affects the pharmacological behaviour of most current use drugs (Schinkel, 2003). The ABC super family of proteins is a diverse collection of evolutionarily conserved proteins that serve as membrane transporters (as mentioned above), ion channels and receptors (Chan, 2004). Most members of the ABC family perform cellular transport functions (Gottesman and Pastan, 1993) and can be divided into a number of subfamilies. The most frequently studied of these ABC transport proteins are: ABCA1, which is a cholesterol and lipid transporter (Attie, 2007); ABCB1 (MDR1 or P-gp), described above; and ABCC1, which refers to the multidrug resistance associated protein (MRP) that transports GSH conjugated cellular metabolites and xenobiotics (Leier, 1994).

Shared sequence homology and domain organization are features common to all ABC proteins (Chan, 2004). The general structure of an ABC transport protein comprises twelve transmembrane domains (TMD) and two nucleotide binding (NBD) domains (Chan, 2004). Within the structure of the P-gp protein, at least two ligand binding sites are known to exist (Callaghan, 2006), an attribute which contributes to the lack of substrate specificity and its ability to efflux a wide variety of structurally unrelated compounds (ligand promiscuity). The TMDs forming the pore through which substrates are exported have been found to be enriched in aromatic amino acids, which may in part account for P-gp's broad substrate specificity (Endicott, 1989; Pawagi, 1994). The analogy of a

“hydrophobic vacuum cleaner” has been used to describe the actions of P-gp (Higgins, 1992). Two other models have also been described, these are the “classical pore pump model” and the “flippase model” (Li, 2010).

At least two chemical binding sites are known to exist within the P-gp molecule, and there appears to be a positive cooperative interaction between these binding sites that increases the range of substrates that can be transported (Shapiro, 1998). Due to their lipohilic character, P-gp substrates accumulate in the plasma membrane; this is also where the binding sites of P-gp are located (Shapiro, 1998).

The substrate translocation process performed by P-gp has been divided into 4 distinct processes: loading of P-gp with the xenobiotic and nucleotide; reorientation of the chemical binding sites from high to low affinity; nucleotide hydrolysis and finally the resetting phase (Callaghan, 2006). Hydrolysis of ATP appears to be the rate limiting step in the catalytic cycle of P-gp (Chan, 2004).

Substrates of P-gp include natural compounds, endogenous metabolites and chemotherapeutic drugs used in the treatment of cancer and other pathophysiological conditions (Gottesman, 2002). P-gp is reported to be a flippase for endogenous simple glycosphingolipids and membrane phospholipids (Eckford, 2005). P-gp substrates are excreted as parent compounds without having undergone functionalization or conjugation reactions, unlike MRPs which export compounds that have been conjugated to either GSH, glucuronide or sulfate (Ambudkar, 2003; Jedlitschky, 1996). The fluorescent cationic dye rhodamine 123 (R123) is a P-gp substrate that is frequently used to quantify P-gp

activity (Bard, 2000). Calcein AM and doxorubicin are other fluorescent substrates that have been used to quantify the activity of P-gp (Litman, 2001; Hildebrand, 2008).

1.6 Reactive oxygen species, anti-oxidant defences, oxidative stress and cellular responses to hydrogen peroxide

Oxidative stress has been defined as a shift in the pro-oxidant – anti-oxidant balance in favour of the former (Sies, 1985). Oxidative stress can be the result of increased production and / or impaired elimination of reactive oxygen species (ROS) (Sies, 1985). To minimize the risk that ROS will accumulate to toxic levels, cells possess anti-oxidant defence systems which eliminate ROS, including H_2O_2, by enzymatic and non-enzymatic mechanisms. ROS are naturally and constitutively produced by numerous cellular processes in multiple cellular locations (Kuo, 2009). Frequently measured manifestations of oxidative stress at the cellular level include increased production of lipid peroxides, non-peptide protein carbonyl formation and oxidative damage to DNA evidenced by the production of 8-hydroxy-deoxy-guanosine (Kelly, 1998; Livingstone, 2001).

ROS include both radical (superoxide anions, hydroxyl radicals) and non-radical (hydrogen peroxide, H_2O_2) compounds. Despite being powerful oxidants that are toxic at high concentrations, ROS also serve as signalling molecules in numerous signal transduction pathways (Finkel, 2001). The superoxide anion and H_2O_2 are the most important ROS that perform physiological roles in cell signalling (Queisser, 2010). Nitric oxide can act as a neurotransmitter and regulator of vascular function and other functions (Bird, 2011). The production of

ROS by NADPH-oxidase in leukocytes provides an important line of defence against pathogenic organisms (Quinn, 2006).

Disruption of the intracellular balance between pro-oxidants and anti-oxidants can have important effects that culminate in of a variety of pathological conditions and diseases (Valko, 2007; Kuo, 2009). Oxidative stress can initiate a cascade of cellular events including mitochondrial dysfunction and a switch to glycolytic respiration (Jaeschke, 2002). Production of reactive metabolites and ROS are involved in most adverse drug reactions (Jaeschke, 2002). When compared with other cellular metabolites, highly reactive, electrophilic metabolites of molecular oxygen are an important concern because the cellular damage they cause is associated with aging, neurologic disorders, cancer, alcoholic liver disease and the pathology of numerous other diseases (Kuo, 2009).

The aquatic environment provides a sink for numerous environmental contaminants that have the potential to cause oxidative stress in aquatic organisms (Winston and Di Guilio, 1991; Kelly, 1998). Considerable evidence suggests that increased production of ROS in aquatic organisms results from exposure to anthropogenic pollutants (Winston and Di Guilio, 1991). Exposure to numerous anthropogenic agents has been reported to cause oxidative stress in fish including waste water discharged from hydrocarbon extraction processes (Farmen, 2010); effluents from sewage treatment plants (Sturve, 2008); and diesel oil (Nogueira, 2010). Other common pollutants that have the potential to cause oxidative stress in exposed aquatic organisms include polyaromatic

hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), quinones (Di Guilio, 1989), transition metals (Valko, 2005), pesticides (Banerjee, 2001) and pentachlorophenol (Dong, 2009). Naturally occurring cyanobacterial cyclic peptides have also been shown to cause increased production of ROS in tilapia (*Oreochromis sp.*) exposed under laboratory conditions. (Jos, 2005).

Application of exogenous peroxides, including H_2O_2, has been used in numerous investigations to produce a state of oxidative stress in numerous cell types, including trout hepatocytes (Ploch, 1999). By showing that increased production of ROS and / or decreased levels of anti-oxidants, such as GSH, elicit changes in the expression or activity a protein, one may obtain evidence to suggest that oxidative stress is a regulator of the cellular parameter being investigated.

1.7 P-gp: induction, regulation and the role of oxidative stress

Due to its mechanistic contribution to the MDR phenotype and the many other physiological functions performed by ABC transport proteins, much effort has focused on describing the mechanisms by which P-gp and other ABC proteins are regulated. This regulation can occur at various levels including: transcriptional (Scotto, 2003); post transcriptional (Gomez Martinez, 2007); translational (Gazzin, 2011); post translational (Ho, 2006); and epigenetic (Baker, 2005) levels. Elements of the cellular microenvironment, such as pH and intracellular Ca^{2+} levels have also been investigated as potential regulators of P-gp activity in human prostate carcinoma cells (Thews, 2006).

It has been suggested that several other factors may also be responsible for the modulation of MDR / MXR activity (Saves, 1998; Shtil, 2005). Alterations in the levels of hepatic P-gp in fish species, and other organisms, exposed to various chemicals have been reported by numerous authors; however, there remains much uncertainty in this area. For example, contaminant and chemical exposure has been shown to reduce P-gp expression in teleosts. Increased expression of ABCC and ABCG, but not ABCB (P-gp) transporters, have been observed in killifish (*Fundulus heteroclitus*) inhabiting the Sydney tar ponds in Nova Scotia, Canada (Paetzold, 2009). Similarly, Bard (2002) reported that killifish from the heavily contaminated New Bedford Harbor, (USA) had hepatic P-gp levels that were 40% less than those of fish from a relatively non-polluted reference site. Catfish (*Ictalurus punctatus*) fed a diet spiked with β-naphthaflavone (βNF), tetrachlorobiphenyl (TCB) or benzo[a]pyrene (BaP) all showed decreased hepatic P-gp expression (Doi, 2001). Contrary results have been found by other investigations on the effect on P-gp expression in fish exposed to various chemicals. For example, killifish inhabiting an environment heavily contaminated by creosote were found to have increased hepatic P-gp concentrations (Cooper, 1999). High cockscomb blennies (*Anoplarchus purpurescens*) exposed to crude oil in a laboratory were found to have hepatic P-gp concentrations that were 3 to 5 fold higher than controls (Bard, 2002). Isolated hepatocytes from rainbow trout (*Oncorhynchus mykiss*) exposed *in vitro* to either doxorubicin, chlorpyrifos or N-nitrosodiethylamine were all found to have increased concentrations of P-gp (Albertus, 2001). The results of these studies

indicate that P-gp can be induced by exposure numerous chemicals, but the opposite effect, down regulation of P-gp is also a possible outcome of chemical exposure.

Environmental stress has been suggested to be an important regulator of MDR1 expression (Chin, 1990). Expression of the human mdr1 gene (which codes for human P-gp) is induced by various stress conditions, most of which result in a cellular redox imbalance (Kuo, 2009). Although the regulation of P-gp by chemical induction is unclear, several reports have shown that diverse stimuli can elicit an increase in the expression of P-gp; this supports the claim that P-gp expression forms part of a cellular response, the 'general cellular stress response' (Ledoux et al. 2003). Using various experimental organisms, up regulation of mdr1-mRNA transcription has been shown to result from exposure to diverse stress stimuli including: carcinogens; antineoplastic drugs; heat shock; arsenite (Chin, 1990); retinoic acid; dimethyl sulfoxide (Zhou, 1996); glucose deprivation (Ledoux, 2003) and ionizing radiation (Hill, 1990).

Several nuclear receptors, including the pregnane-X-receptor (PXR) and retinoid-X-receptor α (RXRα), have been implicated in the regulation of P-gp in humans cells (Klaassen, 2005). PXR is expressed at high levels in the liver where it serves as a molecular sensor of toxic by-products derived from metabolism of endogenous and exogenous chemicals (Timsit, 2007). Activation of PXR plays an important role in the induction of hepatic transporters, including P-gp, as well as Phase I and II enzymes (Timsit, 2007). PXR has been shown to regulate expression of P-gp in human HepG2 cells and porcine endothelial cells

of the blood brain barrier (Rigalli, 2011; Ott, 2009). PXR expression has been confirmed in zebra fish (*Danio rerio*) liver (Bresolin, 2005) as well as the liver, intestine, kidney and heart of rainbow trout (Wassmur, 2010).

A mechanism describing the increased expression of P-gp by the generation of ROS and subsequent activation of nuclear factor κB (NF-κB) pathway in rats has been suggested (Deng, 2001). NF-κB proteins are a family of transcription factors that regulate the expression of hundreds of genes involved in regulating cell differentiation, growth, development, apoptosis and protection from ROS accumulation (Morgan, 2011).

It has been reported that P-gp activity can vary without appreciable changes in P-gp expression, suggesting that the activity of this protein can be modulated post-translationally (Thews, 2006). Increased P-gp activity has been linked to decreased intracellular [Ca^{2+}] and decreased pH in human prostate carcinoma cells (Thews, 2006). This increased P-gp activity was suggested to be mediated by decreased protein kinase C (PKC) activity (Thews, 2006). Activation of PKC is a very early response to numerous stressors and PKC isozymes are involved in apoptosis and cell survival following treatment with toxic compounds (Hofmann, 2004). Human P-gp is known to be phosphorylated by PKC (Chambers, 1993). In general, the results of studies examining the link between PKC and the MDR phenotype are contradictory (Hofmann, 2004). The results of studies examining the link between PKC and the mdr1-mRNA are also contradictory (Hofmann, 2004).

General attributes of P-gp inhibitors include ATPase inhibition, rapid re-entry of the transport substrate into the plasma membrane following transmembrane efflux by P-gp and non-competitive inhibition (Litman, 2001). There is a group of chemicals that can block the MDR phenotype which are collectively referred to as the chemosensitizers (Skovsgaard, 1984). By blocking the activity of P-gp, chemosensitizers, such as verapamil, lead to the increased accumulation of cytotoxic drugs, such as vincristine and vinblastine in MDR cells (Tsuruo, 1981). Trifluoperazin and zinc, other calcium channel blockers and quinidine have also been found to behave as chemosensitizers (Tsuruo, 1981; Tsuruo, 1982; Tsuruo, 1983; Tsuruo, 1984).

The expression of numerous genes involved in reducing cellular accumulation of xenobiotics are reported to be regulated by oxidative stress including MRP1, γ-glutamylcysteine synthetase (γ-GCS), P-gp, and Cytochrome P450 (Yamane, 1998; Ziemann, 1999). Investigations seeking to determine whether oxidative stress can regulate the activity and expression of P-gp and other ABC proteins including MRPs have often employed rodent brain endothelial cells (Wu, 2009; Hong, 2006; Felix, 2002) or rodent hepatocytes (Sekine, 2006) and have frequently reported that R123 accumulation is decreased in cells treated with H_2O_2 or GSH depleting agents (Ziemann, 1999; Hirsch, 2000; Hong, 2006; Wu, 2009). Piscine models have received less attention. To date, no investigations have used isolated teleost hepatocyte suspensions to determine whether ROS or oxidative stress can regulate the activity of P-gp.

1.8 Glutathione: roles in cellular metabolism, maintenance of the cellular redox balance and environmentally induced perturbations of GSH levels

The structure and function of many cellular structures and biological macromolecules can be affected by oxidative stress (Valko, 2006). To protect the structure and function of biological macromolecules, the intracellular levels of ROS are tightly regulated by a variety of detoxification mechanisms to ensure that cells remain able to respond appropriately to environmental challenges and metabolic demands (Valko, 2007). The anti-oxidant defence system enables the detoxification of highly reactive molecules, such as highly electrophilic toxicants, including ROS, through enzymatic and non-enzymatic mechanisms (Valko, 2007).

The tripeptide glutathione (GSH), composed of glutamate, cysteine and glycine, GSH is a small molecular weight thiol and the most abundant anti-oxidant in the cytosol, nucleus, and mitochondria (Valko, 2007). GSH can be conjugated to numerous unsaturated compounds by a variety of GSH conjugating enzymes (Boyland, 1967). In most cell types, cytosolic GSH is present at mM concentrations, making it the most abundant intracellular thiol containing compound (Anderson, 1998). Some hepatic GSH is exported to the plasma to protect other organs (Sies, 1985). It has been demonstrated that depletion of hepatic GSH leads to increased generation of ROS by hepatocytes (Tirmenstein, 2000). Diminished GSH levels may impair the activity of GSH dependant enzymes and transport proteins (GST, MRP) and the cellular capacity to regenerate other anti-oxidants, ultimately leading to an altered intracellular redox balance (Valko, 2007).

GSH levels in fish are known to be affected by temperature and exposure to environmental pollutants (Leggatt, 2007; Farmen, 2010). Consequently, measurement of cellular GSH levels may provide a useful biomarker for the purposes of Environmental Risk Assessment (ERA) to indicate whether animals have been exposed to high levels of deleterious agents causing oxidative stress (van der Oost, 2003). Numerous reports have found that GSH levels are depleted in fish tissues (or isolated hepatocytes) following exposure to a variety of exogenous agents including: cyanobacterial toxins (Amado, 2010); $CuSO_4$ (Farmen, 2010; Rau, 2004); agricultural chemicals (Dorval, 2005); endosulfan (Dorval, 2003); pentachlorophenol (Dong, 2009); paraquat (Farmen, 2010). Increased hepatic GSH concentrations have been reported in fish following exposure to benzo(a)pyrene (Feng, 2001); and in fish inhabiting contaminated superfund sites (Meyer, 2003).

1.9 Objectives of study

Multiple environmental, cellular and biochemical parameters have been hypothesized to regulate P-gp expression and activity, however, the exact mechanisms by which this first line of cellular defence is regulated remains unclear (Callaghan, 2006; Bard, 2000). This aim of this work is determine if P-gp is regulated by ROS through the *in vitro* treatment of rainbow trout (*Oncorhynchus mykiss*) hepatocytes with model chemicals that either promote oxidative stress (H_2O_2; Felix, 2002) or reduce GSH concentrations (diethyl maleate [DEM]; Tirmenstein, 2000). Based on the existing literature, it is hypothesized that either increased ROS or decreased GSH levels will increase

P-gp activity (Hong, 2006). The results of this work will increase the understanding of P-gp function and regulation and has relevance to both human health and understanding how aquatic organisms function in contaminated environments.

2: MATERIALS AND METHODS

2.1 Fish

Sexually mature male and female rainbow trout weighing 548 ± 35 g (mean ± SE, range 398 – 944 g) were purchased from Miracle Springs Trout Farms, Mission, BC, Canada. Fish were acclimated in 1000 L tanks (supplied with dechlorinated municipal water at pH 6.9, hardness 6.3 mg/L as $CaCO_3$, and >95% O_2 saturation) at SFU for at least 2 weeks prior to experimentation. Approval to conduct experiments was obtained from the SFU animal care committee. Fish were fed laboratory trout chow daily *ad libitum*. For the *in vitro* H_2O_2 exposure experiments, fish were kept in outdoor tanks and with a water temperature of 3 ± 1˚C for the two week acclimation period. For DEM and GSH exposure experiments, water temperature was 7 ± 1˚C and 13 ± 1˚C, respectively.

2.2 Chemicals

The following products were purchased from Sigma (St. Louis, USA): β-nicotinamide adenine dinucleotide (β-NADH), reduced dipotassium salt, sodium pyruvate, Hank's balanced salts, fatty acid-free bovine serum albumin (BSA), diethyl maleate (DEM) reduced glutathione (rGSH), metaphosphoric acid, triton X-100, Collagenase type IV from *Clostridium histolyticum*, rhodamine 123 (R123), trypan blue, N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES). 3-aminobenzoic acid ethyl ester methane sulphonate (MS-222) and

N-Butanol were purchased from Caledon Laboratories Ltd (Georgetown, ON). Glutathione quantitation kits (GSH-400, Oxis Research, Portland, USA) were purchased from Medicorp (Montreal, QC).

2.3 Hepatocyte isolation

Hepatocytes were isolated as in Moon *et al.* (1985). Three solutions containing Hank's balanced salts (KCl 0.4 g/ L, KH_2PO_4 0.06 g/ L, NaCl 8.0 g/ L, Na_2HPO_4 0.05 g/ L, D-Glucose 1.0 g/ L) were prepared and designated HSSA, HSSB and HSSC (Moon, 1985). HSSA was the starting solution for HSSB and HSSC. HSSC contained collagenase and was used to perfuse the liver. HSSB was used to wash and culture cells. In addition to Hank's balanced salts, HSSA also contained 0.2 M HEPES and 0.4 mM $MgSO_4$. Immediately before commencing isolation, HSSA was placed on ice and aerated with a mixture of 99% O_2/1% CO_2 (Praxair Products Inc., Mississauga, ON) for 30 min. Following aeration of HSSA, 0.5 g of $NaHCO_3$ was added to HSSA and the pH of the HSSA was adjusted to 7.6 using either 1N NaOH or 1N H_2SO_4. HSSB contained 20 g/L of BSA, $CaCl_2$ (1.8 g/L) and 5 mM D-glucose and was prepared from oxygenated HSSA. HSSC was produced by adding 0.6 g/L collagenase to HSSA. All solutions were kept on ice for the duration of an isolation.

Fish were anesthetized with MS-222 (0.2 g/L) buffered with $NaHCO_3$ (0.2 g/L). The mass and sex of the fish were recorded and the fish placed on ice. The liver was exposed by removing the anterior lateral body wall. The portal vein was cannulated and the hepatic veins were severed. The liver was perfused with HSSA using a Cole Parmer model 7013-20 peristaltic pump adjusted to deliver

solution at a flow rate of approximately 2 mL/ min/ g of liver. The liver was perfused with HSSA for less than 5 min in order to clear the blood from the organ. Once cleared of blood (as evidenced by the change in colour) the liver was perfused with HSSC for approximately 20 min in order to promote collagenase digestion and liver dissociation. Small volumes of ice cold HSSA were gently pipetted over the surface of the liver to keep the temperature as constant as possible during perfusion.

Following perfusion with HSSC, the liver was carefully removed from the fish, dissected away from the gall bladder and transferred to an ice cold watch glass containing a small amount (~10 mL) of HSSC to further promote dissociation of liver tissue into isolated hepatocytes and prevent desiccation of cells. In the watch glass, the liver was gently diced and hepatocytes scraped away from the hepatic epithelium using a surgical blade. The resulting liver homogenate was then sequentially passed through 2 nylon meshes having pore diameters of 253 µm and 73 µm, respectively. The filtrate was collected and transferred into 15 mL plastic centrifuge tubes and spun at 42 x *g* for 5 min at 4˚ C in a Beckman Coulter GS-15R centrifuge (Mississauga, ON). Following centrifugation, the supernatant (containing cellular debris and some erythrocytes), was discarded and replaced with ice cold HSSB. Hepatocytes were washed according to the same procedure described above a second time prior adjusting cell suspension concentration to 25 mg/mL (approximately 4.0 x 10^6 viable cells/mL).

2.4 Determination of cell concentration, cell viability, and plasma membrane integrity

To determine the cell concentration of the hepatocyte suspension (in mg/mL), three 1.0 mL aliquots of the twice-washed hepatocyte suspension were transferred into pre-weighed 1.5 mL plastic centrifuge tubes. Tubes containing the hepatocyte suspension were spun in a micocentrifuge (VWR model 18R mini, Edmonton, AB) for 2 min at 5500 x *g* at 22˚C. The supernatant was discarded and the inner surface of the tube carefully dried using a laboratory grade paper towel (Siebert, 1985) to remove all aqueous components while leaving only the cell pellet inside of the tube. The wet masses of the 3 cell pellets were obtained by subtracting the mass of the empty tube from the mass of the tube containing the cell pellet. The average of these 3 values was used to determine the concentration of the hepatocyte suspension in mg/mL. The concentration of the hepatocyte suspension was diluted to 25 mg/mL by combining the initial hepatocyte suspension with the appropriate volume of ice cold HSSB,. For 1 h, the hepatocyte suspension was kept in 15 mL plastic centrifuge tubes on an orbital shaker (VWR, Edmonton, AB) in an incubator (VWR model 1575R, Edmonton, AB) that was maintained at 7˚C. Hepatocyte suspensions were allowed to acclimate for 1 h before initial cell viability was measured and before any treatments were initiated.

The initial viability of hepatocyte suspensions was assessed in triplicate. Viability was assessed again in triplicate at the end of an experiment for all treatments. The trypan blue exclusion test was used to determine cell viability (Gill and Walsh, 1990). 50 µL of trypan blue were combined with 50 µL of the 25

mg/mL cell suspension which was mixed by withdrawing and expelling a small volume of suspension using an automatic pipette. Then 10 µL of this suspension was transferred onto a hemacytometer (Hausser Scientific, Horsham, PA, USA) and the number of live and dead cells was counted. Viability was calculated by dividing the number of live cells by the number of live and dead cells and expressed as a percentage. The criteria for using hepatocyte suspensions for future assays was that initial cell viability ≥ 70% (Gourley, 2009).

Cytotoxicity, and damage to the plasma membrane, may be evidenced by the presence of cytoplasmic enzymes, such as lactate dehydrogenase (LDH), outside of the cytoplasm. At 2h and 7 h following the various treatments, a positive control (TTX [1%]) or control (HSSA only), 500 µL aliquots of each treated hepatocyte suspension were centrifuged at 42 x *g* for 5 min at 4˚ C in a Beckman Coulter GS-15R centrifuge (Mississauga, ON). LDH activity of the supernatants from all treatments was determined using a spectrophotometric microplate reader (Biotek powerwave 340, Winooski, VT, USA) connected to a computer running KC junior software (Biotek, Winooski, VT, USA) that recorded the time and absorption of every plate reading event.

The absorbance at 340 nm (A340) provides an accurate measure to quantitate NADH. LDH activity can be calculated by determining the rate at which NADH is converted to NAD+, which is evidenced by a decrease in the A340. Wells of a 96 well plate were loaded with 200 µL of HSSB, 20 µL of 23 mM pyruvate and 20 µL of 4.3 mM NADH. The initial concentrations of NADH and pyruvate present in the 96 well plates were 0.33 mM and 1.75 mM (Bains,

2004). The absorbance at 340 nm was measured prior to commencing the assay in order to quantify the amount of NADH present in the well prior to sample (treated hepatocyte suspension supernatant) addition. To initiate the assay, a 20 µL aliquot of sample was added to a loaded well in the microplate. The A340 measured immediately following sample addition and again at 1 min intervals following sample addition. Each sample was assayed in triplicate. From the measured quantities of NADH present in the wells at different times (up to 5 min) following sample addition, the average rate of NADH consumption (as evidenced by the decrease in A340) was calculated for all treatments and expressed as a percentage of the control (Pesonen, 1992; Fotakis, 2006).

2.5 Treatment of hepatocyte suspensions

Different pro-oxidant and anti-oxidant compounds were applied to hepatocyte suspensions in order to test the hypotheses that oxidative stress as increased ROS is involved in the regulation of P-gp activity. The three main treatments, H_2O_2, DEM, and GSH were applied to hepatocyte suspensions in an attempt to modulate intracellular concentrations of ROS. For all experiments, hepatocyte incubations contained cell concentrations of 25 mg/ mL in 15 mL conical tubes. Treatment volumes were 7.5 mL and did not differ between treatments within the same experiment. To minimize the possibility of introducing variation in cell density between treatments, cells were not washed following any treatment and were therefore continuously exposed to the modulating chemical (H_2O_2, DEM, or GSH) for the duration of an experiment.

H_2O_2 was applied to hepatocyte suspensions to increase intracellular levels of ROS at concentrations of 3, 30, 300 1200 µM and 10 mM. These were prepared from a 30% stock solution (v/v) diluted with HSSA. For the experiments in which hepatocyte suspensions were treated with 3-1200 µM H_2O_2, 400 µL of the appropriate H_2O_2 solution was added to 8 mL of hepatocyte suspension to achieve final concentrations of 3-1200 µM H_2O_2 in the hepatocyte suspensions. The control consisted of an equal volume of HSSA.

DEM, dissolved in DMSO, was applied to hepatocyte suspensions to decrease cellular GSH concentrations, which can lead to a diminished capacity to inactivate endogenously produced ROS, which could potentially lead to oxidative stress. Final concentrations of DEM in hepatocyte suspensions were 0.025, 0.25 and 2.5 mM DEM. DEM stock solutions were prepared by serially diluting DEM in DMSO. A DMSO control was tested alongside DEM treatments. The amount of DMSO in DEM-treated hepatocyte suspensions, and the DMSO control, was 1% of the volume of the hepatocyte suspension. For the experiments in which hepatocyte suspensions were treated with DEM (0.025, 0.25, 2.5 mM), 75 µl of treatment was added to 7.5 mL of hepatocyte suspension.

GSH dissolved in HSSA was added to hepatocyte suspensions to increase intracellular GSH concentrations, with the expectation that GSH would cross the plasma membrane, increase intracellular GSH concentrations and increase the capacity to inactivate endogenously produced ROS. GSH stock solutions were prepared fresh on each day of an experiment by dissolving GSH in HSSA (0.2 M stock solution). Dilutions of 0.02 and 0.002 M were prepared from the 0.2 M

stock solution. Hepatocytes were incubated with final GSH concentrations of 0.02, 0.2, or 2.0 mM. For the experiments in which hepatocyte suspensions were treated with GSH (0.02, 0.2, 2.0 mM) 75 µl of treatment was added to 7.5 mL of hepatocyte suspension. The controls consisted of an equal volume (1%) HSSA.

The detergent Triton X-100 (TTX) was used as a positive control in cytoplasmic leakage investigations in which 30 µL of TTX was added to 3 mL of hepatocyte suspension to achieve a final TTX concentration of 1%.

2.6 Cellular glutathione concentration measurement

At 30, 60, 150 and 360 min following treatment with either DEM, (0.025, 0.25, 2.5 mM), GSH (0.02, 0.2, 2.0 mM), H_2O_2 (10 mM), or HSSA, triplicate 500 µL aliquots of treated hepatocyte suspension (25 mg/ mL) containing 12.5 mg of cells were removed from each treatment and transferred into 1.5 mL micro centrifuge tubes. Samples following exposure to DMSO were taken at 30 min. Tubes were immediately centrifuged for 2 min at 5500 x *g* at 4˚C; the supernatant was then discarded and replaced with 500 µL of HSSB and centrifuged again under the same conditions. Following the second centrifugation, the supernatant was discarded and the cell pellet frozen at -80˚C.

The GSH concentration of cell pellets was determined according to the manufacturer's instructions (colorimetric glutathione quantitation kits [Oxis Research, GSH-400] Medicorp, Montreal, QC) with slight modifications that allowed for the measurement of GSH concentrations in a smaller quantity of cells. Cell pellets were thawed and then lysed in 500 µL of ice cold

metaphosphoric acid (MPA, 50 g/ L) to release cellular GSH. The resulting hepatocyte lysate and cellular debris were centrifuged at 3000 x *g* for 10 min at 4˚C to separate lysed cell fragments from the supernatant that contained GSH. For each sample, 200 µL of the cell-free lysate was combined with 250 µL of reaction buffer and 25 µL of reagent 1 (4-chloro-1-methyl-7-trifluromethyl-quinolinium methylsulfate) in a small centrifuge tube. Each centrifuge tube was vortexed for 10 s prior to the addition of 25 µL of reagent 2 (30% NaOH). The centrifuge tubes were vortexed a second time and placed into a water bath for 10 min at 25˚C. In duplicate, 200 µL of the contents of each centrifuge tube were transferred to 96 well plates and the absorbance at 400 nm measured (Biotek powerwave 340, Winooski, VT, USA). The average quantity of GSH in all samples was determined by comparing sample absorbance values to absorbance values from a standard curve constructed using known quantities of GSH ranging from 1 to 30 µg/mL. Mean concentrations of GSH are expressed in terms of µg GSH per mg of cells.

2.7 Determination of P-gp activity by rhodamine 123 accumulation

2.7.1 R123 accumulation

In order to determine the P-gp activity of hepatocyte suspensions, R123 accumulation was measured according to Gourley (2009) with minor modifications. Cells were always treated with the appropriate chemical (DEM, GSH, or H_2O_2) prior to the addition of R123. For each treatment group of hepatocytes, 7.5 mL of cell suspension (25 mg/mL) was combined with an equal volume of ice cold HSSB containing 10 µM R123 to achieve a final working cell

concentration of 12.5 mg/ mL. Cells were exposed to 5 µM R123 in 15 mL plastic centrifuge tubes (Fisher, Ottawa, ON, Canada). Hepatocyte suspensions were placed in an incubator on an orbital shaker at low speed to minimize cell settling within the tube during the accumulation assay.

For experiments in which hepatocyte suspensions were treated with 3 to 1200 µM H_2O_2, R123 was added to treated hepatocyte suspensions immediately following treatment with H_2O_2. Triplicate 1.0 mL aliquots of treated hepatocyte suspension were transferred to from the 15 mL conical tubes (containing the H_2O_2 treated hepatocyte suspensions) to 1.5 mL micro centrifuge tubes and immediately centrifuged (Beckman Model GS-15R) at 37× *g* for 4 min at 4 °C following 6, 13, 33 and 60 min of incubation with R123 to stop R123 accumulation. Immediately following centrifugation, the supernatant was discarded and replaced with 1.0 mL of ice cold HSSB that contained no R123 and the cell pellet was then centrifuged again. Following the second centrifugation, the supernatant was removed. Any remaining supernatant was removed by carefully employing the capillary action of a small piece of Kim wipe. Cell pellets were then stored at -20˚C until extraction and fluorometric determination of R123 content.

For experiments in which hepatocyte suspensions were treated with DEM (0.025, 0.25, 2.5 mM) or GSH (0.02, 0.20, 2.0 mM), R123 was added to treated hepatocyte suspensions 45 min following treatment with DEM or GSH. For these experiments, triplicate 1.0 mL aliquots of treated hepatocyte suspension were

removed and R123 accumulation assessed at 15, 30, 45 and 90 min following R123 exposure as described above.

2.7.2 R123 extractions and fluorometric measurement

Cell pellets containing R123 were thawed on ice and extracted twice using a total of 2.0 mL of n-butanol (Bains and Kennedy, 2005). After 1.0 mL of n-butanol was added to each of the samples, cell pellets were vortexed for 10 s to accelerate cell pellet dissociation, and to improve the extraction efficiency of R123. Following vortexing, samples were placed onto an orbital shaker and extracted for at least 60 min at room temperature in the dark. Following this extraction period, samples were vortexed again for 10 s prior to being spun in a micocentrifuge at 5500 x *g* for 2 min at 22˚C to precipitate any cellular debris. For each sample, 900 µL of the n-butanol supernatant containing extracted R123 were carefully transferred into a glass amber vial. Fresh n-butanol (1.0 mL) was added to the pellet and the extraction process repeated. N-butanol extracts from the first and second extractions were combined and kept at -20˚C for future fluorometric analysis.

When fluorometric analyses were conducted, n-butanol extracts were removed from the freezer and kept on ice in the dark. Triplicate n-butanol samples from each cell pellet sample were measured for R123 concentration. For each sample, 200 µL of n-butanol were transferred into a black 96 well plate. Using a spectrofluorometer equipped with KC junior software (Biotek, Winooski, VT, USA), the fluorescence intensity of samples was determined at excitation and emission wavelengths 485 nm (± 20) and 528 nm (± 20), respectively (Bains

and Kennedy, 2005; Sturm et al., 2001). The average fluorescence values of the triplicate samples (each measured in triplicate) were averaged and then converted into R123 concentrations using a standard curve constructed using known quantities of R123 (triplicates) ranging from 1 to 27 pg/mL which was linear over this range. Cellular quantities of R123 in units of ng R123 / mg cells were calculated and presented as such.

2.8 Calculations and statistical analyses

Statistical analyses were performed using JMP version 8.0.2 software. Results are expressed as means ± SE. Differences were considered significant when $p \leq 0.05$.

Cell viability was compared between treatments using a one factor analysis of variance. The factor was treatment group which had 9 levels ([0.02, 0.2, 2.0 mM] GSH, [0.025, 0.25, 2.5 mM] DEM, 10 mM H_2O_2, HSSA or 1% DMSO).

LDH activity was expressed as a percentage of the HSSA control. Relative LDH activity was compared between treatments using a two factor analysis of variance and Tukey-Kramer HSD test in a two factor split plot in time analysis of variance ($\alpha = 0.05$). The two factors were time period, with two levels, (2, 7h) and treatment group which had 8 levels ([0.02, 0.2, 2.0 mM] GSH, [0.025, 0.25, 2.5 mM] DEM, 10 mM H_2O_2, or 1% triton X-100). F ratios and p values from one way ANOVAs are reported in the results section.

Cellular GSH concentrations of GSH treated cells were compared to the HSSA control in a two-factor analysis of variance and Tukey-Kramer HSD test in a two factor split plot in time analysis of variance ($\alpha = 0.05$). The two factors were time period with 4 levels (30, 60, 150, 360 min) and treatment group which had 4 levels (GSH [0.02, 0.2, 2.0 mM] and HSSA). F ratios and p values from one way ANOVAs are reported in the results section.

Cellular GSH concentrations of DEM treated cells were compared to the DMSO control in a two-factor analysis of variance and Tukey-Kramer HSD test in a two factor analysis of variance ($\alpha = 0.05$). The two factors were time period with 1 level (30 min) and treatment group with 4 levels (DEM [0.025, 0.25, 2.5 mM] and DMSO). F ratios and p values from one way ANOVAs are reported in the results section. For DEM-treated hepatocyte suspensions, N = 8 for the 30 min time point, while N = 4 for 60, 150 and 360 min time points.

Cellular GSH concentrations of H_2O_2 treated cells were compared to the HSSA control in a two-factor analysis of variance and Tukey-Kramer HSD test in a two factor analysis of variance ($\alpha = 0.05$). The two factors were time period with 4 levels (30, 60, 150, 360 min) and treatment group which had 1 level (H_2O_2 [10 mM]). F ratios and p values from one way ANOVAs are reported in the results section.

Mean initial rates of R123 accumulation ± SE are expressed in units of pg R123/mg cells/min and were calculated from the linear slope of the accumulation curves. This linear portion of the R123 accumulation curve corresponded to the

first and third time points. For all experiments R123 accumulation was linear between 5 and 30 min (Fig 3, Fig 4, Fig 5).

For H_2O_2 exposed cells, R123 accumulation rates between treatments were compared using a single factor analysis of variance test. The single factor was treatment group which had 5 levels (0, 3, 30, 300 and 1200 µM H_2O_2).

For DEM exposed cells, R123 accumulation rates between treatments were compared using a single factor analysis of variance test. The single factor was treatment group which had 4 levels (0, 0.025, 0.25, 2.5 mM DEM).

For GSH exposed cells, R123 accumulation rates between treatments were compared using a single factor analysis of variance test. The single factor was treatment group which had 4 levels (0, 0.02, 0.2, 2.0 mM GSH).

3: RESULTS

3.1 Cell viability

Results from the trypan blue exclusion test of cell viaility are presented in Table 1. No significant differences in cell viability were measured by trypan blue exclusion between treatments and controls following 6 h of incubation with H_2O_2 (10 mM), GSH (0.02, 0.2, 2.0 mM), DEM (0.025, 0.25, 2.5 mM), HSSA or 1% DMSO were found.

Results from the lactate dehydrogenase leakage (LDH) assay following 2 and 7 h of treatment with GSH (0.02, 0.2, 2.0 mM), DEM (0.025, 0.25, 2.5 mM), H_2O_2 (10 mM), HSSA or 1% Triton X-100 are presented in Table 2 . Values of LDH leakage for each treatment are shown as a percentage of the HSSA control. LDH activity did not differ significantly between treated cells and the control. At 2 h and 7 h incubations with the positive control, 1 % Triton X-100, LDH activity was significantly different from their respective HSSA controls (F ratio = 6.61, $p = 8.7 \times 10^{-5}$ F ratio = 9.03, $p = 6.0 \times 10^{-6}$, respectively). These differences at 2 and 7 h in the mean rate of LDH activity between hepatocyte suspensions treated with 1% TTX and hepatocyte suspensions treated with HSSA were found to be significant ($\alpha = 0.05$).

Table 1. Cell viability (mean ± SE) (as assessed by trypan blue exclusion) of cells treated with GSH (0.02, 0.2, 2.0 mM), DEM (0.025, 0.25, 2.5 mM), H_2O_2 (10 mM), HSSA or 1% DMSO. Viability of cells was measured 6 h following exposure. N ≥ 4 fish for all treatments. N = 8 fish for all DEM concentrations.

Mean cell viability ± SE (%)								
GSH (mM)			DEM (mM)			H_2O_2 (mM)	HSSA	DMSO
0.02	0.20	2.0	0.025	0.25	2.5	10		
83 ± 5	83 ± 5	80 ± 5	84 ± 3	83 ± 3	81 ± 5	85 ± 5	83 ± 5	81 ± 5

Table 2 LDH leakage in hepatocytes at 2 and 7 h of incubation with either GSH (0.02, 0.2, 2.0 mM), DEM (0.025, 0.25, 2.5 mM), H_2O_2 (10 mM), HSSA or 1% TTX. The mean rate of NADH consumption (± SEM) by cell-free supernatants is expressed relative to that of the control (HSSA). N = 4 fish. *Denotes a significant difference at p<0.0001.

	LDH leakage (% control)							
	GSH (mM)			DEM (mM)			H_2O_2 (mM)	TTX (1%)
	0.02	0.2	2.0	0.025	0.25	2.5	10	
2h	98 ± 3	93 ± 4	101 ± 8	101 ± 6	98 ± 6	95 ± 4	105 ± 6	351 ± 97*
7h	110 ± 21	107 ± 15	108 ± 15	105 ± 14	112 ± 13	118 ± 16	97 ± 8	292 ± 48*

3.2 Cellular glutathione measurement

3.2.1 Effects GSH treatment on intracellular GSH concentrations

Cells treated with 0.02 or 0.2 mM GSH had intracellular concentrations of GSH that were not significantly different from the HSSA controls at any of the time points tested. However, treatment with 2 mM GSH for 30, 60, 150 min and 360 min increased GSH concentrations significantly over controls (Figure 1). Increases were approximately two-fold over HSSA controls at all 4 time points examined and were sustained for at least 6 h. Differences between hepatocyte suspensions treated with 2.0 mM GSH and the HSSA control were significant at 30 min (F ratio = 11.72, $p = 0.0003$), 60 min (F ratio = 4.43, $p = 0.03$), 150 min (F ratio = 6.46, $p = 0.008$) and at 360 min (F ratio = 5.06, $p = 0.02$).

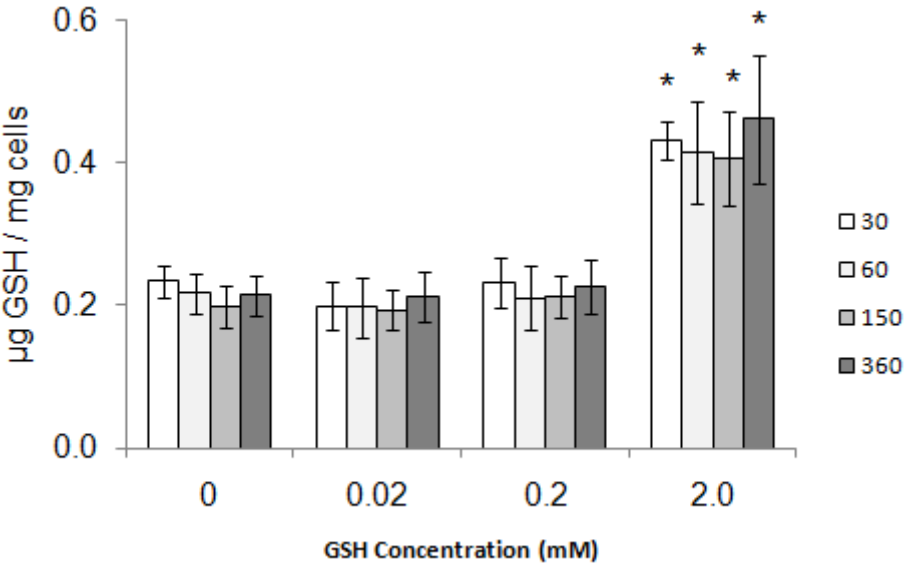


Figure 1. Mean intracellular GSH content in control, and GSH- treated (0.02, 0.20 and 2.0 mM) cells (± SEM) expressed in µg GSH / mg cells at 30, 60, 150 and 360 min post treatment with 0.02, to 2.0 mM GSH. N ≥ 4 fish. *Denotes a significant difference at $p < 0.05$.

3.2.2 Effects of DEM and H_2O_2 treatment on intracellular GSH concentrations

In Figure 2 the effects of DEM and H_2O_2 treatment are shown. Treatment with DEM caused a decrease in mean intracellular GSH concentrations that occurred at the first time point (30 min), and was sustained for the entire incubation period (6 h). When compared with the DMSO control, hepatocyte suspensions treated with 0.025, 0.25 and 2.5 mM DEM had decreased mean cellular GSH concentrations of 14%, 24% and 35%, respectively, following 30 min of exposure, although the only statistically significant decrease in GSH concentration occurred in the 2.5 mM DEM treatment group at 30 min when compared with the DMSO control (F ratio = 3.42, p = 0.02). However, Tukey's HSD test ($\alpha = 0.05$) showed that this difference was not significant. In Figure 2, it can be seen that treatment with 10 mM H_2O_2 did not significantly affect intracellular GSH concentration at any time point.

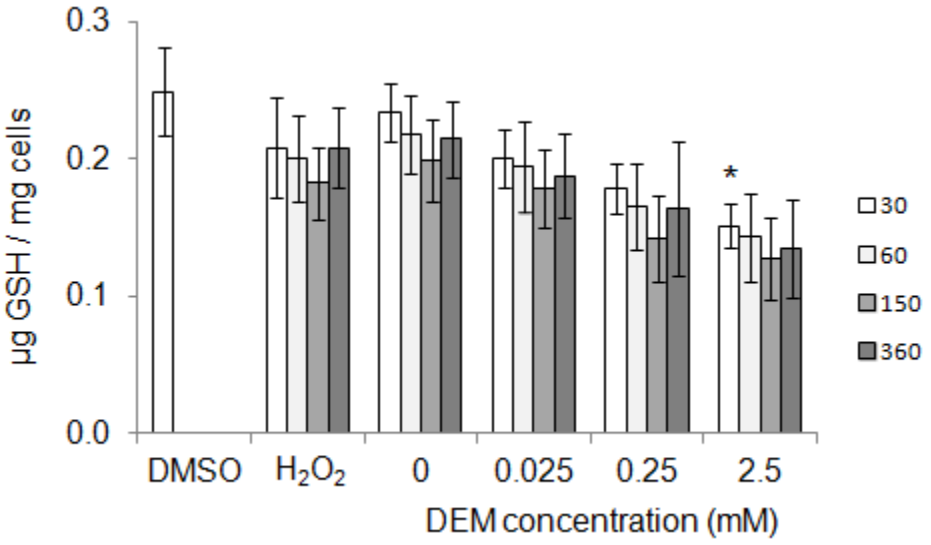
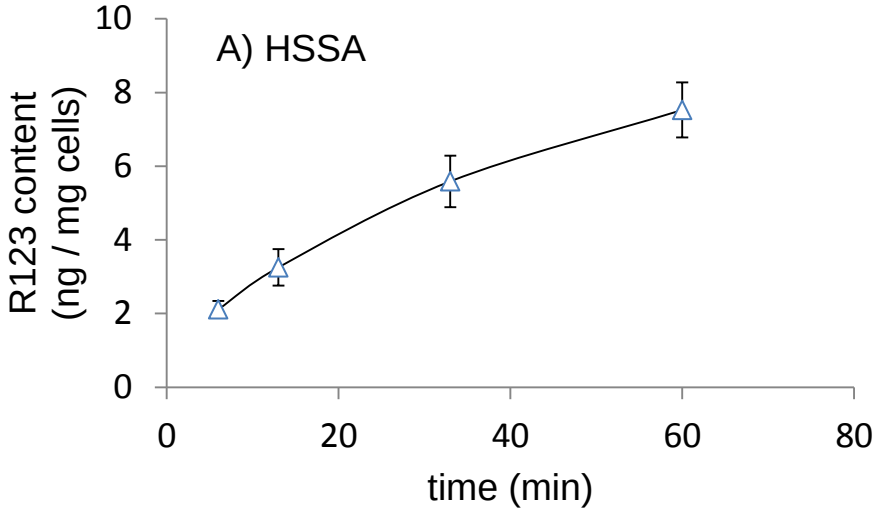


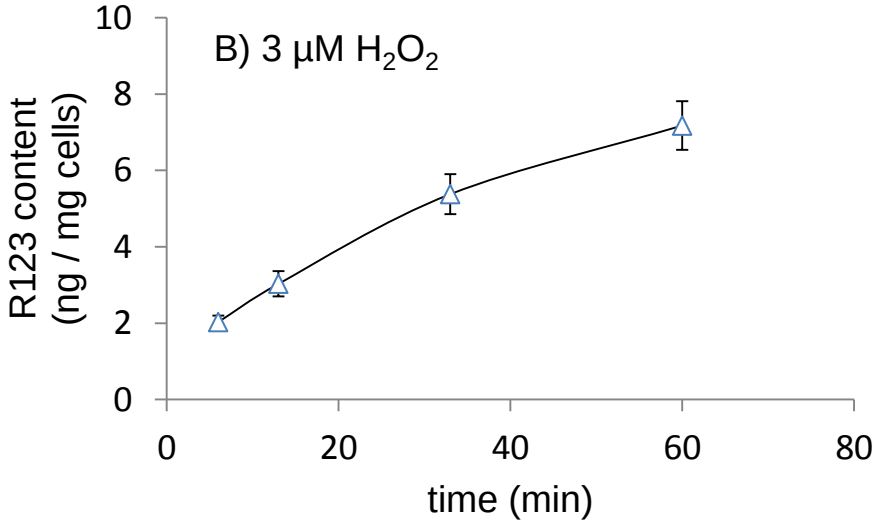
Figure 2. Mean intracellular GSH content in control, DEM- treated (0.025, 0.25 and 2.5 mM) and H_2O_2 (10 mM) treated cells (± SEM) expressed in µg GSH / mg cells at 30, 60, 150 and 360 min post treatment with 0.025 to 2.5 mM DEM or 10 mM H_2O_2. N ≥ 4 fish. *Denotes a significant difference at $p < 0.05$.

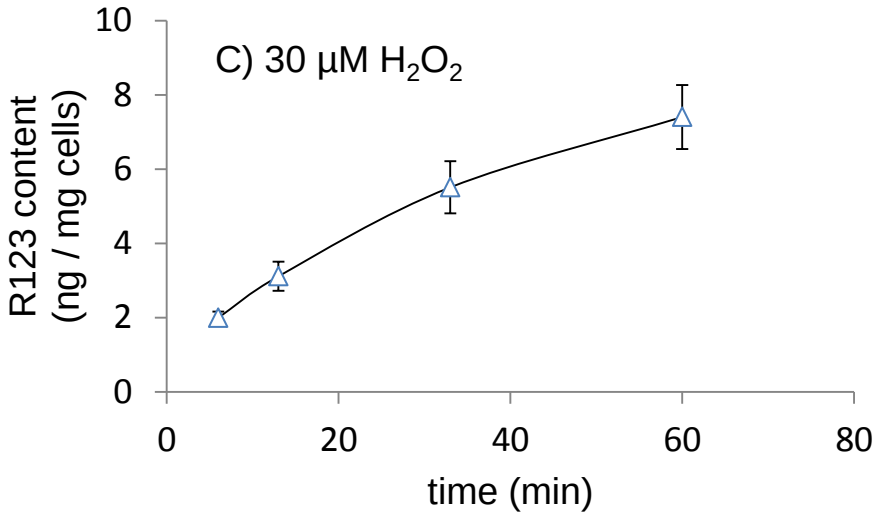
3.3 P-gp activity measured by rhodamine 123 accumulation

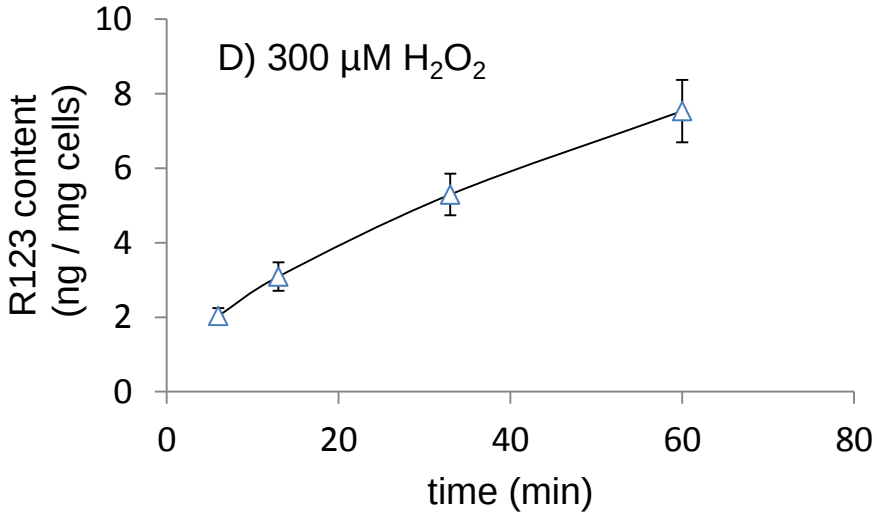
3.3.1 H_2O_2 treatments

Fig 3 shows the accumulation of R123 with time in H_2O_2-treated cells at varying concentrations (3, 30, 300 and1200 µM) at 6, 13, 32 and 60 min. As a surrogate for the measurement of P-gp in hepatocyte suspensions, the initial rate of R123 accumulation was calculated and compared between controls and H_2O_2 treated (3, 30, 300 or 1200 µM) cells. The mean initial rates of R123 accumulation by hepatocytes exposed to 3, 30, 300 or 1200 µM H_2O_2 were not significantly different when compared with each other or the HSSA control (F ratio = 0.049, p = 1.0).


A) HSSA
R123 content (ng / mg cells)
10
8
6
4
2
0
0
20
40
60
80
time (min)


B) 3 µM H_2O_2
R123 content (ng / mg cells)
10
8
6
4
2
0
0
20
40
60
80
time (min)


C) 30 µM H_2O_2
R123 content
(ng / mg cells)
10
8
6
4
2
0
0
20
40
60
80
time (min)


D) 300 µM H_2O_2
R123 content
(ng / mg cells)
10
8
6
4
2
0
0
20
40
60
80
time (min)

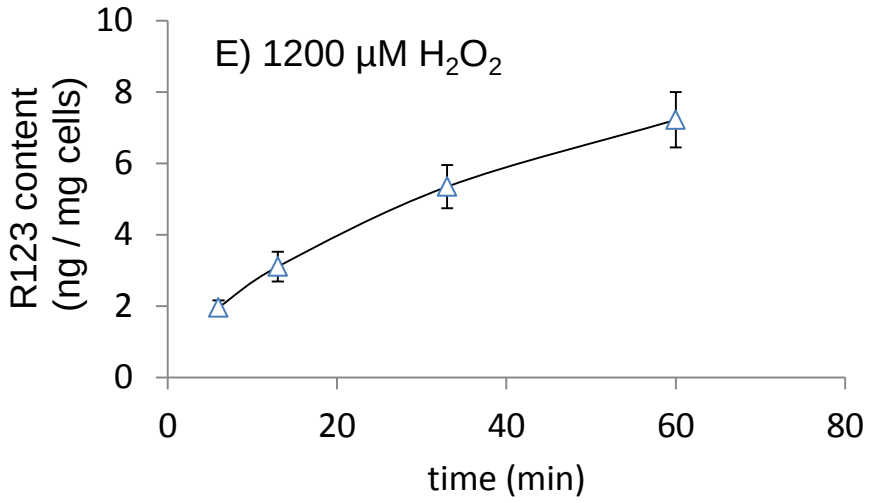


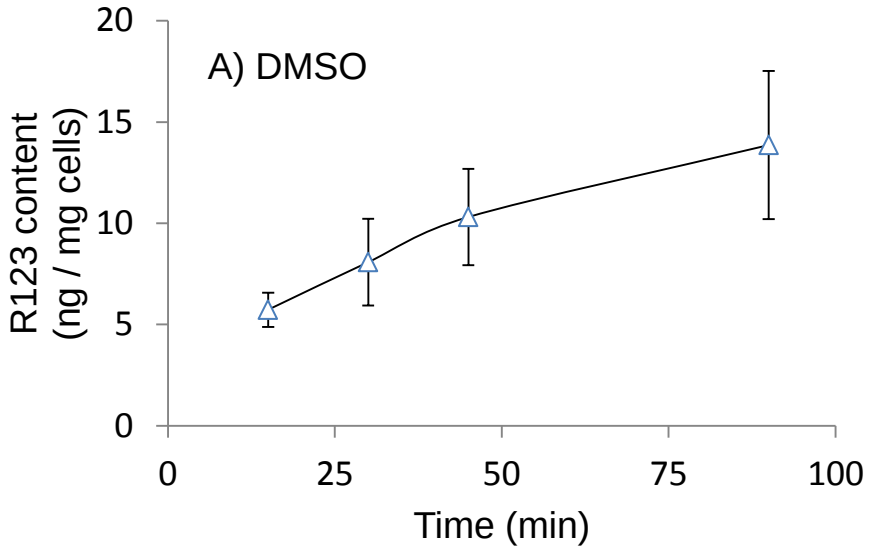
Figure 3. P-gp activity was not affected by treatment with H_2O_2. Accumulation of R123 at 6, 13, 32 and 60 min. Values are means of R123 in ng R123/mg cells ± SE immediately following exposure to 3, 30, 300 or 1200 µM H_2O_2. N = 5 fish.

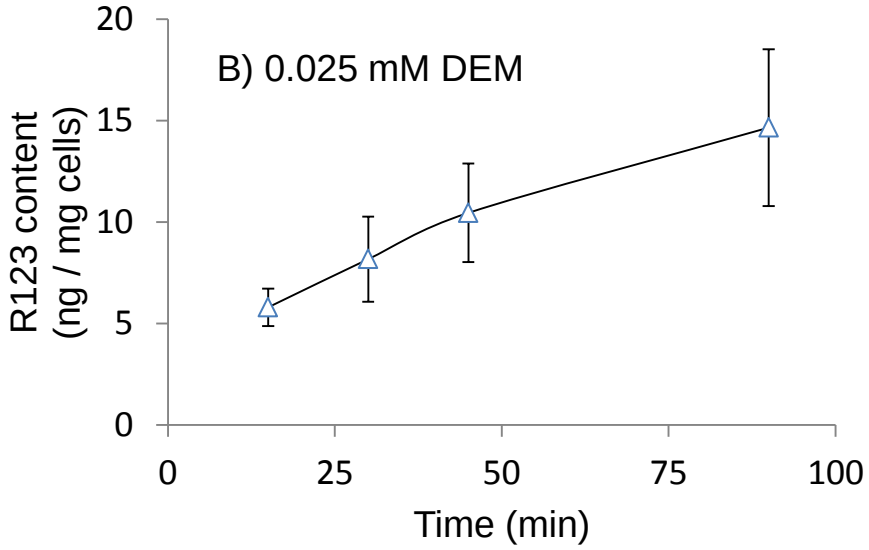
Table 3. Initial rate of R123 accumulation (mean ± SE) in units of pg R123/mg cells/min by hepatocytes immediately following exposure to 3 to 1200 µM H_2O_2. N = 5 fish. No significant differences were found between treatments and the control at $p<0.05$.

Rate of R123 accumulation ± SE (pg R123/mg cells/min)				
HSSA	H_2O_2 (µM)			
	3	30	300	1200
132 ± 19	127 ± 15	133 ± 22	123 ± 15	128 ± 18

3.3.2 DEM treatments

Fig 2 shows the accumulation of R123 with time in control (0 mM DEM) and DEM-treated (0.025, 0.25 and 2.5 mM) cells. Initial rates of R123 accumulation in control and DEM-treated hepatocytes calculated from data in Fig 2 are given in Table 4. There were no significant differences in the mean initial accumulation rates of R123 between control and DEM-treated cells (F ratio = 0.0132, p = 1.0).


A) DMSO
R123 content (ng / mg cells)
20
15
10
5
0
0
25
50
75
100
Time (min)


B) 0.025 mM DEM
R123 content (ng / mg cells)
20
15
10
5
0
0
25
50
75
100
Time (min)

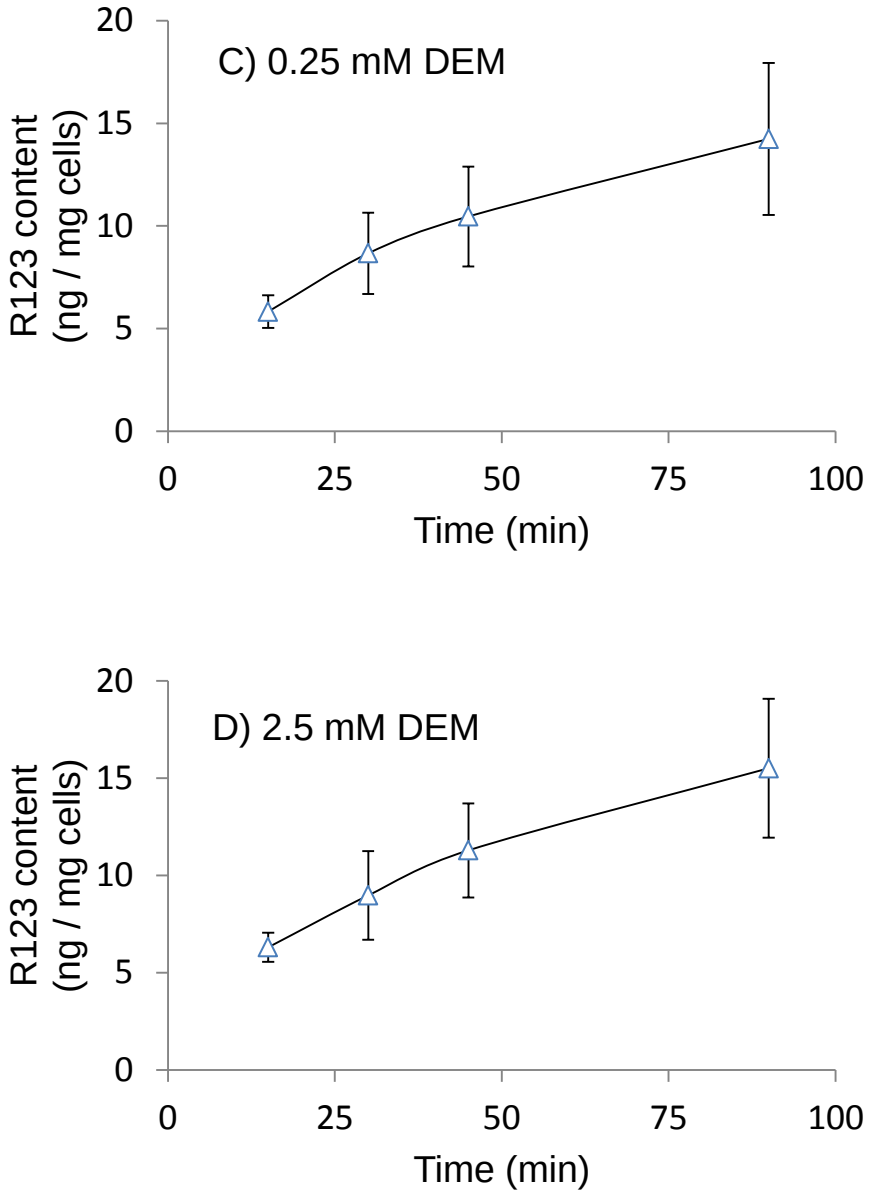


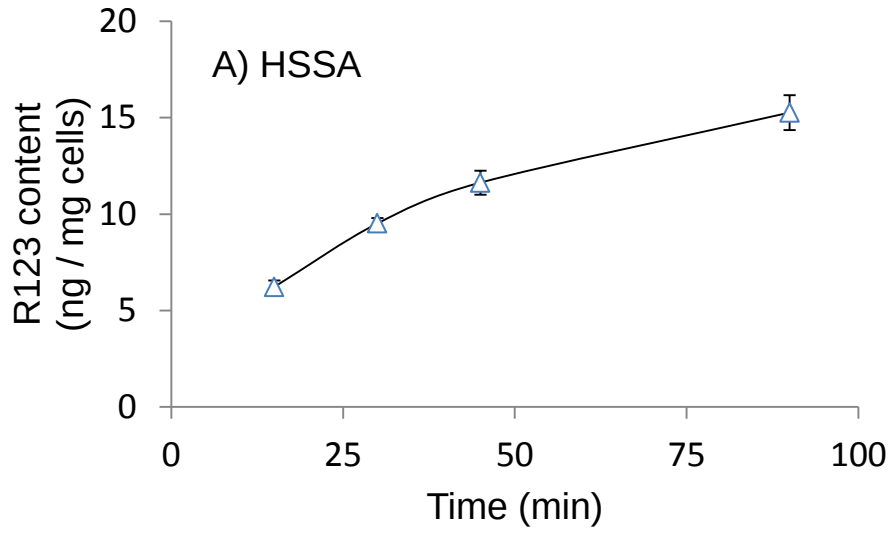
Figure 4. P-gp activity was not affected by treatment with DEM. Accumulation of R123 at 15, 30, 45 and 90 min. Values are means of R123 in ng R123/mg cells ± SE. Accumulation commenced 45 min following treatment with DEM (0.025, 0.25 or 2.5 mM) or the negative control (DMSO). N=4 fish.

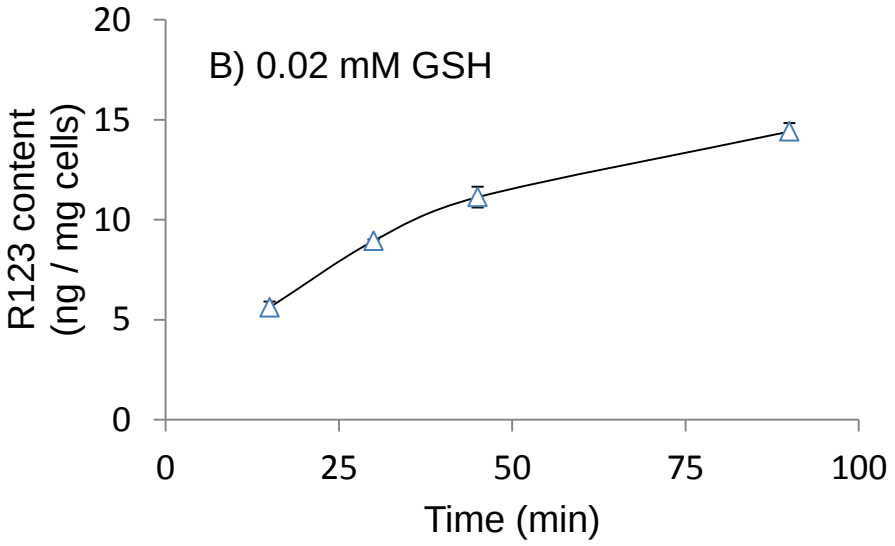
Table 4. Initial rate of R123 accumulation (mean ± SE) in units of pg R123/mg cells/min by hepatocytes 45 min following treatment with 0.025 to 2.5 mM DEM. N = 4 fish. No significant differences were found between treatments and the control at $p<0.05$.

Rate of R123 accumulation ± SE (pg R123/mg cells/min)			
DMSO	DEM concentration (mM)		
	0.025	0.25	2.5
153 ± 53	155 ± 51	155 ± 55	166 ± 56

3.3.3 GSH treatments

Figure 5 shows the mean (± SE) accumulation of R123 at 15, 30, 45 and 90 min following 45 min treatment with either 0, 0.02, 0.20 or 2.0 mM GSH. There were no significant differences in the mean initial accumulation rates of R123 (Table 5) calculated from the quantities of R123 accumulated between 15 and 45 min in each of the treatment groups. There were no significant differences in the mean initial accumulation rates of R123 between control and DEM-treated cells (F ratio = 0.173, p = 0.9)


A) HSSA
R123 content (ng / mg cells)
20
15
10
5
0
0
25
50
75
100
Time (min)


B) 0.02 mM GSH
R123 content (ng / mg cells)
20
15
10
5
0
0
25
50
75
100
Time (min)

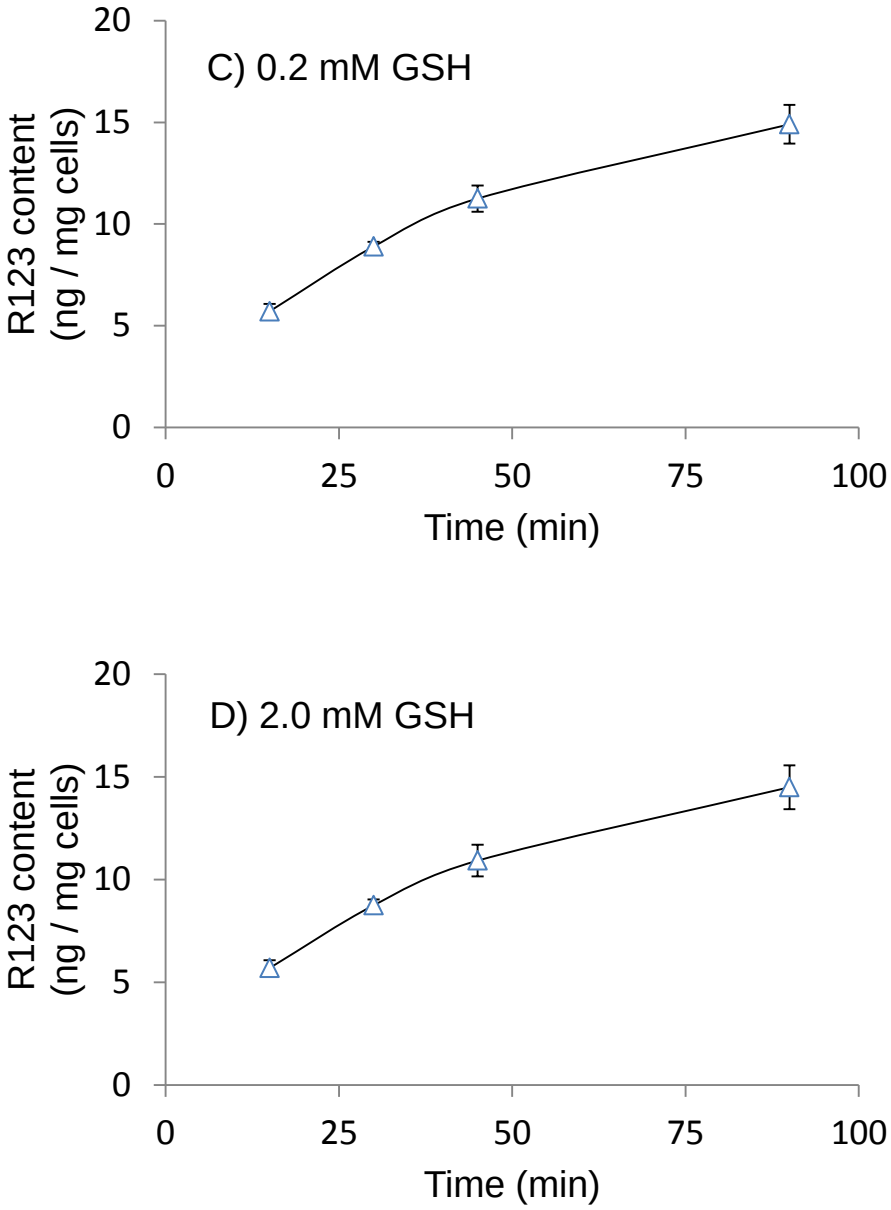


Figure 5. P-gp activity was not affected by treatment with GSH. Accumulation of R123 at 15, 30, 45 and 90 min. Values are means of R123 in ng R123/mg cells ± SE. Accumulation commenced 45 min following treatment with GSH (0.02, 0.20 or 2.0 mM) or the negative control (HSSA). N = 3 fish.

Table 5. Initial rate of R123 accumulation (mean ± SE) in units of pg R123/mg cells/min by hepatocytes 45 min following treatment with 0.02 to 2.0 mM GSH. N = 4 fish. No significant differences were found between treatments and the control at $p<0.05$.

Rate of R123 accumulation ± SE (pg R123/mg cells/min)			
HSSA	GSH concentration (mM)		
	0.02	0.20	2.0
204 ± 15	207 ± 18	210 ± 15	195 ± 15

4: DISCUSSION

4.1 Overview

Oxidative stress (OS) has been defined as a shift in the pro-oxidant – anti-oxidant balance within cells in favor of the former (Sies, 1985). This investigation sought to determine whether OS affects P-gp function as measured by R123 accumulation in freshly isolated rainbow trout hepatocytes as a model fish cell system. To achieve this, freshly isolated hepatocytes were treated with several chemicals in order to achieve various levels of OS. First, cells were exposed to varying concentrations of H_2O_2 to increase intracellular levels of reactive oxygen species (ROS). Second, to promote the accumulation of endogenous ROS, hepatocytes were treated with DEM to deplete intracellular concentrations of the ROS scavenging anti-oxidant GSH (Wu, 2009). Third, cells were treated with GSH to increase intracellular concentrations of GSH with the expectation that accumulation of endogenous ROS would be decreased as a result.

4.2 Hepatocytes as a model metabolic system in research

Fish are the dominant vertebrate group used for the purposes of regulatory evaluation in ecotoxicology and are often less expensive to maintain than mammals (Yanhong, 2008; Lushchak, 2011). Isolated and cultured hepatocytes provide a useful and relevant *in vitro* experimental model because they serve as a link between immortalized cell lines and the *in vivo* condition (Yahong, 2008). Primary hepatocyte cultures provide a useful experimental model for

investigating the metabolism of endogenous and foreign compounds (Moon, 1985; Sies, 1985; Hewitt, 2007). Much of the biological complexity of an organ system and organism is sacrificed by moving from the *in vivo* condition to the use of isolated hepatocytes, however, use of the isolated hepatocyte model provides the ability to specifically manipulate a single variable and potentially achieve a relatively high throughput system (Hewitt, 2007). The freshly isolated primary trout hepatocytes used in this study had excellent viability and appear to be an acceptable model system to investigate cellular responses to ROS modulating chemicals and regulation of P-gp. Freshly isolated primary trout hepatocytes may also be useful for the purposes of regulatory toxicology and predicting the ecotoxicological effects of environmental pollution (Lilius, 1996). Research has demonstrated that mammalian and piscine systems exhibit similar responses to oxidative stress, therefore fish systems may be useful for the purposes of elucidating basic toxicological mechanisms, including links between environmental pollution and neoplasia (Kelly, 1998), responses to oxidative stress and the regulation of P-gp.

4.3 Cell viability and plasma membrane leakage

In order to attribute potential changes in the rate of R123 accumulation to actual changes in P-gp activity, it was necessary to first determine whether GSH modulating treatments affected cell viability, and plasma membrane integrity.

Trypan blue exclusion and LDH leakage assays indicated that all DEM, GSH and H_2O_2 treatments had excellent viability and plasma membrane integrity. The finding that the average cell viability of hepatocyte suspensions treated with

concentrations of H_2O_2 up to 10 mM was unique and differed from expectations and precluded the derivation of an estimate for an LC_{50} value in the present investigation. The LC_{50} for H_2O_2 applied directly to primary cultures of rat hepatocytes has been reported to be approximately 0.5 mM (Rubin and Farber, 1984), which is considerably lower than the highest concentration of H_2O_2 found to have no adverse effect on average cell viability in the present investigation. It is expected that the difference in incubation temperature (7˚C for piscine cells vs. 37˚C for mammalian cells) may be partly responsible for the decreased toxicity of H_2O_2 to trout hepatocytes reported here. Using fish cell lines maintained at 34˚C, Babich (1993) reported H_2O_2 LC_{50} values of 0.9 mM for bluegill sunfish BF-2 fibroblasts, and 1.6 mM for fathead minnow (FHM) epithelioid cells. Given the liver high GSH content and role in xenobiotic elimination, it is not surprising that hepatocytes are more resistant to H_2O_2 than fibroblasts and epithelial cells (Otto, 1995; Luschak, 2005). Increased hepatic anti-oxidant concentrations, including GSH, are expected to play a protective role in the increased tolerance of H_2O_2 because the oxidative loss of protein thiols has been implicated in the mechanism of hepatocyte damage from H_2O_2 (Kyle, 1989).

Lipid peroxidation is an important potential confounding factor associated with treatments such as DEM (Wofford, 1988), and caution is warranted when using DEM to produce GSH depleted models of oxidative stress (Gallagher, 1992). The possibility that GSH modulating treatments may have caused cytotoxicity at the concentrations used in the present study indicated that treatment with DEM, GSH, or H_2O_2 did not affect LDH leakage or membrane

integrity as measured by trypan blue exclusion. Treatment with the positive control, 1% TTX, caused an increase in extracellular LDH concentrations, suggesting cytotoxicity. Similar to the present findings, *in vitro* treatment of isolated adult male Sprague–Dawley rat hepatocytes with 2.5 mM DEM (but not 5 mM) has been shown to have no effect on LDH leakage (Tirmenstein, 2000). Employing the same rat hepatocyte model, Esteban Pretel (2006) also reported that no signs of cytotoxicity occurred following exposures of up to 0.5 mM DEM (but not 1.0 mM).

Several methods have been described to increase cellular anti-oxidant concentrations including treatment with GSH precursors, highly membrane permeable forms of GSH and GSH itself. Rainbow trout hepatocytes treated with 2 mM GSH displayed a significant increase in GSH concentrations when compared to the HSSA control. The increased cellular GSH concentrations observed here are similar to those reported by Esteban Pretel (2006) who found that GSH concentrations in rat hepatocytes were increased approximately 1.5 fold following 1 h of incubation with 5 mM GSH.

Electrophillic unsaturated compounds including aldehydes, ketones, lactones, nitriles and nitro compounds are excreted from cells following conjugation with GSH (Boyland, 1967) which can lead to severe GSH depletion following high doses of compounds containing these functional groups (Comporti, 1991). DEM is a potent GSH depleting agent (Wofford and Thomas, 1988; Winston, 1991) whose toxicity is reported to be dependent on ROS mediated lipid peroxidation (Tirmenstein, 2000). It has also been suggested that GSH

depletion, and loss of protein thiols can lead to dysregulation of Ca^{2+} compartmentalization (which can further promote ROS production and GSH depletion) culminating in cell death (Brookes, 2004; Comporti, 1991).

Treatment with DEM caused a decrease in cellular GSH content that was sustained for 6 h. Statistical significance was achieved only for the highest concentration of DEM at 30 min following treatment. It was believed that decreasing GSH concentrations would cause cells to become more susceptible and responsive to ROS treatments, yet still remain in a physiologically relevant state. The rapid depletion of GSH concentrations reported here parallel the findings of Esteban Pretel (2006), who reported maximal GSH depletion in rat hepatocytes within 60 min of DEM exposure (0.1, 0.25 and 1.0 mM). These observations that DEM treatment decreased cellular GSH concentrations for at least 6 h without causing observable cytotoxicity suggest that DEM treatment produces an acceptable model to study the effects of oxidative stress on P-gp activity.

The 35% decrease in GSH concentration of cells treated with 2.5 mM DEM was statistically significant but less than what might have been predicted based on existing literature. Dalich (1985) and Gallagher (1992) treated trout and channel cat fish *in vivo* with 0.6 mL DEM / kg bw and both reported that GSH concentrations were maximally depleted by approximately 80% of pre-treatment concentrations following 2 h of DEM exposure. It has been reported that trout hepatocytes suffer significant GSH depletion as a result of the collagenase isolation procedure (Ferraris, 2002) but GSH returns to normal concentrations

within 48 h following isolation, when cultured in supplemented L-15 media. The findings of Ferraris (2002) provide a possible explanation for the lessened efficacy of DEM observed in the present investigation, compared to other investigations. It is possible that cellular GSH concentrations could not be depleted any further than they were in the present investigation because significant loss of GSH likely occurred during the isolation procedure. Cellular GSH concentrations of the HSSA controls in the present report were approximately 40% less than those reported by Stephensen, (2002) and 75% less than those reported by Otto (1995).

4.4 P-gp function measured by rhodamine 123 accumulation

There are several reports of altered P-gp expression and P-gp activity following pro-oxidant treatment. Modulation of P-gp levels in response to oxidative stress has been suggested to be an important regulatory mechanism promoting survival following acute environmental changes (Wu, 2009). For example, an *in vivo* study reporting increased P-gp activity and mdr1 mRNA concentrations in gill tissue of the freshwater mussel *Dreissena polymorpha* following 1 h of exposure to the cyanobacteria toxin microcystin-LR (MC-LR) provided evidence suggesting that the disruption of anti-oxidant defences leads to increased ROS accumulation and P-gp activity (Contardo, 2008).

Two different studies using rat hepatocytes have suggested that increased levels of ROS, or dissolved O_2, increase P-gp activity and mdr1 expression. P-gp activity and mdr1b mRNA concentrations were both increased when rat hepatocytes were cultured for 3 days under an atmosphere containing 16%

oxygen when compared with hepatocytes cultured under an atmosphere containing only 8% O_2 (Hirsch-Ernst, 2000). Another study that employed the rat hepatocyte model reported increased P-gp activity following 72 h of exposure to 0.1-1 mM H_2O_2 (Ziemann, 1999). However, the same study found that initial rates of R123 accumulation by pre-cultured rat hepatocytes treated with 1 mM H_2O_2 were not significantly different from controls when accumulation assays were conducted 20, 60 and 120 min following exposure. The results of these studies suggest that increases in mdr1 dependant transport activity in H_2O_2 treated hepatocytes are due to induction of P-gp expression, and not increased activity of pre-existing P-gp (Ziemann, 1999).

The initial rates of R123 accumulation found in the present study are comparable with those reported by others (Gourley, 2009; Bains & Kennedy, 2005). Initial rates of R123 accumulation measured under different H_2O_2 concentrations were not significantly different from controls suggesting that P-gp function was unaffected by increased levels of H_2O_2 (ROS). The findings of this report, along with published reports from other authors, suggest that in the short term (hours) the *in vitro* activity of P-gp in rat and rainbow trout hepatocytes, as measured by the initial rate of R123 accumulation, is insensitive to exogenously applied H_2O_2 over a broad range of concentrations (3-1200 µM).

The effects of oxidative stress on P-gp activity and P-gp expression have been investigated by depleting GSH concentrations to promote accumulation of endogenous ROS. Increased P-gp activity in isolated rat brain endothelial cells has been reported following 24 h of exposure to 200 µM of the GSH synthesis

inhibitor buthionine sulfoximine (BSO) (Hong, 2006). Another study reported that following 4 h of exposure to 912 mg/kg bw DEM, concentrations of GSH in rat brain endothelial cells were decreased, P-gp expression was increased and R123 accumulation in brain homogenate was decreased relative to controls (Wu, 2009). The findings of these studies suggest that GSH depletion, and potentially increases in ROS, cause an increase in P-gp expression and activity.

Contrary results that suggest ROS down regulate P-gp expression and activity have been reported in studies employing human prostate tumor spheroids (Wartenberg, 2000; Wartenberg, 2001). Different pro-oxidant treatments H_2O_2 (200 µM), menadione (10 µM) and glyceraldehyde (10 µM) were all found to decrease P-gp expression and increase doxorubicin retention, suggesting decreased P-gp activity (Wartenberg, 2000). These effects on P-gp expression and activity were abolished when 1 µM of the free radical scavenger ebselen was added to pro-oxidant treatments. In another experiment, elevation of endogenous ROS concentrations by depletion of GSH with BSO treatment (50 µM, 7 days) was found to decrease P-gp expression and activity. These ROS-induced increases in P-gp expression and activity were abolished when tumor spheroids were treated with ebselen (Wartenberg, 2001). Other studies have reported that treatment with anti-oxidants, such as GSH, or GSH precursors (N-acetyl cysteine) can partially reverse pro-oxidant induced increases in P-gp activity or expression (Hong, 2006; Wu, 2009). Treatment of prostate tumor spheroids for 24 h with H_2O_2 was reported to have variable effects on relative P-gp expression. Tumor spheroids treated with 1 and 200 µM H_2O_2 displayed

decreased P-gp expression while tumor spheroids treated with 750 µM H_2O_2 displayed increased P-gp expression (Wartenberg, 2001).

In the present study, the initial rate of R123 accumulation by hepatocyte suspensions treated with 2.5 mM DEM was not significantly different compared to that of the control, further suggesting that P-gp activity in isolated rainbow trout hepatocytes is not regulated by ROS or oxidative stress in the short term. Similarly, treatment of hepatocyte suspensions with 2.0 mM GSH did not cause any significant change in P-gp activity.

The observation that GSH concentrations in hepatocyte suspensions treated with 10 mM H_2O_2 were not decreased relative to the HSSA control may provide a possible explanation for the insensitivity of P-gp activity to exogenously administered H_2O_2 over such a wide range of concentrations (3 - 1200 µM): Due to the protective effects of anti-oxidants, including GSH, concentrations of ROS may not have been sufficiently elevated for a long enough period to bring about a measurable and significant effect on P-gp.

Use of R123 accumulation to measure the activity of P-gp is a good model because R123 is not reported to cause any toxic effects (Bains & Kennedy, 2005). However, an important confounding factor associated with the use of R123 may be the transport of R123 by other hepatic transporters. R123 is reported to be a substrate of multiple hepatic efflux proteins including P-gp and BCRP (ABCG2) (Litman, 2001). R123 has been used to measure the activity of BCRP (Doyle, 1998) and the existence of the BCRP ortholog in rainbow trout hepatocytes has been reported (Zaja, 2008). Hepatic concentrations of BCRP

mRNA in rainbow trout hepatocytes were more than 40 times greater than hepatic P-gp concentrations, but actual BCRP protein levels have not yet been reported (Zaja, 2008). Multiple ABC transporters contribute to the clinically important MDR phenotype and multiple ABC transporters contribute to the MXR phenotype observed in aquatic species. The importance of ABC transporters other than P-gp is widely under-appreciated in aquatic ecotoxicology (Fischer, 2010).

Using isolated hepatocytes to measure cellular parameters associated with the MDR / MXR phenotype, including increased P-gp activity and expression, have been proposed as biomarkers of environmental contamination (Kurelec, 1992; Albertus, 2001) because they are induced by exposure to a wide variety of contaminants (Minier, 1999). Some authors have suggested that the degree of MXR expression exhibited by marine invertebrates can be reliably regarded as an indicator of contaminant exposure (van der Oost, 2003). However, the complexity, and uncertainty regarding the mechanisms of P-gp induction and regulation make interpretation of environmentally induced MXR measurements difficult (Minier, 1999).

In summary, using several experimental models including mammalian and non-mammalian organisms, as well as normal and cancerous cell types, variable responses in P-gp expression and activity to ROS and oxidative stress have been reported. Regulation of P-gp has been suggested to occur through several mechanisms and may be tissue specific (Ho, 2006). Studies that used tumor spheroids found that ROS and oxidative stress decreased mdr1 mRNA levels, P-

gp expression and P-gp activity. Studies using rat hepatocytes and endothelial cells, suggest that ROS, or oxidative stress, up regulate mdr1 mRNA levels, P-gp expression and P-gp activity. However, this increase in P-gp activity is due to increased expression of P-gp and not activation of existing P-gp. The initial rates of R123 accumulation presented in this report suggest that acute, short term production of oxidative stress conditions caused by direct application of exogenous H_2O_2 or depletion of cellular GSH concentrations by DEM, are insufficient to cause a significant change in the quantity of R123 accumulated by hepatocytes in suspension over the time frame examined here.

4.5 Future work

The data presented here provide a good starting point to further evaluate the possibility that oxidative stress and ROS may regulate the activity of P-gp in isolated rainbow trout hepatocytes. It has been shown that cellular GSH concentrations can be depleted in rainbow trout hepatocytes using DEM without causing cytotoxicity and that R123 loading kinetics do not differ significantly between control and oxidatively stressed cells. Future studies should employ concentrations of DEM greater than 2.5 mM, higher incubation temperatures and longer exposure periods. An expected consequence of incubating cells at 7˚C is a decrease in the rates of all cellular metabolic events, including adaptive changes in P-gp expression and activity. Hepatocyte suspensions incubated at higher temperatures may show evidence of altered P-gp activity sooner than hepatocyte suspensions incubated at 7˚C.

Pre exposing cells to DEM prior to application of exogenous oxidants may be a useful treatment to further evaluate whether oxidative stress and ROS are important regulatory factors controlling P-gp activity and expression in rainbow trout hepatocytes. Future studies investigating the regulation of P-gp in teleost hepatocytes should employ longer exposure periods and focus on steady state concentrations of R123 by allowing cells be exposed to R123 for 3 h prior to centrifugation and pellet washing (Cooper, 1999; Hong, 2006; Ziemann, 1999).

Reports of R123 accumulation rates by trout hepatocytes have been published (Gourley, 2009), but this parameter describing R123 kinetics may be a less sensitive indicator compared to measurements taken once a steady state has been achieved in the hepatocyte suspension owing to the relative magnitudes of the forces controlling R123 partitioning between the intracellular and extracellular components of hepatocyte suspensions. Future studies examining the potential for OS to regulate P-gp activity should focus on efflux rates, now that it has been determined that R123 accumulation by hepatocyte suspensions is not significantly affected by treatment with H_2O_2, DEM or GSH.

4.6 General conclusions

Oxidative stress is an important mechanism of toxicity that results following exposure to a variety of environmentally relevant contaminants of natural, and human origin (Livingstone, 2001). During states of oxidative stress, when cellular anti-oxidant defences are depleted, biological macromolecules are more likely to be damaged by pro-oxidant chemicals resulting in loss of structural and functional integrity (Valko, 2007). P-gp is a critical contributor to the cellular

ability to remove endogenous and exogenous compounds from the cell. The expression of P-gp has previously been shown to be regulated by conditions of oxidative stress (Felix, 2002; Hong, 2006; Wu, 2009). However, the results of this work suggest that the activity of P-gp, as evidenced by the accumulation of R123, is not affected by oxidative stress conditions.

Disrupted cellular efflux capabilities can lead to increased accumulation of deleterious compounds, and ultimately cause adverse impacts on health at a cellular, organism and ecosystem level. By understanding the mechanisms by which this cellular defence mechanism is regulated across phylogenetic boundaries, insights may be gained into the basic biology of P-gp and perhaps diverse physiological events which may provide information that is relevant for the processes of environmental risk assessment (Livingstone, 2001) and setting water quality guidelines that are sufficiently protective.

LITERATURE CITED

Albertus, J., Laine, R. O., 2001. Enhanced xenobiotic transporter expression in normal teleost hepatocytes: response to environmental and chemotherapeutic toxins. J. Exp. Biol. 204, 217-227.

Ambudkar, S. V., Dey, S., Hrycyna, C. A, Ramachandra, M., Pastan, I., Gottesman M. M., (1999). Annu. Rev. Pharmacol. Toxicol., 39, 361–398.

Ambudkar, S. V., Kimchi-Sarfaty, C., Sauna, Z. E., Gottesman, M. M., 2003. P-glycoprotein: from genomics to mechanism. Oncogene. 22,7468-2485.

Anderson, M. E., 1998. Glutathione: an overview of biosynthesis and modulation. Chem. Biol. Interact. 111-112, 1-14.

Annilo, T., Chen, Z. Q., Shulenin, S., Costantino, J., Thomas, L., Lou, H., Stefanov, S., Dean, M., 2006. Evolution of the vertebrate ABC gene family: analysis of gene birth and death. Genomics 88, 1-11.

Attie, A. D., 2007. ABCA1: at the nexus of cholesterol, HDL and atherosclerosis. Trends Biochem. Sci. 32, 172-179.

Babich, H., Palace, M. R., Stern, A., 1993. Oxidative stress in fish cells: in vitro studies. Arch. Environ. Contam. Toxicol. 24, 173-178.

Bains, O. S., Kennedy, C. J., 2005. Alterations in respiration rate of isolated rainbow trout hepatocytes exposed to the P-glycoprotein substrate rhodamine 123. Toxicology 214, 87-98.

Bains, O. S., Kennedy, C. J., 2004. Energetic costs of pyrene metabolism in isolated hepatocytes of rainbow trout, *Oncorhynchus mykiss*. Aquat. Toxicol. 67, 217-226.

Baker, E. K., Johnstone, R. W., Zalcberg, J. R., El-Osta, A., 2005. Epigenetic changes to the MDR1 locus in response to chemotherapeutic drugs. Oncogene 24, 8061-8075.

Banerjee, B. D., Seth, V., Ahmed, R. S., 2001. Pesticide-induced oxidative stress: perspectives and trends. Rev. Environ. Health 16, 1-40.

Bard, S. M., 2000. Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms. Aquat. Toxicol. 48, 357-389.

Bard, S. M., Bello, S. M., Hahn, M. E., Stegeman, J. J., 2002. Expression of P-glycoprotein in killifish (*Fundulus heteroclitus*) exposed to environmental xenobiotics. Aquat. Toxicol. 59, 237-251.

Bard, S. M., Woodin, B. R., Stegeman, J. J., 2002. Expression of P-glycoprotein and cytochrome p450 1A in intertidal fish (*Anoplarchus purpurescens*) exposed to environmental contaminants. Aquat. Toxicol. 60, 17-32.

Barnhart, J. L., Combes, B., 1978. Choleresis associated with metabolism and biliary excretion of diethyl maleate in the rat and dog. J. Pharmacol. Exp. Ther. 206, 614-623.

Bird, I. M., 2011. Endothelial nitric oxide synthase activation and nitric oxide function: new light through old windows. J. Endocrinol. 210, 239-241.

Boyland, E., Chasseaud, L. F., 1967. Enzyme-catalysed conjugations of glutathione with unsaturated compounds. Biochem. J. 104, 95-102.

Bresolin, T., Rebelo M. F., Bainy A. C. D., 2005. Expression of PXR, CYP3A and MDR1 genes in liver of zebrafish. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 140, 403-407.

Brookes, P. S., Yoon, Y., Robotham, J. L., Anders, M. W., Sheu, S. S., 2004. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. Am. J. Physiol. Cell. Physiol. 287, 817-33.

Callaghan, R., Crowley, E., Potter, S., Kerr, I. D., 2008. P-glycoprotein: so many ways to turn it on. J. Clin. Pharmacol. 48, 365-378.

Caminada, D., Zaja, R., Smital, T., Fent, K., 2008. Human pharmaceuticals modulate P-gp1 (ABCB1) transport activity in the fish cell line PLHC-1. Aquat. Toxicol. 90, 214-222.

Chambers, T. C., Pohl, J., Raynor, R. L., Kuo, J. F., 1993. Identification of specific sites in human P-glycoprotein phosphorylated by protein kinase C. J Biol Chem. 268, 4592-5.

Chan, L. M., Lowes, S., Hirst, B. H., 2004. The ABCs of drug transport in intestine and liver: efflux proteins limiting drug absorption and bioavailability. Eur. J. Pharm. Sci. 21, 25-51.

Chin, K. V., Tanaka, S., Darlington, G., Pastan, I., Gottesman, M. M., 1990. Heat shock and arsenite increase expression of the multidrug resistance (MDR1) gene in human renal carcinoma cells. J. Biol. Chem. 265, 221-226.

Comporti, M., Maellaro, E., Del Bello, B., Casini, A. F., 1991. Glutathione depletion: its effects on other anti-oxidant systems and hepatocellular damage. Xenobiotica 21, 1067-1076.

Contardo-Jara, V., Pflugmacher, S., Wiegand, C., 2008. Multi-xenobiotic-resistance a possible explanation for the insensitivity of bivalves towards cyanobacterial toxins. Toxicon 52, 936–943.

Cooper, P. S., Vogelbein, W. K., Veld, P. A. V., 1999. Altered expression of the xenobiotic transporter P-glycoprotein in liver and liver tumours of mummichog *Fundulus heteroclitus* from a creosote-contaminated environment. Biomarkers 4, 48-58.

Dalich, G. M., Larson, R. E., 1985. A comparative study of the hepatotoxicity of monochlorobenzene in the rainbow trout (*Salmo gairdnerii*) and the Sprague-Dawley rat. Comp. Biochem. Physiol. C. 80, 115-122.

Deng, L., Lin-Lee, Y. C., Claret, F. X., Kuo, M. T., 2001. 2-acetylaminofluorene up-regulates rat mdr1b expression through generating reactive oxygen species that activate NF-kappa B pathway. J. Biol. Chem. 276, 413-420.

Di Giulio, R. T, Washburn, P, C, Wenning, R. J, Winston, G. W, Jewell, C. S. 1989. Biochemical responses in aquatic animals: a review of determinants of oxidative stress. Environ. Toxicol. Chem. 8, 1103-1123.

Doi, A. M., Holmes, E., Kleinow, K. M., 2001. P-glycoprotein in the catfish intestine: inducibility by xenobiotics and functional properties. Aquat. Toxicol. 55, 157-170.

Dong, Y. L., Zhou, P. J., Jiang, S. Y., Pan, X. W., Zhao, X. H., 2009. Induction of oxidative stress and apoptosis by pentachlorophenol in primary cultures of *Carassius carassius* hepatocytes. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 150, 179-185.

Dorval, J., Leblond, V., Deblois, C., Hontela, A., 2005. Oxidative stress and endocrine endpoints in white sucker (*Catostomus commersoni*) from a river impacted by agricultural chemicals. Environ. Toxicol. Chem. 24, 1273-1280.

Dorval, J., Leblond, V. S., Hontela, A., 2003. Oxidative stress and loss of cortisol secretion in adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*) exposed in vitro to endosulfan, an organochlorine pesticide. Aquat. Toxicol. 63, 229-241.

Doyle, L. A., Yang, W., Abruzzo, L. V., Krogmann, T., Gao, Y., Rishi, A. K., Ross, D. D., 1998. A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc. Natl. Acad. Sci. U. S. A. 95, 15665-15670.

Eckford, P.D., Sharom, F.J., 2005. The reconstituted P-glycoprotein multidrug transporter is a flippase for glucosylceramide and other simple glycosphingolipids. Biochem J. 389(Pt 2),517-26.

Endicott, J. A., Ling, V., 1989. The biochemistry of P-glycoprotein-mediated multidrug resistance. Annu. Rev. Biochem. 58, 137-171.

Epel, D., Luckenbach, T., Stevenson, C. N., Macmanus-Spencer, L. A., Hamdoun, A., Smital, T., 2008. Efflux transporters: newly appreciated roles in protection against pollutants. Environ. Sci. Technol. 42, 3914-3920.

Esteban-Pretel, G., Pilar Lopez-Garcia, M., 2006. An experimental design for the controlled modulation of intracellular GSH levels in cultured hepatocytes. Free Radic. Biol. Med. 41, 610-619.

Felix, R. A., Barrand, M. A., 2002. P-glycoprotein expression in rat brain endothelial cells: evidence for regulation by transient oxidative stress. J. Neurochem. 80, 64-72.

Feng, T., Zheng, W. Y., Guo, X. Q., Zhang, Y., 2001. Reduced-glutathione concentrations in *Boleophthalmus pectinirostris* tissues exposed to benzo(a)pyrene. J. Environ. Sci. (China) 13, 308-310.

Ferraris, M., Radice, S., Catalani, P., Francolini, M., Marabini, L., Chiesara, E., 2002. Early oxidative damage in primary cultured trout hepatocytes: a time course study. Aquat. Toxicol. 59, 283-296.

Finkel, T., 2001. Reactive oxygen species and signal transduction. IUBMB Life 52, 3-6.

Fischer, S., Pietsch, M., Schirmer, K., Luckenbach, T. 2010. Identification of multi-drug resistance associated proteins MRP1 (ABCC1) and MRP3 (ABCC3) from rainbow trout (*Oncorhynchus mykiss*). Mar. Environ. Res. 69, Suppl, S7-S10.

Ford, J. M., Hait, W. N., 1990. Pharmacology of drugs that alter multidrug resistance in cancer. Pharmacol. Rev. 42, 155-199.

Gallagher, E. P., Hasspieler, B. M., Di Glulio, R. T., 1992. Effects of buthionine sulfoximine and diethyl maleate on glutathione turnover in the channel catfish. Biochem. Pharmacol. 43, 2209-2215.

Gazzin, S., Berengeno, A., Strazielle, N., Fazzari, F., Raseni, A., Ostrow, J. D., Wennberg, R., Ghersi-Egea, J., Tiribelli, C., 2011. Modulation of Mrp1 (ABCC1) and P-gp (ABCB1) by bilirubin at the blood-CSF and blood-brain barriers in the Gunn rat. PLoS One 6, e16165.

Gill, K.A., Walsh, P.J., 1990. Effects of temperature on metabolism of benzo[a]pyrene by toadfish (*Opsanus beta*) hepatocytes. Can.J. Aquat. Sci. 47, 831–837.

Gomez-Martinez, A., Garcia-Morales, P., Carrato, A., Castro-Galache, M. D., Soto, J. L., Carrasco-Garcia, E., Garcia-Bautista, M., Guaraz, P., Ferragut, J. A., Saceda, M., 2007. Post-transcriptional regulation of P-glycoprotein expression in cancer cell lines. Mol. Cancer. Res. 5, 641-653.

Gottesman, M. M., Pastan, I., 1993. Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu. Rev. Biochem. 62, 385-427.

Gottesman, M.M, Fojo, T., Bates, S.E., 2002. Multidrug resistance in cancer: role of ATP-dependant transporters. Nat. Rev. Cancer 2, 48-58.

Gourley, M. E., Kennedy, C. J., 2009. Energy allocations to xenobiotic transport and biotransformation reactions in rainbow trout (*Oncorhynchus mykiss*) during energy intake restriction. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 150, 270-278.

Hemmer, M. J., Courtney, L. A., Ortego, L. S., 1995. Immunohistochemical detection of P-glycoprotein in teleost tissues using mammalian polyclonal and monoclonal antibodies. J. Exp. Zool. 272, 69-77.

Hewitt, N. J., Lechon, M. J., Houston, J. B., Hallifax, D., Brown, H. S., Maurel, P., Kenna, J. G., Gustavsson, L., Lohmann, C., Skonberg, C., Guillouzo, A., Tuschl, G., Li, A. P., LeCluyse, E., Groothuis, G. M., Hengstler, J. G., 2007. Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. Drug Metab. Rev. 39, 159-234.

Higgins, C. F., Gottesman, M. M., 1992. Is the multidrug transporter a flippase? Trends Biochem. Sci. 17, 18-21.

Higgins, C. F., 2007. Multiple molecular mechanisms for multidrug resistance transporters. Nature 446, 749-757.

Hildebrand, J. L., Bains, O. S., Lee, D. S., Kennedy, C. J., 2009. Functional and energetic characterization of P-gp-mediated doxorubicin transport in rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 149, 65-72.

Hill, B. T., Deuchars, K., Hosking, L. K., Ling, V., Whelan, R. D., 1990. Overexpression of P-glycoprotein in mammalian tumor cell lines after fractionated X irradiation in vitro. J. Natl. Cancer Inst. 82, 607-612.

Hirsch-Ernst, K, I., Kietzmann, T., Ziemann, C., Jungermann, K., Kahl, G. F., 2000. Physiological oxygen tensions modulate expression of the mdr1b multidrug-resistance gene in primary rat hepatocyte cultures. Biochem J. 350 Pt 2: 443-51.

Ho, E. A., Piquette-Miller, M., 2006. Regulation of multidrug resistance by pro-inflammatory cytokines. Curr. Cancer. Drug Targets 6, 295-311.

Hong, H., Lu, Y., Ji, Z. N., Liu, G. Q., 2006. Up-regulation of P-glycoprotein expression by glutathione depletion-induced oxidative stress in rat brain microvessel endothelial cells. J. Neurochem. 98, 1465-1473.

Jaeschke, H., Gores, G. J., Cederbaum, A. I., Hinson, J. A., Pessayre, D., Lemasters, J. J., 2002. Mechanisms of hepatotoxicity. Toxicol. Sci. 65, 166-176.

Jedlitschky, G., Leier I., Buchholz U., Barnouin K., Kurz G., Keppler, D., 1996. Transport of glutathione, glucuronate and sulfate conjugates by the MRP gene-encoded conjugate export pump. Cancer Res. 56, 988–994.

Jos, A., Cameán, A. M., Pflugmacher, S., Segner, H., 2009. The anti-oxidant glutathione in the fish cell lines EPC and BCF-2: Response to model pro-oxidants as measured by three different fluorescent dyes. Toxicology in Vitro. 23, 546-553.

Jos, A., Pichardo, S., Prieto, A. I., Repetto, G., Vazquez, C. M., Moreno, I., Camean, A. M., 2005. Toxic cyanobacterial cells containing microcystins induce oxidative stress in exposed tilapia fish (*Oreochromis sp.*) under laboratory conditions. Aquat. Toxicol. 72, 261-271.

Juliano, R. L., Ling, V., 1976. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochimica et Biophysica Acta (BBA) - Biomembranes 455, 152-162.

Klaassen, C. D., 1988. Intestinal and hepatobiliary disposition of drugs. Toxicol. Pathol. 16, 130-137.

Klaassen, C. D., Slitt, A. L., 2005. Regulation of hepatic transporters by xenobiotic receptors. Curr. Drug Metab. 6, 309-328.

Kelly, K. A., Havrilla, C. M., Brady, T. C., Abramo, K. H., Levin, E. D., 1998. Oxidative stress in toxicology: established mammalian and emerging piscine model systems. Environ. Health. Perspect. 106, 375-384.

Kleinow, K. M., Doi, A. M., Smith, A. A., 2000. Distribution and inducibility of P-glycoprotein in the catfish: immunohistochemical detection using the mammalian C-219 monoclonal. Mar. Environ. Res. 50, 313-317.

Krug, P. J., 2006. Defense of benthic invertebrates against surface colonization by larvae: a chemical arms race. Prog. Mol. Subcell. Biol. 42, 1-53.

Kuo, M. T., 2009. Redox regulation of multidrug resistance in cancer chemotherapy: molecular mechanisms and therapeutic opportunities. Antioxid. Redox Signal. 11, 99-133.

Kurelec, B., 1992. The multixenobiotic resistance mechanism in aquatic organisms. Crit. Rev. Toxicol. 22, 23-43.

Kyle, M. E., Nakae, D., Sakaida, S., Serroni, A., Farber, J. L., 1989. Protein thiol depletion and the killing of cultured hepatocytes by hydrogen peroxide. Biochem. Pharmacol. 38, 3797-3805.

Leão, P. N., Vasconcelos, M. T., Vasconcelos, V. M., 2009. Allelopathy in freshwater cyanobacteria. Crit Rev Microbiol. 35, 271-82.

Ledoux, S., Yang, R., Friedlander, G., Laouari, D., 2003. Glucose depletion enhances P-glycoprotein expression in hepatoma cells: role of endoplasmic reticulum stress response. Cancer Res. 63, 7284-7290.

Leggatt, R. A., Brauner, C. J., Schulte, P. M., Iwama, G. K., 2007. Effects of acclimation and incubation temperature on the glutathione anti-oxidant system in killifish and RTH-149 cells. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 146, 317-326.

Leier, I., Jedlitschky, G., Buchholz, U., Cole, S. P., Deeley, R. G., Keppler, D,. 1994. The MRP gene encodes an ATP-dependent export pump for leukotriene C4 and structurally related conjugates. J. Biol. Chem. 269, 27807-27810.

Li, Y., Yuan, H., Yang, K., Xu, W., Tang, W., Li, X., 2010. The structure and functions of P-glycoprotein. Curr. Med. Chem. 17, 786-800.

Lilius, H., Hastbacka, T., Isomaa, B., 1996. Combination of Fluorescent Probes for Evaluation of Cytotoxicity and Toxic Mechanisms in Isolated Rainbow Trout Hepatocytes. Toxicol. in Vitro 10, 341-348.

Litman, T., Druley, T. E., Stein, W. D., Bates, S. E., 2001. From MDR to MXR: new understanding of multidrug resistance systems, their properties and clinical significance. Cell. Mol. Life Sci. 58, 931–959.

Marchand, J., Quiniou, L., Riso, R., Thebaut, M. T., Laroche, J., 2004. Physiological cost of tolerance to toxicants in the European flounder *Platichthys flesus*, along the French Atlantic Coast. Aquat. Toxicol. 70, 327-343.

Minier, C., Eufemia, N., Epel, D., 1999. The multi-xenobiotic resistance phenotype as a tool to biomonitor the environment. Biomarkers 4, 442-454.

Moon, T. W., Walsh, P. J., Mommsen, T. P., 1985. Fish hepatocytes: a model metabolic system. Can. J. Fish. Aquat. Sci. 42, 1772-1782.

Moore, M. N., 1992. Molecular cell pathology of pollutant-induced liver injury in flatfish: use of fluorescent probes. Mar. Ecol. Prog. Ser. 91, 127-133.

Morgan, M. J., Liu, Z. G., 2011. Crosstalk of reactive oxygen species and NF-kappaB signaling. Cell Res. 21, 103-15.

Nogueira, L., Rodrigues, A. C., Tridico, C. P., Fossa, C. E., de Almeida, E. A., 2010. Oxidative stress in Nile tilapia (*Oreochromis niloticus*) and armored catfish (*Pterygoplichthys anisitsi*) exposed to diesel oil. Environ. Monit. Assess. 80, 243-55.

Ott, M., Fricker, G., Bauer, B., 2009. Pregnane X receptor (PXR) regulates P-glycoprotein at the blood-brain barrier: functional similarities between pig and human PXR. J. Pharmacol. Exp. Ther. 329, 141-149.

Otto, D. M., Moon, T. W., 1995. 3,3',4,4'-tetrachlorobiphenyl effects on anti-oxidant enzymes and glutathione status in different tissues of rainbow trout. Pharmacol. Toxicol. 77, 281-287.

Paetzold, S. C., Ross, N. W., Richards, R. C., Jones, M., Hellou, J., Bard, S. M., 2009. Up-regulation of hepatic ABCC2, ABCG2, CYP1A1 and GST in multixenobiotic-resistant killifish (*Fundulus heteroclitus*) from the Sydney Tar Ponds, Nova Scotia, Canada. Mar. Environ. Res. 68, 37-47.

Patel, T., Roberts, L. R., Jones, B. A., Gores, G. J., 1998. Dysregulation of apoptosis as a mechanism of liver disease: an overview. Semin. Liver Dis. 18, 105-114.

Pawagi, A. B., Wang, J., Silverman, M., Reithmeier, R. A., Deber, C. M., 1994. Transmembrane aromatic amino acid distribution in P-glycoprotein. A functional role in broad substrate specificity. J. Mol. Biol. 235, 554-564.

Ploch, S. A., Lee, Y., MacLean, E., Di Giulio, R. T., 1999. Oxidative stress in liver of brown bullhead and channel catfish following exposure to tert-butyl hydroperoxide. Aquat. Toxicol. 46, 231-240.

Queisser, N., Fazeli, G., Schupp, N., 2010. Superoxide anion and hydrogen peroxide-induced signaling and damage in angiotensin II and aldosterone action. Biol. Chem. 391, 1265-1279.

Quinn, M. T., Ammons, M. C., Deleo, FR., 2006. The expanding role of NADPH oxidases in health and disease: no longer just agents of death and destruction. Clin. Sci. 1, 1-20.

Rebbeor, J. F., Connolly, G. C., Henson, J. H., Boyer, J. L., Ballatori, N., 2000. ATP-dependent GSH and glutathione S-conjugate transport in skate liver: role of an Mrp functional homologue. Am. J. Physiol. Gastrointest. Liver Physiol. 279, 417–425.

Rigalli, J. P., Ruiz, M. L., Perdomo, V. G., Villanueva, S. S., Mottino, A. D., Catania, V. A., 2011. Pregnane X receptor mediates the induction of P-glycoprotein by spironolactone in HepG2 cells. Toxicology 285, 18-24.

Rost, D., Kartenbeck, J., Keppler, D., 1999. Changes in the localization of the rat canalicular conjugate export pump Mrp2 in phalloidin-induced cholestasis. Hepatology 29, 814-821.

Rubin, R., Farber, J. L., 1984. Mechanisms of the killing of cultured hepatocytes by hydrogen peroxide. Arch. Biochem. Biophys. 228, 450-459.

Saves, I, Masson, J. M., 1998. Mechanisms of resistance to xenobiotics in human therapy. Cell. Mol. Life. Sci. 54, 405-426.

Schinkel, A. H., Jonker, J. W., 2003. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. Adv. Drug Deliv. Rev. 55, 3-29.

Seibert, H., 1985. Viability control and oxygen consumption of isolated hepatocytes from thermally acclimated rainbow trout (*Salmo gairdnerii*). Comp. Biochem. Physiol. B. 80, 677-683.

Sekine, S., Ito, K., Horie, T., 2006. Oxidative stress and Mrp2 internalization. Free Radic. Biol. Med. 40, 2166-2174.

Shtil, A. A., Azare, J., 2005. Redundancy of biological regulation as the basis of emergence of multidrug resistance. Int. Rev. Cytol. 246, 1-29.

Sies, H., Cadenas, E., 1985. Oxidative stress: damage to intact cells and organs. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 311, 617-631.

Singhal, S.S., Yadav, S., Roth, C., Singhal, J. 2009. RLIP76: A novel glutathione-conjugate and multi-drug transporter. Biochem Pharmacol. 77, 761–769.

Skovsgaard, T., Danø, K., and Nissen, N. I., 1984. Chemosensitizers counteracting acquired resistance to anthracyclines and vinca alkaloides in vivo. A new treatment principle. Cancer Treat. Rev. 11, 63–72.

Stephensen, E., Sturve, J., Forlin, L., 2002. Effects of redox cycling compounds on glutathione content and activity of glutathione-related enzymes in rainbow trout liver. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 133, 435-442.

Sturm, A., Ziemann, C., Hirsch-Ernst, K. I., Segner, H., 2001. Expression and functional activity of P-glycoprotein in cultured hepatocytes from *Oncorhynchus mykiss*. Am. J. Physiol. Regul. Integr. Comp. Physiol. 281, 1119-1126.

Sturve, J., Almroth, B. C., Forlin, L., 2008. Oxidative stress in rainbow trout (*Oncorhynchus mykiss*) exposed to sewage treatment plant effluent. Ecotoxicol. Environ. Saf. 70, 446-452.

Taylor, M., Feyereisen, R., 1996. Molecular biology and evolution of resistance to toxicants. Mol. Biol. Evol. 13, 719-734.

Thews, O., Gassner, B., Kelleher, D. K., Schwerdt, G., Gekle, M., 2006. Impact of extracellular acidity on the activity of P-glycoprotein and the cytotoxicity of chemotherapeutic drugs. Neoplasia 8, 143-152.

Timsit, Y. E., Negishi, M., 2007. CAR and PXR: The xenobiotic-sensing receptors. Steroids 72, 231-246.

Tirmenstein, M.A., Nicholls-Grzemski, F.A., Zhang, J.-G., Fariss, M.W., 2000. Glutathione depletion and the production of reactive oxygen species in isolated hepatocyte suspensions. Chem. Biol. Interact. 127, 201–21.

Tsuruo, T., Iida, H., Tsukagoshi, S., Sakurai, Y., 1981. Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. Cancer Res. 41, 1967–1972.

Tsuruo, T., Iida, H., Tsukagoshi, S., Sakurai, Y., 1982. Increased accumulation of vincristine and adriamycin in drug resistant P388 tumor cells following incubation with calcium antagonists and calmodulin inhibitors. Cancer Res. 42, 4730–4733.

Tsuruo T., Iida H., Nojiri M., Tsukagoshi S., Sakurai Y., 1983. Circumvention of vincristine and adriamycin resistance in vitro and in vivo by calcium channel blockers. Cancer Res. 43, 2905–2910.

Tsuruo, T., Iida H., Kitatani, Y., Yokota, K., Tsukagoshi, S., Yakurai Y,. 1984. Effects of quinidine and related compounds on cytotoxicity and cellular accumulation of vincristine and adriamycin in drug-resistant tumor cells. Cancer Res. 44, 4303–4307.

Valko, M., Morris, H., Cronin, M. T., 2005. Metals, toxicity and oxidative stress. Curr. Med. Chem. 12, 1161-1208.

Valko, M., Leibfritz, D., Moncola, J., Cronin, M.T., Mazura, M., Telser, J., 2007. Free radicals and anti-oxidants in normal physiological functions and human disease. Int. J. Biochem. Cell Biol. 39, 44-84.

van der Oost, R., Beyer, J., Vermeulen, N. P., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57-149.

Vander Borght, S., Libbrecht, L., Blokzijl, H., Faber, K. N., Moshage, H., Aerts, R., Van Steenbergen, W., Jansen, P. L., Desmet, V. J., Roskams, TA., 2005. Diagnostic and pathogenetic implications of the expression of hepatic transporters in focal lesions occurring in normal liver. J. Pathol. 207, 471-482.

Versantvoort, C. H., Broxterman, H. J., Bagrij, T., Scheper, R. J., Twentyman, P. R., 1995. Regulation by glutathione of drug transport in multidrug-resistant human lung tumour cell lines overexpressing multidrug resistance-associated protein. Br. J. Cancer. 72, 82-89.

Wartenberg, M., Fischer, K., Hescheler, J., Sauer, H., 2000. Redox regulation of P-glycoprotein-mediated multidrug resistance in multicellular prostate tumor spheroids. Int. J. Cancer. 85, 267-274.

Wartenberg, M., Ling, F. C., Schallenberg, M., Baumer, A. T., Petrat, K., Hescheler, J., Sauer, H., 2001. Down-regulation of intrinsic P-glycoprotein expression in multicellular prostate tumor spheroids by reactive oxygen species. J. Biol. Chem. 276, 17420-17428.

Wassmur, B., Grans, J., Kling, P., Celander, M. C., 2010. Interactions of pharmaceuticals and other xenobiotics on hepatic pregnane X receptor and cytochrome P450 3A signaling pathway in rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 100, 91-100.

Whittaker, R. H., Feeny, P. P., 1971. Allelochemics: chemical interactions between species. Science. 171, 757-70.

Williams, R. T., 1971. Detoxification mechamisms, 2nd ed. New York: Wiley.

Winston, G. W., Di Giulio, R. T., 1991. Prooxidant and anti-oxidant mechanisms in aquatic organisms. Aquat. Toxicol. 19, 137-161.

Wofford, H. W., Thomas, P., 1988. Peroxidation of mullet and rat liver lipids in vitro: effects of pyridine nucleotides, iron, incubation buffer, and xenobiotics. Comp. Biochem. Physiol. C. 89, 201-205.

Wofford, H. W., Thomas, P., 1988. Effect of xenobiotics on peroxidation of hepatic microsormal lipids from striped mullet (*Mugil cephalus*) and Atlantic croaker (*Micropogonias undulatus*). Mar. Environ. Res. 24, 285-289.

Xu, C., Li, C. Y., Kong, A. N., 2005. Induction of phase I, II and III drug metabolism/transport by xenobiotics. Arch. Pharm. Res. 28, 249-268.

Yamane, Y., Furuichi, M., Song, R., Van, N. T., Mulcahy, R. T., Ishikawa, T., Kuo, M. T., 1998. Expression of multidrug resistance protein/GS-X pump and gamma-glutamylcysteine synthetase genes is regulated by oxidative stress. J. Biol. Chem. 273, 31075-31085.

Yanhong, F., Chenghua, H., Guofang, L., Haibin, Z., 2008. Optimization of the isolation and cultivation of *Cyprinus carpio* primary hepatocytes. Cytotechnology 58, 85-92.

Zaja, R., Munic, V., Klobucar, R. S., Ambriovic-Ristov, A., Smital, T., 2008. Cloning and molecular characterization of apical efflux transporters (ABCB1, ABCB11 and ABCC2) in rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Aquat. Toxicol. 90, 322-332.

Zhou, W. D., Zhang, H. Q., Fang, M., He, Q. Y., Pang, D. B., Xue, S. B., 1996. Induction of expression of MDR 1 gene by retinoic acid and DMSO and effects on rhodamine-123 efflux in HL-60 cell lines and resistant sublines. Yao Xue Xue Bao. 31, 1-5.

Zhou, S. F., 2008. Structure, function and regulation of P-glycoprotein and its clinical relevance in drug disposition. Xenobiotica 38, 802-832.

Ziemann, C., Burkle, A., Kahl, G. F., Hirsch-Ernst, K. I., 1999. Reactive oxygen species participate in mdr1b mRNA and P-glycoprotein overexpression in primary rat hepatocyte cultures. Carcinogenesis 20, 407-414.