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P-glycoprotein and membrane permeability as determinants of xenobiotic bioavailability and bioaccumulation

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P-GLYCOPROTEIN AND MEMBRANE PERMEABILITY AS DETERMINANTS OF XENOBIOTIC BIOAVAILABILITY AND BIOACCUMULATION

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ABSTRACT

The ABC transporter P-glycoprotein (Pgp) and membrane permeability are determinant factors for absorption and disposition of xenobiotics. These studies investigated the effects of potential modulators of Pgp and/or membrane function on the disposition, bioavailability and bioaccumulation of environmentally relevant pharmaceuticals, tetracycline and ivermectin, and the carcinogen benzo[a]pyrene in catfish. The pesticide dieldrin and surfactant linear alkylbenzene sulfonate (LAS) were selected as mixture components thought to have potential interactions with these determinants of disposition. Initial *in situ* experiments demonstrated dieldrin and tetracycline both significantly inhibited biliary excretion of Pgp prototypic substrate Rhodamine-123 in isolated perfused livers. Further, dieldrin (20μM) reduced movement of ^{3}H-tetracycline (8.8nM) into bile (55%) to a greater extent than Pgp prototypic competitive inhibitor verapamil. In contrast to inhibitory effects *in situ*, a 4-week dietary dieldrin preexposure (0.1mg/day/kg body weight) increased plasma clearance (17%) and reduced tissue concentrations of ^{3}H-tetracycline equivalents (parent and metabolites) for a single intra-aortic administration of ^{3}H-tetracycline (8.31μg/kg body weight) in catfish. A 23% increase in the immunoreactive Pgp level in the hepatic membranes following chronic dietary dieldrin exposure was correlated with *in vivo* changes in disposition. Additional *in situ* studies demonstrated LAS treatments (1, 5, and 20μM) reduced movement of Rhodamine-123 (1μM) into bile in isolated perfused livers (18.6, 38.1 and 66.7%, respectively). Fluorescent anisotropy measurements of the corresponding hepatic membranes showed a 29.7% decrease (increase in membrane fluidity) at the 1μM LAS concentration, with little additional change evident at higher concentrations. In sequential *in vivo* experiments, following six daily diet administrations of ^{3}H-ivermectin (10

µg/day/kg body weight) or ^{3}H-benzo[a]pyrene (40 µg/day/kg body weight) starting on day 7 during a twelve-day waterborne LAS exposure (0, 100 and 300µg/l), ^{3}H-ivermectin and ^{3}H-benzo[a]pyrene equivalent remaining in catfish and their blood and tissue concentrations increased in a dose-dependent fashion with increasing LAS concentrations. The first study indicates dieldrin inhibited Pgp transport at the high concentration with the inductive effect upon Pgp expression predominating at the low concentration exposures *in vivo*. Findings of the second study suggest that LAS at environmental concentrations altered membrane permeability and/or transporter function so to increase bioaccumulation of other xenobiotics from the diet.

INTRODUCTION

A wide range of anthropogenic contaminants are found in the aquatic environment. Fish living in those environments may be exposed to various contaminants as well as pharmaceutical drugs entering the water from domestic, agricultural and aquaculture applications. Many factors are known to influence bioavailability and bioaccumulation of these chemicals in fish (Leblanc, 1995; Ivanciuc et al., 2006). Membrane permeability is generally considered as a key determinant in the extent of absorption, distribution and elimination of chemicals in the body. Exposure concentration and duration, compound lipophilicity and the susceptibility of the chemical to biotransformation also play a role in dictating contaminant body burdens. Recently, studies in mammals suggest that Phase III transport processes by ABC (ATP binding cassette) transporters such as P-glycoprotein (Pgp) may have a significant effect on xenobiotic bioavailability and bioaccumulation (Kurata et al ., 2002; Liu and Hu, 2000; Fromm, 2000; Shitara, 2006).

Membrane permeability may affect the transfer of the xenobiotics in the body in various ways (Malkia et al., 2004). The gastrointestinal tract provides an efficient barrier to the permeability of some xenobiotics, while chemicals are readily permeable as a function of physicochemical characteristics of the compound and the membrane (Smith et al., 1996; Lin, 2003). In addition, the gastrointestinal tract is lined with transporters which “pump” absorbed xenobiotics back into the intestinal lumen. Such an action reduces systemic bioavailability in a compound specific manner. Increases in gastrointestinal tract membrane permeability may facilitate xenobiotic uptake from the diet as a means to circumvent the transport barrier function. In the liver, the compound enters into the hepatocyte from the blood and is actively transported from the hepatocyte

across the canalicular membrane into bile for clearance of the compound. In this case, increased membrane permeability in the liver short-circuits active transport and clearance by allowing the compound to move from the higher concentration of the bile back into the hepatocyte. This action promotes compound retention in the body.

The transfer of xenobiotics in the body is generally controlled by their permeability across tissue membranes. However, increased diffusion across the biological membranes is not always predicative increased permeability due to the contribution of active efflux transporters (Ayrton and Morgan, 2001). Pgp, an ATP-dependent transmembrane transporter, is best known for efflux movement of various therapeutic agents actively out of the cell. First observed and overexpressed in multidrug resistance (MDR) tumor cells, Pgp has been found in normal mammalian tissues, especially in the luminal face of intestine, the biliary canalicular membrane of hepatocytes, the apical surface of epithelial cells of kidney and as part of the blood-brain barrier (Wacher et al., 2001; Frohlich et al., 2004). Owing to its location in organs of absorption, the blood-brain interface and organs of elimination, the functionality of the Pgp transporter in conjunction with membrane permeability may be a significant factor in compound absorption, distribution and elimination.

A Pgp-mediated multixenobiotic resistance (MXR) mechanism has also been associated with modulation of xenobiotic bioavailability and bioaccumulation in aquatic organisms (Kurelec, 1995). Fish have been shown to have Pgp “like” protein which displays a high degree of sequence homology (Chan et al., 1992), a similar tissue distribution pattern (Kleinow et al., 2000), and transport function consistent with those known in mammals (Schramm et al., 1995; Smital et al., 2004). One of the most remarkable features for the Pgp transporter in both mammals and fish is its broad

substrate specificity. Substrates which have been identified include various therapeutic agents, certain food constituents (Balayssac et al., 2005), hormones (Leslie et al., 2005), as well as a number of contaminants (Galgani et al., 1996; Toomey et al., 1996; Bard, 2000; Leslie et al., 2005). With the exception of moderate hydrophobicity or amphiphilicity, few common chemical characteristics have been identified among Pgp substrates (Gottesman and Pastan, 1993; Kusuhara et al., 1998).

A variety of environmental xenobiotics have been shown to inhibit Pgp transport activity either as competitive or noncompetitive inhibitors. Four hydrophobic pesticides including dacthal, chlorbenside, pentachlorophenol, and sulfallate, for example, have been shown to reduce Pgp-mediated Rho-123 efflux from special gill cells in the mussel *Mytilus galloprovincialis* (Cornwall et al., 1995; Galgani et al., 1996). Similarly, in mussels (*M. galloprovincialis*), exposure to Diesel-2 oil increased accumulation of the Pgp substrate, ^{3}H-vincristine (VCR) threefold (Kurelec, 1995). On the opposite extreme, induction of Pgp has also been observed following exposure to environmental contaminants. Polycyclic aromatic hydrocarbons, including benzo[*a*]pyrene (BaP) and 3-methylcholanthrene (3-MC) (Fardel et al., 1996), and the carcinogen 2-acetylaminofluorene (AAF) (Chieli et al., 1995; Tateishi et al., 1999) increased expression of Pgp in rat hepatocytes. As a consequence, absorption and disposition of Pgp mediated xenobiotics may be altered by a variety of compounds in environmental mixtures, which inhibit or induce Pgp.

In the aquatic environment, certain contaminants may have the potential to interact with the lipid membrane and/or Pgp transporter, leading to altered bioavailability and bioaccumulation of other pollutants and drugs from the environment. In the present studies, the pesticide dieldrin and surfactant linear alkylbenzene sulfonate (LAS) were

selected as model contaminants to investigate their effects on xenobiotic bioavailability and bioaccumulation because of their possible interactions with the lipid membrane and/or Pgp transporter, environmental relevance, and their frequent exposure to fish and other wildlife.

Dieldrin is an extremely persistent and toxic organochlorine pesticide, which has been shown to readily bioaccumulate in fish, wildlife and humans (ASTDR 2002). It is found in all environmental media. Fish are at high risk of being exposed to dieldrin through the food chain and direct contact (Jorgenson, 2001). Studies have shown that dieldrin is a potent activator of human pregnane X receptor (PXR) (Coumoul et al., 2002; Lemaire et al., 2004), which is the master regulator for Pgp and CYP 3A4 expression (Veau et al., 2002; Matheny et al., 2004). In addition, chronic dieldrin treatment altered hepatic disposition of ^{14}C-dieldrin and ^{3}H-7, 12-dimethylbenz[a]anthracene (DMBA) (Gilroy et al., 1993 and 1996), and stimulated biliary excretion of ^{3}H-DMBA and ^{14}C-BaP in rainbow trout (Donohoe et al., 1998; Barnhill et al., 2003). These results were evident without induction of xenobiotic metabolizing enzymes, suggesting that transporter interactions may be operative with these findings.

Surfactants, common environmental pollutants and mixture components in aquatic systems (Ahel et al., 1993; Todorov et al., 2002), generally exhibit a low order of direct toxicity to aquatic organisms, however, several early studies suggest that surfactants may have an effect upon the bioavailability and disposition of environmentally relevant compounds in fish (Mann, 1962; Solon, et al., 1969; Pärt et al., 1985). Contemporary studies on the involvement of ABC transporters in cancer multidrug resistance have shown that surfactants are able to inhibit Pgp's contribution to this phenomenon (Kabanov et al., 2002). Recently, surfactants used in drug formulations

have been shown to increase intestinal absorption and decreased the elimination of drugs through alteration of membrane permeability (Drori et al., 1995; Hendrich and Michalak, 2003) and/or modulation of ABC transporters (Woodcock et al., 1992; Nerurkar et al., 1996; Regev et al., 1999; Alqawi and Georges, 2003). Therefore, through these actions, it is of interest to determine if surfactants at environmentally relevant concentrations may influence bioavailability or bioaccumulation of other more hazardous xenobiotics present in environmental mixtures.

The overall objective of these studies was to investigate the influence of the pesticide, dieldrin, and the widely used surfactant, linear alkylbenzene sulfonate (LAS) on xenobiotic bioavailability and bioaccumulation through the alteration of ABC transporters and/or membrane permeability. The first hypothesis examined in the *in situ* isolated perfused liver and *in vivo* in the catfish if the disposition and pharmacokinetics of the drug tetracycline may be altered by dieldrin through Pgp inhibition or induction. Tetracycline was chosen for its structural similarity to Rho-123, the prototypic Pgp substrate, its minimal metabolism in the body (Aronson, 1980) and its widespread use in humans and animals. The second hypothesis examined if C18 LAS at environmentally relevant concentrations may alter membrane permeability and/or the efflux activity of Pgp in the bile canniculi of the catfish liver and influence the *in vivo* dietary bioaccumulation of the pharmaceutical drug ivermectin (IVM) and pro-carcinogen benzo(a)pyrene (BaP) in catfish. The widely used veterinary pharmaceutical ivermectin and the PAH and environmental contaminant benzo[a]pyrene (BaP) were selected as model compounds based on their possible involvement with ABC transporters, environmental relevance and their otherwise limited bioaccumulation.

In order to address the above questions, the specific objectives of these studies are as follows:

1. To characterize the effects of dieldrin and tetracycline on Pgp transport activity using isolated perfused liver preparations.

2. To evaluate the effect of dieldrin on biliary excretion of ^{3}H-tetracycline in the *in situ* isolated perfused livers.

3. To examine the effects of chronic dieldrin pretreatment on the *in vivo* pharmacokinetics and disposition of ^{3}H-tetracycline in catfish.

4. To evaluate Pgp expression in association with alterations of pharmacokinetics and disposition of ^{3}H-tetracycline in dieldrin pretreated catfish.

5. To investigate the influence of C18 LAS at environmentally relevant concentrations on movement of the Pgp prototypic substrate Rhodamine-123 into bile in the *in situ* isolated perfused livers.

6. To assess fluidity changes in the corresponding hepatic membranes and their relationship with inhibitory effects of LAS on movement of Rhodamine-123 into bile in the *in situ* isolated perfused livers.

7. To investigate the effects of the C18 LAS surfactant at environmentally relevant levels on the *in vivo* bioavailability and bioaccumulation of ^{3}H-IVM and ^{3}H-BaP in catfish.

The first chapter of this dissertation presents a literature review of the discovery, structure, action mechanisms and substrate specificity of ABC transporters Pgp and Mrp, a discussion of possible mechanisms that may modulate transport function or induce the overexpression of these ABC transporters, membrane permeability as well as relevant xenobiotics in these studies. The second chapter examines effects of dieldrin upon pharmacokinetics and disposition of tetracycline through interactions with Pgp in *in situ*

isolated perfused livers and *in vivo* catfish. The third chapter investigates the effect of C18 LAS on the function of Pgp and membrane permeability in the isolated perfused livers and the influence of LAS at environmentally relevant concentrations on the *in vivo* bioaccumulation and bioavailability of ^{3}H-IVM and ^{3}H-BaP. The fourth chapter presents an overall summary and general conclusions.

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CHAPTER 1: LITERATURE REVIEW

ABC TRANSPORTERS: PGP AND MRP

Background: The Multidrug Resistance (MDR)

The phenomenon of multidrug resistance (MDR) was first discovered in the early 1970s. After a course of chemotherapy, tumor cells were found to exhibit reduced sensitivity to the administered drugs and a variety of other compounds. These cross-resistant agents appeared to have no common chemical structure, pharmacological function or pathway of biotransformation (Kessel et al., 1968; Biedler et al., 1975). Subsequent studies have shown that this form of resistance can be attributed to an active, ATP-dependent efflux of various agents from inside the cell across the plasma membrane to the outside (Higgins et al., 1998; Dean et al., 2001). P-glycoprotein (Pgp) was the first and best studied membrane transporter involved in this resistance phenotype in cancer cells (Juliano and Ling, 1976). The second major protein responsible for MDR is the multidrug resistance-related protein (Mrp). Pgp and Mrp both belong to the ABC (ATP binding cassette) protein superfamily, containing conserved sequences for ATP binding and hydrolysis (Doige and Ames, 1993; Higgins, 1992). Therefore, these proteins have been proposed to actively transport chemical agents across the plasma membrane using the energy released by ATP binding and hydrolysis (Doige and Ames, 1993).

Although, the physiological role of Pgp and Mrp is unknown, it has been suggested that they are associated with an ancient-type cellular immune apparatus, providing resistance to endogenous and exogenous toxicants (Sarkadi et al., 1996). In addition to tumor cells, these proteins are located in intestine, liver, kidney, adrenal gland, placenta, testes and blood-brain barrier (Cordon-Cardo et al., 1989, Thiebaut et al., 1987). The anatomical localization of Pgp and Mrp suggests that these MDR transporters

may play an important role in absorption, distribution, and excretion of endogenous and exogenous chemicals (Lin, 2003).

MDR in Aquatic Organisms

ABC transporters are members of a superfamily of transmembrane (TM) proteins. This family is one of the largest and most ancient protein families with representatives in all living organisms from prokaryotes to mammals (Higgins and Gottesman, 1992; Ambudkar et al., 1999). Highly conserved MDR genes (encode Pgp) or Pgp-like proteins have been observed in different aquatic organisms including sponges, oysters, clams, worms and fish (Toomey and Epel, 1993; Waldmann et al., 1995; Minier et al., 1996; Kurelec et al., 1992; Kleinow et al., 2000). Studies with fish have shown a high sequence homology (Chan et al., 1992), a similar tissue distribution pattern (Kleinow et al., 2000), and transport function consistent with that demonstrated in mammals (Schramm et al., 1995). Mrp-related genes or proteins have also been found in Atlantic killifish (*Fundulus heteroclitus*), dogfish shark (*Squalus acanthias*) and red mullet (*Mullus barbatus*) (Miller et al., 1998; Sauerborn et al., 2004; Miller, 2003).

The MDR-like phenomenon occurring in aquatic organisms is referred to as multixenobiotic resistance (MXR). Pgp and Mrp in aquatic organisms may constitute a "first line of defense" to prevent absorption and facilitate elimination of endogenous and exogenous toxicants (Epel, 1998; Kurelec, 1995). This role in chemical defense may contribute to the observation that many aquatic species are able to survive and reproduce successfully in highly contaminated environments with multiple anthropogenic pollutants and natural toxins (Smital et al., 2004).

Structure and Mechanisms of Action

The classification of ABC proteins is based on the sequence and organization of their conserved nucleotide binding domain(s) as well as the homology in the gene structure (Leslie et al., 2005). Typical ABC transporters contain a four-domain structure with two conserved nucleotide binding domains (NBDs) and two membrane spanning domains (MSDs) each consisting of six membrane spanning helices. In this structure, each MSD is followed by a NBD (MSD-NBD-MSD-NBD) (Fig. 1. B) (Loo and Clarke, 1999). The 170 kDa Pgp (encoded by ABCB1 or MDR gene) is a typical ABC protein, whereas the 190 kDa Mrp1 and Mrp2 (encoded by ABCC gene) have an atypical structure with five domains. They contain a typical four domain structure, preceded by an extra NH_2-proximal MSD domain composed of five membrane spanning helices (MSD-MSD–NBD–MSD–NBD) (Fig. 1. A) (Bakos et al., 1996; Hipfner et al., 1997).

NBDs, considered as the sites for ATP recognition, binding and hydrolysis, are located on the cytoplasm side of the membrane (Higgins et al., 1986). For most ABC transporters, each NBD contains three highly conserved sequence motifs: the Walker A, the Walker B and the Signature C. The specific amino acid residues in either the Walker A or the Walker B are considered to be important for the ATP hydrolysis. Studies showed that changes in these residues would abolish the ATP hydrolysis of Pgp (Hrycyna et al., 1999). The signature motif has been linked to the conduction of energy from ATP binding and hydrolysis to the transmembrane regions, leading to the conformation changes and the substrate translocation in the transmembrane regions (Hyde et al., 1990).

MSDs contain the substrate binding sites. The structural divergence in these sites may result in differences in substrate specificities among ABC transporters (Chang, 2003). Studies have shown that Pgp contains at least two different binding sites or one

large complex binding bucket (Boer et al., 1996; Pascaud et al., 1998; Ferte, 2000), which may interact in a positively cooperative fashion (Shapiro and Ling, 1997). Mutually stimulated transport has been observed in Pgp for transport of Rhodamine and progesterone (Shapiro and Ling, 1997; Shapiro, 1999).

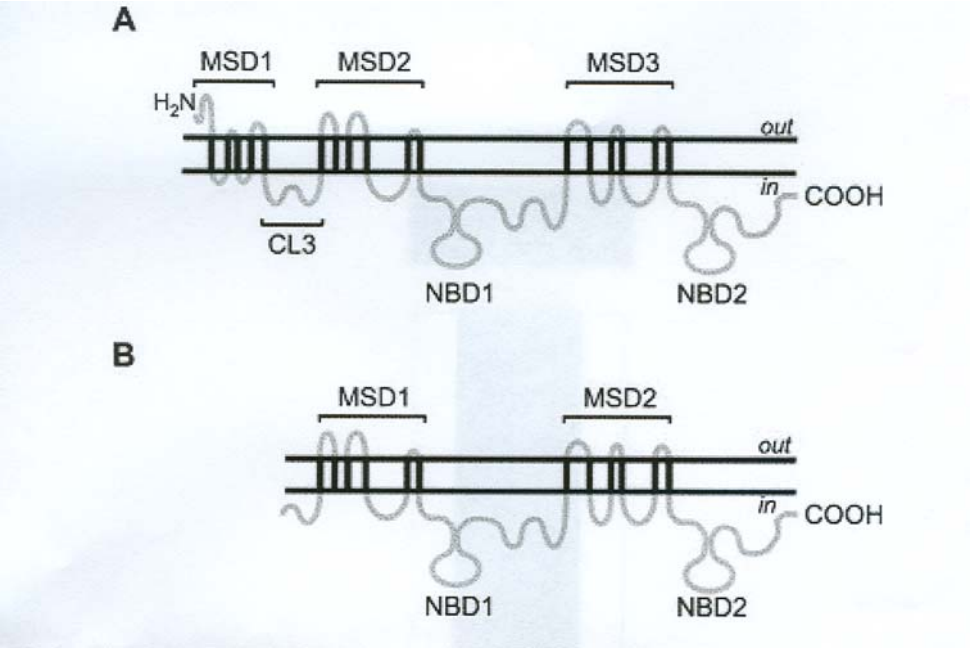

Figure 1.1 - Predicted secondary structures of ABC transporters for (A) Mrp1 and Mrp2 and (B) P-glycoprotein. MSD, membrane spanning domain; NBD, nucleotide binding domain; CL, cytoplasmic loop (Modified from Leslie et al., 2005).

Classic membrane transporters usually move the substrate from the aqueous cytosol to the extracellular aqueous space via an aqueous pore. This pump model is not supported by Pgp mediated transport across the plasma membrane. It is clear that Pgp takes hydrophobic substrates from the lipid phase within the inner leaflet of the bilayer membrane (Stein et al., 1994). The substrate must interact with the lipid phase of the bilayer membrane before reaching Pgp. Based on these findings, a hydrophobic vacuum-cleaner model has been proposed for Pgp transport in which chemicals are extracted directly from the membrane to the extracellular medium (Higgins and Gottesman, 1992). But this model can not adequately explain the means by which Pgp specifically binds to

different substrates (Liu and Sharon, 1996). Thus, another hypothesis has been proposed that Pgp acts as a flippase, moving chemicals against an intramembrane concentration gradient from the inner leaflet to the outer of the plasma membrane where they can diffuse to the aqueous medium (Gottesman and Pastan, 1993). This model provides some basis for understanding the specificity of Pgp by considering compound interactions with lipid membranes.

Substrate Specification

One unique characteristic of Pgp and Mrp is their broad substrate specificity. The therapeutic drugs transported by Pgp include many anti-cancer agents (Gottesman and Pastan, 1993), HIV protease inhibitors (Lee et al., 1987), immunosuppressants (Meador et al., 1987), antibiotics (Horio et al., 1989) and steroids (Ueda et al., 1992). Besides drugs, a number of environmental contaminants have been identified as substrates of Pgp, including carcinogen 2-acetylaminofluorene (AAF), the hydrophobic xenobiotics Aroclor 1254, DDT, DDD, and DDE, and the hydrophobic pesticides dacthal, chlorbenside, sulfallate and pentachlorophenol (Kurelec and Pivcevic, 1991; Cornwall et al., 1995; Galgani et al., 1996; Kurelec et al., 1996). The most common properties shared by these compounds are moderately hydrophobic or amphiphilic, low molecular weight, positively charged or neutral compounds with a planar structure (Gottesman and Pastan, 1993; Kusuhara et al., 1998). The prototypic substrates of Pgp, such as Vinca alkaloids, anthracyclines, epiodophyllotoxins, rhodamine dyes (Argast and Beck, 1984; Sharom, 1997) and doxorubicin (Mealey et al., 2002), are amphiphilic compounds with a four-ringed structure. This structure and physical properties may enhance partitioning into the cell membrane and the active interaction with Pgp (Bain et al., 1997).

Mrp mainly effluxes conjugates of organic anions with glutathione, glucuronide and sulfate (Konig et al., 1999; Leslie et al., 2001). Pgp primarily transports drugs to which it confers resistance, while Mrp acts cooperatively with conjugate enzymes such as the glutathione S-transferases and UDP-glucronosyltransferases to confer resistance to drugs (Depeille et al., 2004; Smitherman et al., 2004). The conjugates of environmental toxins and drugs identified as Mrp substrates include aflatoxin B, metolachlor, chelerythrine, arsenic and vincristine (Leslie et al., 2004; Loe et al., 1997; Lou et al., 2003). The conjugated metabolites are generally thought to be less reactive and toxic as compared to unconjugated toxins. However, Mrp efflux prevents cellular accumulation of these conjugated chemicals, which may result in reformation of the active parent compounds, or direct inhibition of conjugation enzymes and other important enzymes for the cell viability (Leslie et al., 2005). In humans, the lack of Mrp2 transport function may lead to Dubin–Johnson syndrome, associated with impaired biliary excretion of bilirubin glucuronides and other anionic conjugates (Kajihara et al., 1998; Toh et al., 1999).

MDR/MXR Inhibition

Broad substrate specificity provides an advantage for Pgp to deal with a wide range of potentially toxic agents. However, Pgp transport may be inhibited when too many substrates or noncompetitive inhibitors are present. Pgp inhibition may result in an increase in the systemic bioavailability and bioaccumulation as well as changes in tissue distribution.

Pgp is thought to have more than one substrate binding site and two ATP binding domains, which interact cooperatively as a functional unit (Lin, 2003). Inhibitors may interfere with Pgp at different sites. Verapamil has been shown to inhibit transport

function of Pgp in a substrate competitive manner (Ford, 1996). Some modulators are not in fact substrates of Pgp but may inhibit Pgp transport indirectly. Certain organophosphorus and organochlorine pesticides which have high affinity to bind Pgp, but are not transported can disrupt the Pgp activity (Bain and Leblanc, 1996). Vanadate binds with the ATP-binding sites of Pgp without blocking substrate binding, while cyclosporine A inhibits Pgp activity by interfering with both substrate recognition and ATP hydrolysis (Ramachandra et al., 1998; Tamai et al., 1991).

Recent studies indicated that Pgp transport activity is particularly sensitive to changes in the physicochemical state of membrane lipids, especially those surrounding Pgp (Callaghan et al., 1997; Doige et al., 1993). ATPase activity of Pgp, directly linked to Pgp transport activity, could be changed by addition of membrane fluidizers such as ethanol and detergents (Doige et al., 1993; Regev et al., 1999). The modulation of Pgp ATPase activity here may result from conformation changes induced in the transmembrane region and transmitted to ATP binding domains (Ferté, 2000). Furthermore, some evidence suggests that ATP binding sites, which were usually depicted as extending to the cytosol, could be physically close to the membrane surface. Alterations of the membrane state may directly affect ATPase activity of Pgp (Liu and Sharom, 1998).

Environmental contaminants have been found to inhibit Pgp activity in marine and freshwater concentrates, sediment, soil, industrial or household waste extracts, hospital waste, Diesel-2 oil and algal extract (Smital et al., 2004). These chemicals classified as chemosensitizers may increase bioaccumulation of environmental toxicants and toxicity sensitivity in cells and organisms through inhibition of Pgp transport (Kurelec, 1995). Studies with mussels (*M. galloprovincialis*) showed that exposure to

Diesel-2 oil resulted in accumulation of ^{3}H-vincristine (VCR) threefold greater than upon exposure to VCR alone (Kurelec and Pivcevic, 1991). Likewise, mussel embryos (*M. edulis*) which were exposed to vinblastine, mitomycin-C, cytocholasin D, chloroquine, or colchicine showed an increase in number and severity of deformities after verapamil addition (McFadzen et al., 1999).

MDR/MXR Induction

Exposure to drugs or contaminants frequently leads to the acquisition of xenobiotic resistance mechanisms, which may act as a generalized response to cellular stress (Bard, 2000). A wide range of chemicals have been observed to induce MDR1 mRNA and Pgp, regardless if they are Pgp substrates or not. These agents include anticancer drugs (Chaundhary and Roninson, 1993), DNA-damaging agents (Fardel et al., 1998), tumor necrosis factor alpha (TNF-α) (Hirsch-Ernst et al., 1998) as well as environmental contaminants such as 2-acetylaminofluorene, aflatoxin B1, Benzo[a]pyrene and 3-methylcholanthrene (3-MC) (Fairchild et al., 1987; Chin et al., 1990; Gant et al., 1991; Doi et al., 2000).

Regulation of Pgp expression in response to inducers has been extensively studied *in vitro* and *in vivo* (Moore et al., 2000; Staudinger et al., 2001). Most evidence points to an orphan nuclear receptor, pregnane X receptor (PXR), which plays a central role in the regulation of expression of Pgp as well as CYP2 and CYP3A (Kretschmer and Baldwin, 2005; Handschin and Meyer, 2005). The PXR was named after a class of steroid, pregnane, which can activate this receptor. Besides pregnane, a broad range of structurally unrelated compounds, including drugs rifamipicin, dexamethasone and heperofin, as well as environmental contaminants dieldrin, DDT, DDE and endosulfan (Lemaire et al., 2004; Coumoul et al., 2002; Wyde et al., 2003), activate PXR. PXR is

expressed in the liver, intestine and brain (Harmsen et al., 2007). In response to activation, PXR forms a heterometer with the retinoid X receptor (RXR), which subsequently binds to the promoter region of the target genes such as MDR1, Mrp and CYP3A genes, and stimulates transcription (Timsit and Negishi, 2007).

Although PXR plays a key role in Pgp induction, other mechanisms such as gene amplification, transcriptional control and post translation modifications, are all possibly involved in increased expression of Pgp in response to inducers (Bard, 2000; Lin, 2003). MDR1 gene amplification has been observed in some resistant cell lines (Roninson et al., 1986), but is not required for increased expression of Pgp (Shen et al., 1986). Post-transcriptional controls, such as phosphorylation state and increased mRNA stability/decreased mRNA half-life, are also important for MDR1 expression. Studies have demonstrated that phosphorylation of serines and threonines on Pgp is associated with elevated drug resistance in mammalian cell lines (Ratnasinghe et al., 1998).

MEMBRANE PERMEABILITY

Membrane permeability is the ability of compounds to move across the biological membrane. Permeation across the cell membranes takes place by three main mechanisms: passive diffusion, passive transport (channels or carriers) and active transport (carriers). Diffusion across the cell membrane constitutes the most important mechanism, by which xenobiotics cross the membrane (Malkia et al., 2004).

The extent of membrane permeability is largely governed by the physiochemical properties of xenobiotics. Lipophilicity is a crucial factor for membrane permeability of chemicals (Ayrton and Morgan, 2001). Other molecular characteristics such as molecular size, charge, volume, hydrogen-bonding capacity may alter the diffusion rate across the biological membrane (Hilgers et al., 1990, Camenisch et al., 1996). On the other hand,

diffusion of xenobiotics is also influenced by membrane lipid composition, structural integrity, membrane thickness and cytosolic characteristics (Fine et al., 2001; Kleinow, 2006).

Membrane Permeability and MDR

Membrane permeability and Pgp transport function are interdependent and for all practical purposes inseparable. The functional vectorial transport of substrates in an efflux fashion by Pgp has been clearly shown to be dependent on the passive permeability of the membrane to the transported substrate in the opposite direction (Eytan et al., 1996 and 1997). Compounds considered to be non-transported modulators of Pgp exhibit greater permeability and inward diffusability (Scala et al., 1997; Mülder et al., 1995). For these compounds, if efflux transported by Pgp, movement may be unrecognizable due to the membrane's inability to maintain the transporter generated gradient and will allow concentration dependent compound back-diffusion (Barnes et al., 1996; Fert'e, 2000). Rho-123, a prototypic substrate of Pgp, on the other hand, demonstrates restricted membrane movement and vectorial efflux transport is evident (Speelmans et al., 1994; James et al., 1977). Ccompetitive Pgp modulators like verapamil are more lipophilic and diffusible than other substrates effluxed by Pgp, allowing diffusion back into the cell (Sharom, 1997). Therefore, the degree of membrane permeability for a compound is a determinant of net transport.

XENOBIOTICS: ENVIRONMENTAL RELEVANCE AND INTERACTIONS WITH ABC TRANSPORTERS

Dieldrin

Dieldrin is a chlorinated cyclodiene compound belonging to a class of synthetic organochlorine pesticides (Fig. 1.2) (Hassell, 1990). It was first synthesized in 1946 and

distributed commercially as an alternative to DDT (Dichloro-Diphenyl-Trichloroethane) in 1950. Dieldrin was broadly used as an insecticide around the world in the middle of 1970s, mainly for the control of tsetse flies and soil pests and for the treatment of seeds. Dieldrin was listed as one of the 12 most persistent bioaccumulative and toxic (PBT) pollutants by the US Environmental Protection Agency (US EPA, 2001) and one of the top 20 hazardous substances to humans (ATSDR, 2005). Due to its extreme persistence and toxicity, US EPA finally banned the use of this insecticide in 1987. However, it is still used in a few developing countries (Suwalsky et al., 2002).

Even though banned from use for decades in most of the world, significant levels of dieldrin continued to be found in soil, water, and biota because of its low volatility and chemical stability (ASTDR, 1987). An epidemiological study conducted in Taiwan revealed dieldrin at concentrations of 0.12–5.8 μg/kg sediment (dry weight) in the impacted river (Doong et al., 2002). In Japan, low levels of dieldrin and other pesticide residues were observed in 32% of domestic agricultural products and 51% of imported products (Akiyama et al., 2002). In India, high levels of dieldrin (> 1 μg/g) in vegetables were reported (Kannan et al., 1997), suggesting higher contamination levels in developing countries. Animals and humans may be exposed to this compound through the food chain and direct contact (ASTDR, 2002). Recently, the high levels of dieldrin have been found in farmed salmon fish worldwide (Hites et al., 2004). An investigation on human exposure to dieldrin demonstrated that farmers in Iowa have significantly higher serum dieldrin concentrations. In this case, dietary dieldrin is a major route for human exposure (Brock et al., 1998).

Effects of organochlorine compounds on Pgp transport have been investigated, but the results were equivocal. Organochlorine compounds including DDT, DDD and

DDE, have been shown to reduce the efflux transport of the prototypic Pgp substrate Rhodamine B from the gills in the marine mussel *M. galloprovinvialis* (Galgani et al., 1996). However, DDT, DDE and dieldrin had no effect on the intracellular accumulation of another Pgp substrate doxorubicin in the murinne melanoma cell lines infected with human MDR1 gene (Bain and Leblanc, 1996). Inconsistency was also observed with *in vitro* studies in gill cells of different mussel species. DDT, DDD, DDE, and polychlorinated biphenyl--Aroclor 1254 all inhibited the efflux of Rhodamine dye from the gill cells in one species of mussel (*Mytilus galloprovincialis*) (Galgani et al., 1996), but showed no effect on Pgp transport activity in another species (*Mytilus californianus*) (Cornwall et al., 1995). The reasons for this inconsistency are still not clear, and may result from tissue, dosage, species, or other experimental differences.

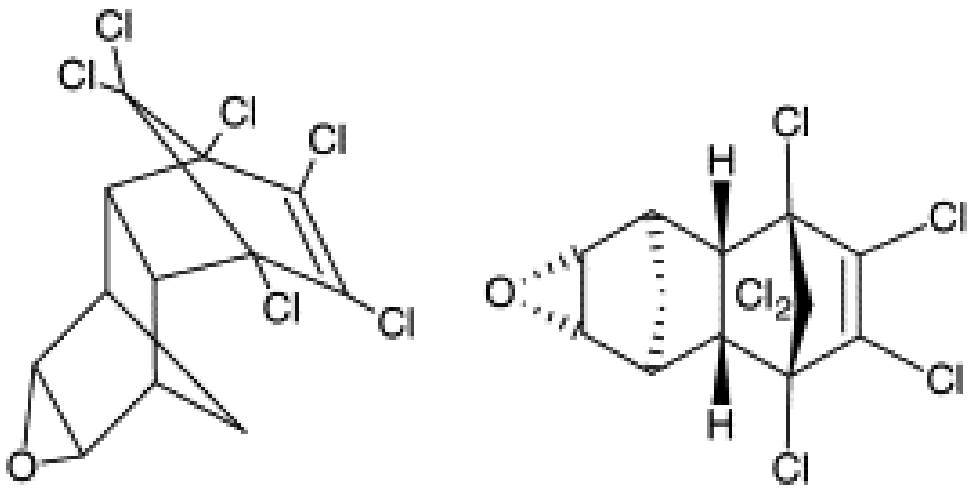

Figure 1.2 - Chemical structure of dieldrin (Modified from Hassell, 1990).

Previous studies demonstrated that chronic dieldrin treatment altered hepatic disposition of ^{14}C-dieldrin and ^{3}H-7, 12-dimethylbenz[a]anthracene (DMBA) (Gilroy et al., 1993 and 1996) and increased biliary excretion of ^{3}H-DMBA and ^{14}C-B(a)P in rainbow trout (Donohoe et al., 1998; Barnhill et al., 2003). These results were evident

without induction of xenobiotic metabolizing enzymes, suggesting that another mechanism, such as transport actions, may be operative with these findings. In addition, *in vivo* studies have shown increased expression of Pgp following exposure of another organochlorine compound DDE in the gill tissues of the mussel *Mytilus californianus* (Eufemia and Epel, 2000). These findings suggest that dieldrin may induce Pgp expression.

Furthermore, dieldrin and other organochlorine compounds, such as DDT, DDE and endosulfan have been shown to interact with pregnane X receptor (PXR), which plays a central role in the regulation of induction of CYP2 and CYP3A as well as Pgp (Kretschmer and Baldwin, 2005; Handschin and Meyer, 2005). Exposure to these compounds has resulted in induction of CYP3A and CYP2B in mammary cells (Lemaire et al., 2004; Coumoul et al., 2002; Wyde et al., 2003). These findings suggest that dieldrin may have a potential to induce Pgp expression through activation of PXR.

In summary, dieldrin as one of the persistent organic contaminants may have potential to inhibit or induce Pgp.

Tetracycline

The tetracycline antibiotics, a group of four-ringed amphoteric compounds (Fig. 1.3), are commonly used as a broad-spectrum antimicrobial agent to inhibit the growth of bacteria, protozoa and intracellular organisms in human and veterinary medicine. In 1970, oxytetracycline was approved by the US FDA for use to treat bacterial infections in salmons and catfish consumed by humans (Plakas et al., 1998). Tetracycline may be administered through an intravenous, intramuscular, or oral pathway. Once absorbed, tetracycline is widely distributed throughout the body and then accumulated in the liver and kidneys. Tetracycline is not significantly metabolized in the body and mainly

eliminated as parent compounds through feces and urine (Aronson, 1980). Enterohepatic circulation may occur with tetracycline (Kunin and Finland 1961).

Bacterial resistance to antibiotics often occurs during the clinical treatment. It involves two major mechanisms: the bacterial cell wall permeability barrier and the active multidrug transport proteins (De Rossi et al., 2006). The active transport proteins found in bacteria exhibit a high degree of sequence homology with Pgp (Chen et al., 1986; Neyfakh 1997) and similar characteristics to the multidrug resistance system in mammalian cells (Neyfakh et al., 1991). Studies performed in bacteria revealed that MDR gene transfection resulted in reduced intracellular accumulation of tetracycline (Levy, 1992; George et al., 1996; Chollet et al., 2004). In addition, cyclosporine A and verapamil, the competitive inhibitors of Pgp, completely reversed tetracycline resistance and increased accumulation of tetracycline in the human leukemic cells expressing MDR1 gene (Kavallaris et al., 1993). A tetracycline derivative doxycycline has also been shown to potentially inhibit transport activity of Pgp and induce overexpression of Pgp in MCF-7 breast carcinoma cells (Kavallaris et al., 1993).

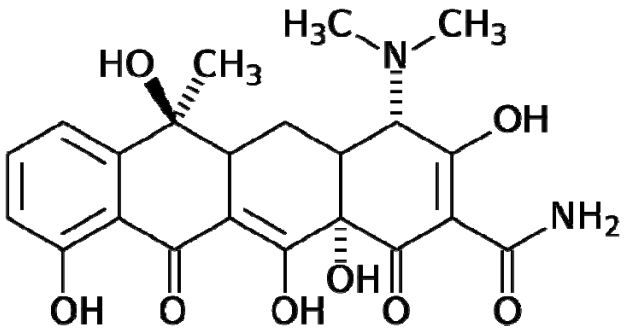

Figure 1.3 – Chemical structure of tetracycline.

Surfactants

Surfactants (surface-active agents) are a diverse group of chemicals designed for cleaning and/or solubilization. They are usually amphiphilic organic compounds

containing both hydrophobic parts (their nonpolar tails) and hydrophilic parts (their polar heads). Therefore, they are readily soluble in both organic solvents and water. These compounds are widely used for domestic and industrial applications, such as household cleaning, personal care products, paints, and paper industries. These agents are also employed as adjuvants in pesticide and pharmaceutical formulations and as dispersants in the remediation of soil contaminated by heavy metals and PAHs. (Burchfield et al., 1994, Baran Jr. et al., 1998; Cowell et al., 2000). Due to their widespread use, the world consumption of synthetic surfactants reached up to 7.2 million tons annually (Di Corcia, 1998).

Linear Alkylbenzene Sulfonates (LAS)

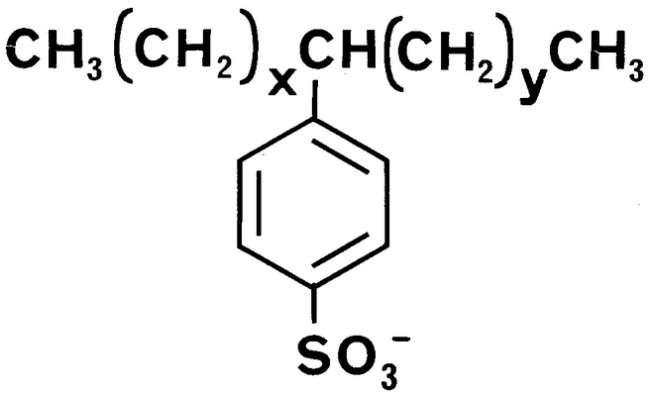

Figure 1.4 - Chemical structure of linear alkylbenzene sulfonate (LAS) where *x* plus *y* equals 7 to 11 depending on the length of the alkyl chain (Adapted from Trehy et al., 1996).

Linear alkylbenzene sulfonate (LAS), the most popular synthetic anionic surfactant, constitutes more than 40% of total surfactants in the detergent industry (Verge et al., 2001). The basic structure of LAS is a benzene ring connected to an alkyl chain with different lengths ranging from 9 to 13 carbon units (the nonpolar tail) and a sodium

sulfate group (the polar head) (Fig. 1.4) (Trehy et al., 1996). Commercial products of LAS are very complex mixtures consisting of different homologues.

After use and disposal, LAS may directly reach surface water, sediment and soil, or be discharged as residuals from sewage treatment plants (Jensen, 1999). LAS are readily biodegradable under aerobic conditions (Perales et al., 1999). However, due to the widespread use and high consumption, various levels of LAS have been detected in the aquatic environments. LAS has been found in sewage effluents and in surface waters in the UK (1090 and 416 μg/l) (Holt et al., 1998; Fox et al., 2000) as well as in the effluents of wastewater treatment plants in the US (4 to 94 μg /l) (Trehy et al., 1996). LAS has also been reported at concentrations of 20 to 1000 μg/l in outlets to estuaries and near-shore marine waters in the North Sea (Berna et al., 1991; Stalmans et al., 1991), and 0.1 to 2.8 μg/l in Mississippi River water (Tabor and Barber, 1996). LAS levels in sediments have been reported to be much higher than in water due to much lower degradation rates under anaerobic conditions. 49-129 μM LAS/kg dry weight (dw) is reported in sediments in the Tsurumi River, Japan (International Program on Chemical Safety, 1996), and 0.11-0.30 μM LAS/kg dw in the German Bight of the North Sea (Berna et al., 1991; Stalmans et al., 1991).

Studies in fish revealed that LAS is mainly absorbed through gills, rapidly distributed throughout the body, highly concentrated in gills, blood, hepatopancreas and kidneys, and primarily eliminated through bile (Kikuchi et al., 1978). In general, surfactants cause disruptions of the biological membrane and denaturation of membrane proteins (Swisher, 1987; Schwunger and Bartnik, 1980). Exposure to high concentrations of LAS results in inhibition of mobility, reproduction and growth, and even death of fish (Verge et al., 2001; Utsunomiya et al., 1997; Hofer et al., 1995; Singh et al., 2002). The

No Observed Effect Concentration (NOEC) and the Predicted No-Effect (PNEC) of LAS for freshwater were 200 and 360 µg/l, respectively. As the thresholds of the defined acute and chronic toxicity for LAS in aquatic organisms are much higher than the measured environmental levels, the direct toxic effects of LAS are assumed to be low in the environment (Ying GG, 2005; Scott and Jones, 2000). As a common environmental pollutant, LAS often exists in mixtures with other contaminants. Mixtures of toxicants containing LAS may result in additive or even synergistic effects on fish and other organisms (Lewis, 1992). Several early studies have shown that LAS exposure resulted in enhanced uptake and altered disposition of other compounds such as phenol (Mann, 1962), pesticide parathion (Solon et al., 1969) and metals (Pärt et al., 1985, Calamari and Marchetti, 1973). These findings are linked to alterations of membrane permeability by surfactants.

Surfactants and Multidrug Resistance

Surfactants have been extensively employed as excipients in the pharmaceutical formulations to facilitate drug absorption (Rege et al., 2002). Initially, they were considered as physiologically insert compounds for enhancing drug solubility. However, recent studies suggested that these compounds may insert into the lipid membrane to varying degrees, and increase membrane fluidity (Sinicrope et al., 1992; Woodcock et al., 1992; Swenson et al, 1994), disrupt tight junctions between the epithelial cells (Aungst 2000; Anderberg et al., 1992), and interact with metabolic enzymes (Mountfield et al., 2000). Moreover, they have been shown to inhibit the efflux activity of ABC transporters Pgp and Mrps (Bobrowska-Hagerstrand et al., 2003; Ellis et al., 1996; Orlowski et al., 1998; Strugala et al., 2000). The modulation of Pgp and related ABC transporters is of

particular interest as their critical role in xenobiotic accumulation and disposition in the body.

Surfactants including Cremophor EI, Solutol HS15, Triton X-100, Nonidet P-40 and Pluronic block copolymers, have been observed to increase accumulation and cytotoxicity of antineoplastic agents in tumor cells overexpressing Pgp and *in vivo* mice carrying with MDR tumors (Batrakova et al., 1996; Webster et al., 1993; Dudeja et al., 1995; Buckingham et al., 1995; Zordan-Nudo et al., 1993). Recently, pluronic polymer bound doxorubicin has been developed and undergone phase I clinical trials to treat MDR tumors (Danson et al., 2004). Surfactant-induced MDR inhibition not only occurred in MDR tumor cells, but also in normal tissues such as in liver (Ellis et al., 1996), intestine (Strugala et al., 2000) and blood brain barrier (Miller et al., 1997).

The mechanism by which Pgp transport is inhibited by surfactants has been investigated. Tween 80 and nonylphenol ethoxylates (NPEs) have been shown to inhibit Pgp transport by blocking the substrate binding to Pgp (Yamazaki et al., 2000; Friche et al., 1990; Doo et al., 2005). Batrakova discovered that surfactant Pluronic P85 was able to permeate the membrane of mitochondria, disrupt oxidative phosphorylation and lead to ATP depletion in the cell (Batrakova et al., 2001). Krylova and Pohl demonstrated that Puronic L61-induced ionic flux in the plasma membrane could result in compensatory ATP consumption by Na^+-K^+ ATPase and decreased cellular ATP levels (Krylova and Pohl, 2004). Studies with lipids and detergents indicated that perturbation of the membrane environment may alter Pgp transport function (Ferté, 2000; Doige et al., 1993). These agents may change lipid mobility and structure in the plasma membrane, and consequently affect conformational changes of membrane bound protein Pgp (Rege

et al., 2002; Regev et al., 1999), which may be associated with Pgp's ATPase activity (Lu et al, 2001).

Likewise, surfactants may modulate Mrp and other membrane enzymes since they have potential to interfere with Pgp through membrane perturbation. Studies have shown that surfactant pluronic copolymer has inhibitory effects on Mrp transport (Miller et al., 1999). Activities of membrane enzymes such as CYP3A and glutathione-S-transferase (GST) system were also decreased in the presence of surfactants (Johnson et al., 2002; Batrakova et al., 2004).

In addition, substrates of Pgp and Mrp are lipophilic or amphiphlic, which enter the cell via passive diffusions through the plasma membrane. The cellular accumulation of these compounds in MDR cells relies on membrane permeation properties as well as on the handling by the ABC transporters (Ferté, 2000). Surfactants, as amphipathetic molecules, whose structures imitate that of endogenous glycerophospholipids, are able to insert themselves between lipophilic tails of membrane bilayers, resulting in disorder of the lipid-packing arrangement of membranes. The net result is that the membrane becomes fluidized, facilitating the passive diffusion of compounds across the membrane (Lo, 2003), and thereby increasing membrane permeability and cellular accumulation (Drori et al., 1995; Hendrich and Michalak, 2003).

Surfactants are common environmental pollutants and mixture components in aquatic systems. Therefore, it is of interest to determine if surfactants at environmentally relevant concentrations may influence bioavailability or bioaccumulation of other more hazardous xenobiotics present in environmental mixtures through these actions.

Ivermectin

Ivermectin (IVM) (22, 23-dihydroavermectin B_{1a} + B_{1b}) are the semi-synthetic derivatives from *Streptomyces avermilities*, consisting of a disaccharide linked to a macrocyclic lactone (Fig. 1.5) (Roberts and Hutson, 1999). It is a broad-spectrum antiparaside extensively used in veterinary and human medicine. In United States, IVM is approved for use in swine, cattle, sheep, goats, bison, reindeer, and horse. Recently, it has been applied to treat the sea lice in salmon (Stone et al., 2000). IVM primarily acts on the nervous system of parasites. It binds to glutamate-gated chloride channels and GABA (*c*-amino butyric acid)-gated chloride channels, increases chloride influx, and results in hyperpolarization in nerves and muscle cells (Martin 1997). This disrupts neural signal transmission and paralyzes neuro-muscular junctions in organisms ultimately leading to death (Mellin et al., 1983).

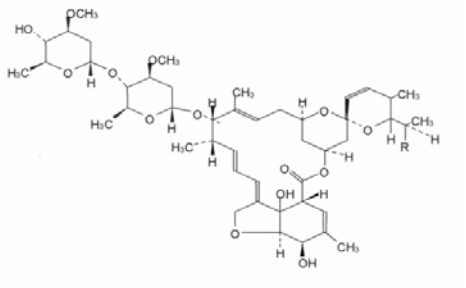

Figure 1.5 - Chemical structure of ivermectin (IVM) (Adapted from Roberts and Hutson, 1999).

At therapeutic doses, IVM does not affect mammals since glutamate and GABA synapses are located in the central nervous system (CNS), which is largely protected from the blood-brain barrier (Campbell et al., 1983). However, some dog species, most notably the collie, exhibit signs of CNS toxicity at IVM doses much lower than the normal therapeutic dose. The cause of CNS toxicity in susceptible dog species has been linked to

a mutation in a MDR1 gene (Mealy et al. 2001). High expression of Pgp in the blood brain barrier may protect the brain tissues from the penetration of IVM and its neurotoxicity (Schinkel et al., 1994). Several studies demonstrated that IVM interferes with Pgp as a substrate (Alvinerie et al., 1999; Ballent et al., 2006). Pgp modulators including verapamil, loperamide, PSC833 and itraconazole, have been shown to increase its IVM plasma and tissue concentrations after co-administration (Alvinerie et al., 1999; Lifschitz et al., 2004; Ballent et al., 2006). On the other hand, multidrug resistance in tumor cells has been reversed by IVM (Korystov et al., 2004). All these findings suggested that Pgp plays a critical role in absorption, dispositions and bioavailability of IVM.

Since its action mode is not limited to parasites, IVM may affect other organisms in the environment. In fish studies, it has been reported that IVMs were distributed extensively to the brain (Høy et al., 1990), indicating that fish have poorly developed blood brain barrier as compared to mammals and may be susceptible to IVM toxicity. IVM has also been shown to produce adverse effects on reproduction, biological function and survival of non-target aquatic and terrestrial organisms (Halley et al., 1993; Steel and Wardhaugh, 2002).

As a widely used veterinary drug, IVM may enter environmental waterbodies and fish through aquaculture practices, runoff /leaching from concentrated on-ground fecal material and municipal sources (Daughton and Ternes, 1999). Following administration, IVM is excreted mainly as a parent compound or active metabolites through faeces (McKellar and Benchaoui, 1996; Lumaret and Errouissi, 2002). The physicochemical properties of IVM are low water solubility, high molecular weight and high Kow (McKellar, 1997). Therefore, once discharged in the environment, it is readily absorbed

by organic matter, soil, sediment particles and fish. It may be rapidly photodegraded in water to less bioactive compounds by oxidative mechanisms (Halley et al. 1993). However, due to its high volume and frequent use, IVM is of concern for its environmental toxic effects and potential risks to human health through the food chain (Tisler et al., 2006).

Benzo[a]pyrene

Benzo[a]pyrene (BaP) is a ubiquitous procarcinogen, belonging to the family of the polycyclic aromatic hydrocarbons (PAHs). BaP and other PAHs occur as a byproduct of incomplete fossil fuel combustion such as in coke ovens, automobile exhaust, cigarette smoke, and charcoal grilling of food. Spills and accidents during the production and the transport of oil constitute another major source of BaP in the environment (ASTDR, 1990). PAHs are frequently found in impacted aquatic environments, especially in contaminated sediments (EPA, 1991). In a designated superfund site of New Bedford Harbor, USA, concentrations of PAHs have been reported to up to 170 μg/g dry weight in sediments (Pruell et al., 1990).

Dietary BaP is an important route of exposure for aquatic species and humans as a consequence of movement of BaP and its metabolites along the food chain (Kleinow et al., 1998; Hattemer-Frey and Travis, 1991). Human exposure to BaP through the food chain has been shown to account for about 97% of the total daily intake (Hattemer-Frey and Travis, 1991). Fish inhabiting polluted waters containing PAH are likely to be exposed in the diet to PAHs and their metabolites in the food item (e.g. smaller fish or plants) (James et al., 2001). High concentrations of BaP in fish food products have been detected over the past decades (Loutfy et al., 2007).

BaP is a moderately lipophilic neutral compound. It is readily absorbed by aquatic animals from the gastrointestinal tract (Varanasi et al., 1989) and rapidly distributed to well-perfused organs in the body (Tyler et al., 1981). BaP is primarily eliminated as water-soluble metabolites through hepatobiliary excretion and feces. Biotransformation of BaP can occur in two main phases. The phase I reaction involves interactions with mixed-function oxidases, especially the cytochrome P450s, and formations of diol-epoxides, including B(a)P-7,8-dihydrodiol-9,10-oxide (BPDE), the major carcinogenic metabolite. In phase II reactions, various polar moieties such as glutathione (GSH), sulfate and glucuronic acids are conjugated to the phase I intermediate metabolites (Di Giulio et al., 1995; Leonard et al., 2001). As a result, only minor parent BaPs remain in body tissues (Di Giulio et al., 1995). The liver is regarded as the major site of metabolism for BaP, although the significant metabolism may occur in gastrointestinal tract, skin and lung (Perera, 1981).

BaP is carcinogenic, mutagenic, teratogenic, and cytotoxic to various cells and organisms (Conney et al., 1994 ; Miller and Ramos, 2001). BPDE, the major reactive metabolite of BaP, is able to interact covalently with biological macromolecules (e.g., DNA, proteins) and form adducts (Hall and Grover 1990; Varanasi et al, 1989). Alternatively, BaP's ultimate carcinogenic metabolite (BPDE) may be detoxified by GSH conjugation, which is catalyzed by glutathione S-transferase (Robertson et al., 1986).

Previous studies suggest that BaP itself is probably not a substrate of Pgp (Schuetz et al., 1998). However, BPDE conjugates have been shown to be actively transported across the canalicular plasma membrane and intestinal Caco-2 cells (Buesen et al., 2003; Srivastava et al., 1998). The accumulation of BPDE and its GSH conjugates is enhanced by reduction of MRP2 transporter function (Srivastava et al., 2002),

suggesting that MRP2 may play an important role in elimination of conjugated BaP metabolites. Therefore, in addition to GSH conjugation, the efflux of the conjugates of BPDE is essential for the cellular protection against BPDE-induced toxicity.

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CHAPTER 2: EFFECTS OF DIELDRIN ON THE PHARMACOKINETICS AND DISPOSITION OF TETRACYCLINE IN *IN SITU* ISOLATED LIVER PREPARATIONS AND *IN VIVO* CHANNEL CATFISH VIA INTERACTIONS WITH P-GLYCOPROTEIN

INTRODUCTION

P-glycoprotein (Pgp) is an ATP-dependent efflux membrane protein belonging to the ATP-binding cassette (ABC) transporter superfamily, which plays a pivotal role in absorption, disposition and excretion of various drugs (Juliano et al., 1976; Ambudkar et al., 1999). It is found in a variety of tissues, including the luminal face of intestine and brain capillaries, and the apical membrane of liver and kidneys (Aryton and Morgan, 2000). Its location in organs of absorption, the blood-brain interface and elimination suggests Pgp plays a role in the barrier function, tissues distribution and excretion of drugs (Wacher et al., 2001; Frohlich et al., 2004). Pgp transports various therapeutic drugs (Lin, 2003), certain food constituents (Balayssac et al., 2005), as well as a variety of contaminants (Galgani et al., 1996; Toomey et al., 1996; Bard, 2000; Leslie et al., 2005).

Numerous environmental xenobiotics have been shown to increase accumulation of Pgp substrates via inhibition of Pgp transport activity either as substrates or as noncompetitive inhibitors. Four hydrophobic pesticides including dacthal, chlorbenside, pentachlorophenol, and sulfallate have been shown to reduce Pgp-mediated Rho-123 efflux from special gill cells in the mussel *Mytilus galloprovincialis* (Cornwall et al., 1995; Galgani et al., 1996). Likewise, in mussels (*M. galloprovincialis*), exposure to Diesel-2 oil increased accumulation of the Pgp substrate, [^{3}H]-vincristine (VCR) threefold (Kurelec, 1995). Induction of Pgp has been observed following exposure to

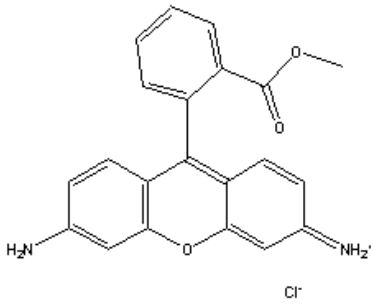
environmental contaminants. Polycyclic aromatic hydrocarbons, including benzo[*a*]pyrene (BaP) and 3-methylcholanthrene (3-MC) (Fardel et al., 1996), and the carcinogen 2-acetylaminofluorene (AAF) (Chieli et al., 1995; Tateishi et al., 1999) increased expression of Pgp in rat hepatocytes. Similarly, *in vivo* studies have shown induction of Pgp in intestine following exposure to the xenobiotic β-naphthoflavone (BNF) in catfish (Kleinow et al., 2000) and rat and human (Lown et al., 1996). As a result, pharmacokinetics, efficacy and bioavailability of drugs by which disposition is mediated by Pgp may be altered by environmental compounds, which inhibit or induce Pgp.

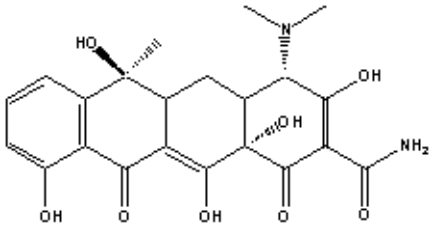
Drug to drug interactions due to inhibition or induction of Pgp are widely recognized to be of clinical concern in that they may lead to altered pharmacokinetics, efficacy and bioavailability of Pgp transported drugs (Yu, 1999; Balayssac et al., 2005). The most overwhelming evidence of this phenomenon in humans is the interaction of digoxin, a Pgp substrate, with other drugs. When administered a daily dose of verapamil (160 mg), a substrate and competitive inhibitor of Pgp, a 40% increase in plasma concentration and a reduction of renal elimination of digoxin were evident (Pedersen, 1985; Verschraagen et al, 1999). In contrast, a 14-day St. John's exposure led to a significant increase in fexofenadine clearance, consistent with Pgp induction (Moore et al., 2000).

As an extremely persistent and toxic organochlorine, dieldrin readily bioaccumulates in fish, wildlife and humans (ASTDR 2002). Even though banned for all uses within the United States by EPA (U.S. EPA, 1971 and 1990), it still exists in all environmental media (U.S. Department of interior, 1999). Humans and animals are exposed to this compound though the food chain and direct contact (ASTDR 2002).

Studies have shown that dieldrin is a potent activator of human pregnane X receptor (PXR) (Coumoul et al, 2002; Lemaire et al., 2004), which is the main regulator for Pgp and CYP 3A4 expression (Veau et al., 2002; Matheny et al., 2004). In addition, chronic dieldrin treatment in rainbow trout alters hepatic disposition of [^{14}C]-dieldrin and [^{3}H]-7, 12-dimethylbenz[a]anthracene (DMBA) (Gilroy et al., 1993 and 1996), and stimulate biliary excretion of [^{3}H]-DMBA and [^{14}C]-benzo[a]pyrene (Donohoe et al., 1998; Barnhill et al., 2003). These results, without induction of xenobiotic metabolizing enzymes, suggest that another mechanism, such as transport action, may be operative with these findings. However, there is still no direct evidence pointing to interactions between dieldrin and Pgp.

Little evidence exists regarding interactions between environmental contaminants and drugs via Pgp mechanisms, especially regarding the effects of contaminants on the pharmacokinetics and bioavailability of Pgp mediated drugs. We hypothesize that the pharmacokinetics and disposition of Pgp mediated drugs may be altered by contaminants, which inhibit or induce Pgp. The goal was to investigate the effects of dieldrin on the pharmacokinetics and disposition of tetracycline, a broad spectrum anti-bacterial drug. Specific objectives include: 1). to characterize the effect of dieldrin and tetracycline on Pgp transport activity at the bile canniculi using the prototypic Pgp substrate Rhodamine 123 (Rho-123) in the isolated perfused liver preparation; 2). to evaluate the effect of diedlrin on biliary excretion of tetracycline and 3). to examine the effect of chronic exposure to dieldrin on the pharmacokinetics, distribution and excretion of tetracycline as related to Pgp expression. Tetracycline was chosen in this study for its structural similarity to Rho-123, the prototypic Pgp substrate (Fig. 2.1), its minimal metabolism in





Rhodamine-123 (Rho-123) Tetracycline

Figure 2.1 - Chemical structures of Rhodamine-123 and tetracycline.

body (Aronson, 1980) and its widespread use in humans and animals. Tetracycline and its derivatives have been recognized as substrates of Pgp in bacteria (Levy, 1992, Chollet et al., 2004; Qin et al., 2004) and cultured cancer cells (Mealey et al., 2002). Studies have shown that the Pgp "like" transporter in fish displays a high degree of sequence homology (Chan et al., 1992), a similar tissue distribution pattern (Kleinow et al., 2000), and transport function consistent with that demonstrated in mammals (Schramm et al., 1995). In the environment, fish are at high risk of being exposed to dieldrin and have shown a potential to retain dieldrin at high concentration levels in body (Jorgenson, 2001). Catfish were used in this study due to use of tetracycline as a therapeutic in aquaculture applications (Plakas et al., 1998) and potential exposure to dieldrin.

MATERIALS AND METHODS

Chemicals

Dieldrin (~90%) was obtained from Aldrich Chemical Company, Inc., Milwaukee, WI. [^{3}H]-tetracycline (0.906 or 1.07 Ci/mM, >95%) was purchased from PerkinElmer Life Sciences, Inc., Boston, MA. Tricane methane sulfonate (MS-222) was provided by Argent Chemical Company, Redmond, WA. Dimethyl sulfoxide (DMSO) was supplied by Fisher Scientific, Fair Lawn, NJ. Tetrahydrofuran (C_8H_8O) was provided by Mallinckrodt, Inc., Pairs, Kentucky. Tissue Solubilizer TS-2 was acquired from Research Products International Corp., Mount Prospect, IL. Heparin sodium (1000 or 10000 USP units/ml) was acquired from Elkins-sinn, Inc. Cherry Hill, NJ. Electrophoresis material and the Immuno-Blot™ Assay Kit with Goat Anti-mouse IgG Alkaline Phosphatase (GAM-AP) were provided by Bio-Rad Laboratories, Hercules, CA. The C219 murine monoclonal antibody was obtained from Signet Laboratories Inc.,

Dedham, MA. Rhodamine-123 (Rho-123), tetracycline, verapamil, and all other chemicals used for buffer preparations were purchased from Sigma, St Louis, MO.

Animals

Male and female channel catfish used in these studies were obtained from the Louisiana State University Aquaculture Research Station, Baton Rouge, LA. Animals were grown and maintained at least 2 months prior to use under a flow-through conditions at 19^{o}C in dechlorinated tap water with a 12 h light/dark photoperiod. A commercial chow diet (Silvercup trout chow, Sterling Farms, UT) was fed at 0.5 to 1% body weight per day prior to use in experiments.

[^{3}H]-tetracycline (TET) Dose Preparations

Solid [^{3}H]-tetracycline (TET) (0.906 Ci/mmol or 1.07 Ci/mmol) as obtained from the manufacturer was diluted with 250 µl of DMSO to form stock solutions. The stock solutions were further diluted into [^{3}H]-TET solutions (~16 µCi/ml) used in the *in situ* experiment, and solutions (52-63 µCi/ml) for *in vivo* investigations.

Isolated Liver Perfusion Studies

Surgery Procedure

Catfish of both sexes were fasted 24 h prior to surgery. Isolated livers (19.02 ± 5.01 g) were prepared according to the details previously described (Kleinow et al., 2004). Animals were anesthetized with buffered Tricane methane sulfonate (MS-222) at induction and maintenance doses of 106 and 86 mg/l, respectively. Following ventral midline and transverse incisions exposing the liver and gallbladder, the common bile duct was cannulated with a perforated PE260 tubing with an attached PE50 tubing for bile collections. Liver vessels were isolated and sutures preplaced. An intravenous catheter (13-gauge) was then inserted into the portal vein, ligated in place, and connected to a

gravity fed perfusion system supplying oxygenated and heparinized (10 USP/ml) teleost Ringer's solution (117.2 mM NaCl, 4.8 mM KCl, 0.9 mM $CaCl_2$, 1.5 mM $MgSO_4$, 12.3 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, 5 mM glucose, pH 7.4). Once media flow was established from the afferent cannula to the liver circulation, collateral vessels were ligated, the cardinal veins transected and the liver cleared of blood. After stabilization, the liver was removed from the fish while being perfused and placed on the perfusion apparatus. The perfusion apparatus was set at 12 cm of head pressure to create physiological pressures for oxygenated perfusate delivery during the experimental procedure. The isolated perfused livers were maintained at 20^{o}C.

Experiment Designs

Eight groups of five to seven individual fish (1532.5 ± 311.5 g) were prepared for isolated liver perfusions in two separate experiments. In the first experiment to evaluate effects of tetracycline and dieldrin on Pgp transport activity, livers were pre-perfused with oxygenated teleost Ringers' solutions (1L) containing vehicle alone for 1) control (N=5), or individual treatments 2) TET (20 μM) (N=5) or 3) dieldrin (20 μM) (N=7). This process removed the remaining blood and preexposed livers to specific treatment condition. Following pre-perfusion, the isolated livers were perfused in a recirculating system for 210 min with 250 ml of media, which were compositionally identical to the pre-perfusion mixture, except containing 1 μM Rho-123. A similar procedure was applied to the second experiment to examine effects of verapamil & dieldrin on transport of [^{3}H]-TET into bile. The treatment groups were: 1) control (N=6), 2) verapamil (20 μM) (N=5), 3) dieldrin (20 μM) (N=5), using radiolabel [^{3}H]-TET (8.8 nM) as analytes. Samples of bile and perfusate were harvested at 30 min intervals for the 210 min perfusion.

In Vivo Studies

Dieldrin Treatment

The effect of chronic dieldrin pretreatment upon Pgp expression and the pharmacokinetics and disposition of tetracycline were investigated *in vivo* in the catfish. All animals were acclimatized to experimental conditions for at least 2 weeks before dietary treatment. Control and experimental diets were prepared by coating commercial chow diet using tetrahypofuran (10 ml/kg diet) with or without dieldrin (10 mg/kg diet). Both stocks were made prior to the beginning of the experiments and stored at -20^{o}C. All animals were fed diets with or without dieldrin (0.1 mg/kg body weight per day) at 1% body weight daily for 4 weeks prior to surgery.

Dorsal Aorta Cannulation

The cannulation procedure and animal maintenance after surgery were performed according to previous details (Kleinow, 1991). Following anesthetization, fish were weighed and placed in a dorsal recumbence on an aquatic operating table. The aortic cannula (PE-50 tubing) with an indwelling stylet was placed into the aorta along the dorsal midline of the fish's mouth between the first and third branchial arches. The cannula was attached to the palate mucosa with preplaced sutures and brought dorsally through the cartilage rostral to the nares. An extension of the cannula was anchored to the dorsal fin, and filled with heparinized saline (10 IU/ml, 0.9% NaCl). After surgery, fish were moved to individual holding chambers with flow-through water. Chamber design and cannula placement allowed for sampling from the free swimming fish. Animals were held 24h for recovery from anesthesia and clearance of MS-222 before use.

***In Vivo* Experiment Dosing, Design and Sample Collection**

Two separate experiments were used for *in vivo* investigations using cannulated animals. All animals (807.7 ± 93.7) of either sex were administered a single bolus dose of [^{3}H]-TET at 20 μCi /kg body weight (8.31 μg/kg) through the preplaced aortic cannulas.

The first experiment examined the pharmacokinetics of [^{3}H]-TET with or without dieldrin (0.1 mg/kg body weight per day for 4 weeks) pretreatment using two groups of five or six fish. Following administration of [^{3}H]-TET, 400 μl of blood samples were collected through the cannula at 1, 2, 4, 6, 8, 12, 24, 36, 48, 72 and 96 h after dosing. 120 μl of blood samples were aliquoted and centrifuged for 10 min at 3000×*g* at 4^{o}C. 50 μl of plasma aliquots were prepared for radioactivity measurements.

The second experiment examined the tissue distribution and mass balance of [^{3}H]-TET as well as the hepatic expression of Pgp using two groups of five or six fish one with and one without dieldrin (0.1 mg/kg body weight per day for 4 weeks) preexposure. At 72 h after administration of [^{3}H]-TET, fish were euthanized with MS-222 (250 mg/l) to harvest samples. Bile, liver, spleen, kidneys, heart, intestine, stomach, abdominal lipids, gills, muscle, eggs and carcass were collected and weighed for total mass balance and aliqots radioassayed for [^{3}H]-TET molar equivalents in fish. In addition, intestine and stomach were cut open, inverted, contents rinsed with 0.9% saline; blotted, dried and homogenized before the sample was harvested and analyzed. The mass of the muscle tissue was assumed 50% of carcass in this study (Bosworth et al., 2001). Aliquots of liver tissues (~ 2g) for Pgp expression analysis were harvested immediately following removal and mass balance, suspended in ice-cold modified Sack's preservation buffer (5mM KH_2PO_4, 14 mM $NaHCO_3$, 54mM K_2HPO_4, 5.9mM $KHCO_3$, and 205 mM

mannitol, pH 7.4), snap frozen in liquid nitrogen and finally stored under -80^{o}C until analysis.

Rho-123 Assay

Fluorescence of Rho-123 in bile and perfusate samples from isolated livers were measured with 20 nm slit width at excitation and emission wavelengths of 507 and 529 nm, respectively, on a Hitachi F-2000 Fluorescent Spectrophotometer. Bile samples (20 µl) or those less than 20 µl added zero time-point bile up to a volume of 20 µl, were diluted with teleost Ringer's solution to a final volume of 600 µl. Each perfusate sample (20 µl) was combined with zero time bile (20 µl) and teleost Ringer's solution to a final volume of 600 µl. Concentrations of Rho-123 was calculated in dilutions by interpolation upon a standard curve of Rho-123 concentrations formulated for each fish, using their individual zero time bile to minimize effects of different bile salt concentrations and compositions on fluorescence background. If the samples had reading outside of the standard curve range, they were diluted again up to a final volume of 600 µl, using teleost Ringer's solution containing zero time bile (30:1, v/v).

Radioactivity Measurements

Tissue samples (50-100 mg) were digested in 0.5 ml of TS-2 Tissue Solublizer at 50^{o}C for 24 h. Following cooling, the samples were neutralized with 18 µl of glacial acetic acid and combined with 4.5 ml LSC -cocktail (Ultima Gold, Packard Bioscience, Meriden, CT). Bile, plasma or perfusate samples (20 µl) were not digested prior to adding 4.5 ml LSC -cocktail. All scintillation samples were dark adapted overnight before counting. Samples were counted 30 min each on a Tri-Carb Liquid Scintillation Analyzer (Model 1900 TR). Quench correction, counting efficiency and background were performed for all samples.

Pgp Expression Analysis

Plasma Membrane Protein Preparations

Hepatic plasma membrane proteins were prepared as described by Doi et al, 2000. Hepatic tissue (~ 2 g) were homogenized in 16 ml of ice-cold plasma membrane buffer (5 mM Tris–HCl, pH 7.5, 0.25 M sucrose, and 0.2 mM magnesium chloride), containing 1 mM EDTA and 100 µl of protease inhibitor cocktail (Sigma, St Louis, MO). The homogenates were centrifuged at 1000×g (Rotor type J25.50, Beckman) for 10 min at 4°C and the resulting supernatant reserved. The pellets were resuspended in plasma membrane buffer and recentrifuged. Supernatants from both operations were combined and spun at 15 000×g for 20 min at 4°C. Resulting supernatants were centrifuged at 100000×g in a TI 70 rotor for 1 h at 4°C. Final pellets containing the crude membrane fractions were resuspended in 1 ml of homogenization buffer. Hepatic membrane protein content was determined with folin phenol reagent (Lowry et al., 1951).

Western Blot Analysis

The membrane vesicles were subjected to gel electrophoresis, transfer to a nitrocellulose membrane, blocking and immunoblotting procedures for the detection of Pgp. Membrane vesicles (30 µg) were diluted (1:5) with sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, and 0.001% bromophenol blue) and maintained at room temperature for 20 min. Proteins were loaded on a 7.5% acrylamide gel at a constant voltage of 200 V in running buffer (25 mM Tris–HCl, pH 8.3, 192 mM glycine, and 0.1% SDS running buffer), using Mini-protean™ II Dual Slab gel system (BioRad). After electrophoresis, proteins were transferred at 100 V for 2 h onto a 0.45 µg nitrocellulose membrane with transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, 20% v/v methanol). The membrane was then washed twice each for 5 min in Tris-buffered saline

(TBS) (20 mM Tris, pH 7.5, 500 mM NaCl) before blocked with 0.1 M Levamisole in TBS for 1 h. Following three 5-min washes in TBS, the membrane was blocked again with 3% gelatin in TBS solution and washed for 5 min using 0.05% Tween-20 in TBS (TTBS). Membranes were then incubated overnight with Pgp C219 monoclonal antibody (Signet Laboratories Inc.) (0.2 μg/ml) in antibody buffer (1% gelatin in TTBS). Following two 5-min washes in TTBS, conjugate binding was performed with Goat Anti-mouse IgG Alkaline Phosphatase (GAM-AP) for 90 min in antibody buffer. Following washes with TTBS and TBS, immunoreactivity was color developed using nitroblue tetrazolium in aqueous dimethylformamide with magnesium and 5-bromo-4-chloro-3-indoyl phosphate. Molecular weight was determined with protein markers (Kaleidoscope Prestained Standards, Bio Rad Laboratories Inc.). The staining densities of protein immunoreactivity were quantified using a VersaDoc Imaging System (Quantity One, Bio Rad Laboratories Inc.). An internal standard sample was loaded in all gels for normalization of variability between gels.

Pharmacokinetic Analysis

The plasma concentration versus time course data obtained from administration of [^{3}H]-TET in catfish was plotted on a semilogarithmic scale. Data were fitted by a two-compartment, bolus-input, first-order output model (model 8), using PCNONLIN 3.0 software (Statistical Consultants, Inc., Lexington, KY, USA). Line fitting for individual animal data was optimized using least-squares fit. Statistical moment theory was used for all subsequent pharmacokinetic determinations (Gibaldi and Perrier, 1982). The pharmacokinetic data were reported as mean ± SD. Whereas AUC = $\int_0^{96}$ Cpdt, Cp was plasma concentration at time t.

Statistical Analysis

The presented results are shown as the mean ± SD. All data were processed and graphed with Microsoft Excel 2003 (Redmond, WA). Data analysis was performed with the statistical software SigmaStat for Windows (Version 1.0, Jandal Corporation, San Rafael, CA). One-way ANOVA was employed to examine significant differences at $P < 0.05$.

RESULTS

Effects of Tetracycline and Dieldrin on Pgp Transport

The effect of tetracycline on Pgp transport activity was evaluated using *in situ* prepared, isolated perfused catfish liver preparation. The cumulative excretion of Rho-123 (nM/kg body weight) into bile during 210 min isolated liver perfusions of 1 μM Rho-123 is presented in figure 2.2. In the presence of 20 μM tetracycline, the cumulative biliary accumulation of Rho-123 (10.65 ± 2.45 nM/kg body weight) over 210 min was significantly less than that transported into bile by control livers (19.29 ± 5 nM/kg body weight) ($p < 0.008$). Tetracycline treatment resulted in a 45% decrease in biliary accumulation, as compared with Rho-123 alone.

Similar to tetracycline, dieldrin decreased Rho-123 transport into bile in isolated perfused livers (Fig. 2.3). In the presence of 20 μM dieldrin, biliary accumulation of Rho-123 during the 210 min liver perfusion was significantly lower (40%) than controls ($p < 0.011$), with 210 min cumulative control and treatment values of 11.5 ± 1.75 nM/kg body weight and 6.80 ± 3.01 nM/kg body weight, respectively.

Effects of Verapamil on Biliary Excretion of [^{3}H]-TET

The contribution of Pgp in the transport of [^{3}H]-TET into bile was examined using verapamil, a competitive inhibitor of Pgp, in the isolated perfused liver

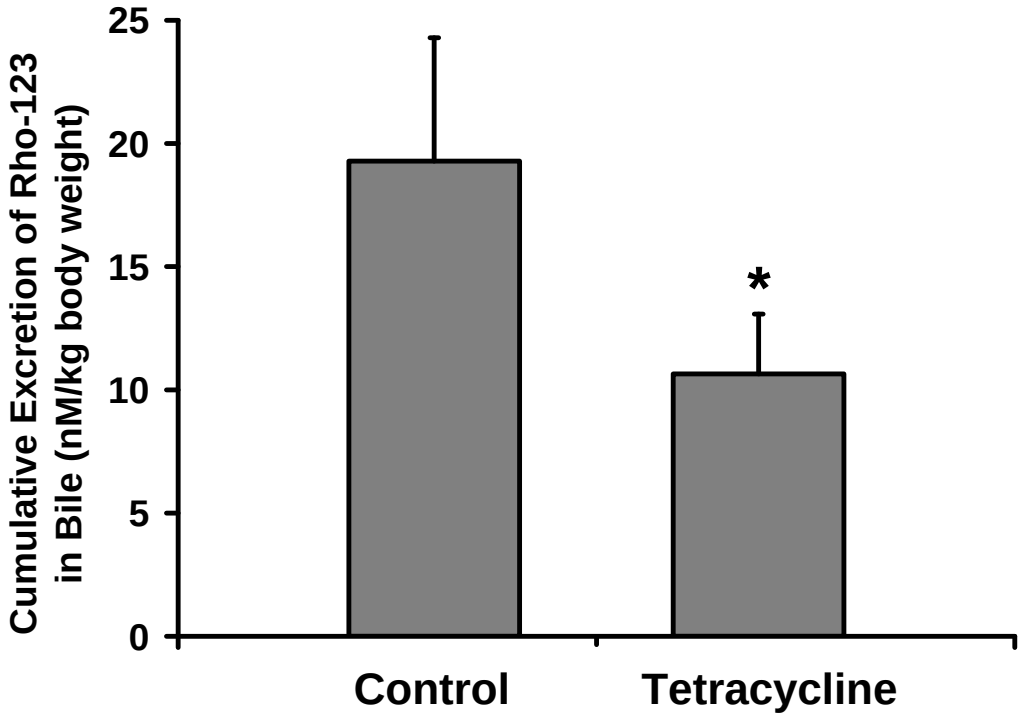


Figure 2.2 - Transport of Rho-123 into bile from isolated perfused catfish livers exposed 210 min to 1 μM Rho-123 with vehicle control or tetracycline (20 μM). Each column represents mean ± SD (nM Rho-123/kg body weight) for controls (n = 6) and tetracycline treatments (n = 5). * = Significantly different from controls at $p < 0.05$.

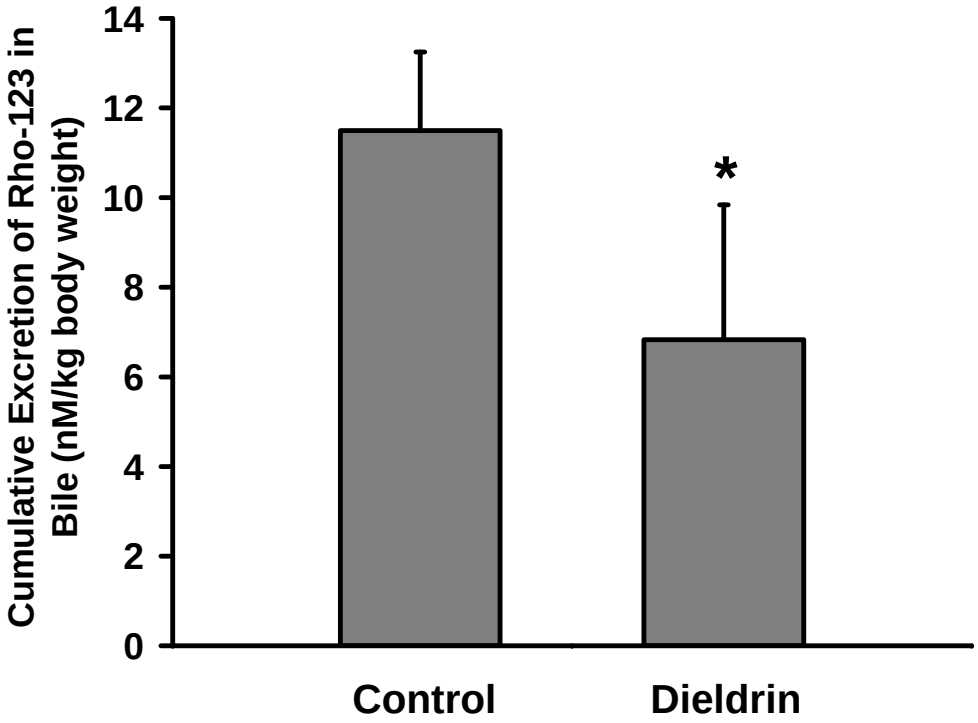


Figure 2.3 - Transport of Rho-123 into bile from isolated perfused catfish livers exposed 210 min to 1 μM Rho-123 with vehicle control or dieldrin (20 μM). Each column represents mean ± SD (nM Rho-123/kg body weight) for controls (n = 5) and dieldrin treatments (TRT) (n = 7). * = Significantly different from controls at $p < 0.05$.

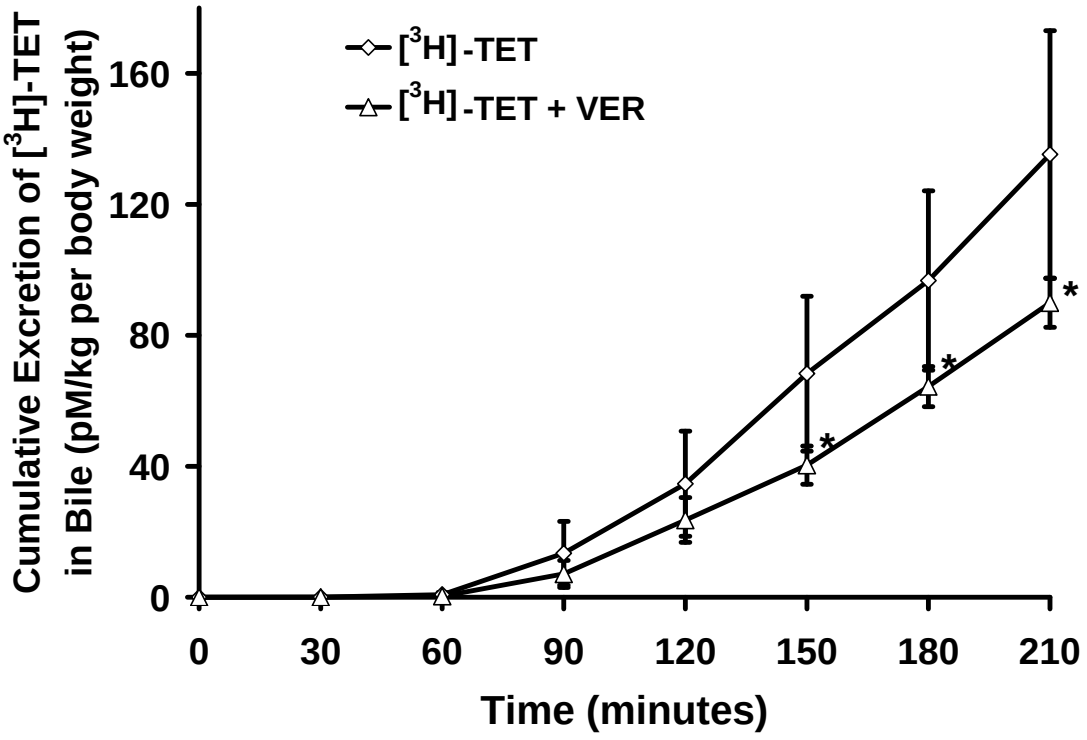


Figure 2.4 - Transport of [^{3}H]-tetracycline (TET) into bile from isolated perfused catfish livers during 210 min exposure to 8.8 nM [^{3}H]-TET with vehicle control or verapamil (VER) (20 μM). [^{3}H]-TET equivalents transported into bile, expressed as pM/kg body weight. Each time point represents mean ± S.D. for controls (CTR) (n = 6) and VER treatments (TRT) (n = 5) (CTR: [^{3}H]-TET; TRT: [^{3}H]-TET + VER). * = Significantly different from controls at $p < 0.05$.

preparations. Biliary accumulation of [^{3}H]-TET equivalents (pM/kg body weight) from isolated perfused livers during exposure to 8.8 nM [^{3}H]-TET with vehicle alone or verapamil (20 μM) is shown in figure 2.4. As compared with [^{3}H]-TET alone, the amount of [^{3}H]-TET equivalents transported into bile during verapamil treatment was lower at all time points. These differences increased over time, with significant differences evident between the two treatments at the 150, 180 and 210 min samplings (Control: 68.29 ± 23.69; TRT: 40.35 ± 5.8) ($p < 0.05$). At 210 min, bile accumulation of [^{3}H]-TET equivalents in the presence of 20 μM verapamil (89.88 ± 7.5) was 34% lower than that with ^{3}H-TET alone (135.29 ± 37.8) ($p < 0.024$).

Effects of Dieldrin on Biliary Excretion of [^{3}H]-TET

Following a similar protocol, the effect of dieldrin on biliary excretion of [^{3}H]-TET was examined. Cumulative [^{3}H]-TET equivalent transport into bile (pM/kg body weight) over time is presented with or without dieldrin (20 μM) (Fig. 2.5). As compared with vehicle alone (104.37 ± 16.96), the cumulative [^{3}H]-TET equivalent content in bile during 210 min dieldrin treatment (50.43 ± 8.99) was significantly lower ($p < 0.002$). Not only was there an overall inhibitory effect on transport of [^{3}H]-TET, but the rate of transport into bile was also reduced by dieldrin treatment. By 120 min, the amount of [^{3}H]-TET equivalents transferred into bile during dieldrin treatment (6.69 ± 2.28) were significantly different from that with [^{3}H]-TET alone (16.6 ± 9.20) ($p < 0.05$). These differences increased over time. At 210 min, dieldrin treatment resulted in a nearly 50% decrease in biliary excretion of [^{3}H]-TET equivalents, a much greater decrease than observed with verapamil (34%).

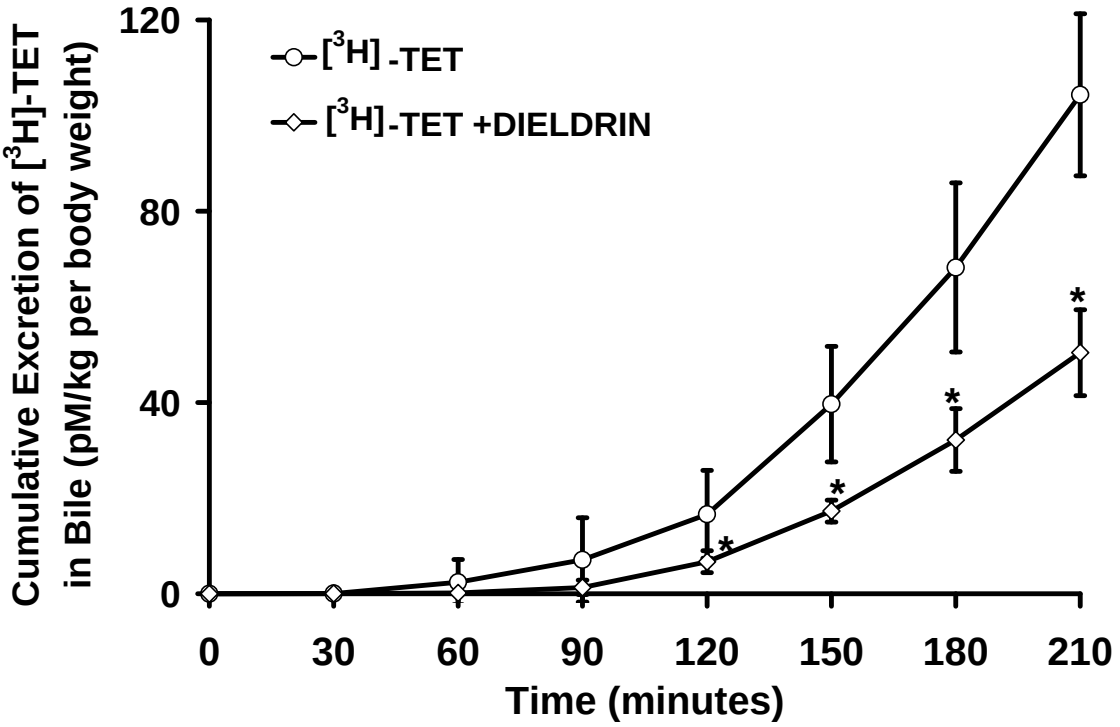


Figure 2.5 – [^{3}H]-tetracycline (TET) equivalents transported into bile from isolated perfused catfish livers during 210 min exposure to 8.8 nM [^{3}H]-TET with vehicle control or dieldrin (20 μM). Data are expressed as pM [^{3}H]-TET equivalents/kg body weight and **e**ach time point represents mean ± SD for controls (CTR) (n = 5) and dieldrin treatments (TRT) (n = 5) (CTR: [^{3}H]-TET; TRT: [^{3}H]-TET + DIELDRIN). * = Significantly different from controls at $p < 0.05$.

Effects of Dieldrin Pretreatment on *In Vivo* Pharmacokinetics of [^{3}H]-TET

Experiments were conducted to investigate the effects of dieldrin pretreatment (0.1 mg/kg body weight per day for 28 days) on the pharmacokinetics of a single intravascular dose of [^{3}H]-TET (20 μCi/kg or 8.31 μg/kg body weight) (Fig. 2.6). Lower plasma concentrations of [^{3}H]-TET equivalents were observed at all time points after dose administration in dieldrin-pretreated fish, as compared to the controls. Significant differences were detected 72 h to 96 h ($p < 0.05$) (Fig. 2.6). Pharmacokinetic parameter values as determined by the two compartment model for administration of a single intravascular dose appear in Table 2.1. The values obtained for AUC_{0-96} were significantly lower 27% in dieldrin treated fish (166.52 ± 26.98 h∗ng/ml) than in the control animals (227.27 ± 50.81 h∗ng/ml) ($p < 0.05$). Consistent with the decreased AUC_{0-96}, the value of the plasma clearance of [^{3}H]-tetracycline in dieldrin pretreated fish (42.12 ± 6.82 ml/h) was elevated by 17% compared to that in controls (31.64 ± 6.21 ml/h) ($p < 0.035$). Significant differences were not observed for the half life of elimination ($t_{1/2}$ k_{el}) between two groups.

Tissue Distribution and Mass Balance of [^{3}H]-TET

[^{3}H]-TET molar equivalents were widely distributed to most tissues of the catfish after the intravascular administration in both control and dieldrin treated groups (Table 2.2). The highest concentrations of [^{3}H]-TET equivalents were found in the bile, liver, kidneys and intestine. Concentrations of [^{3}H]-TET equivalents in liver, kidneys, fat and eggs were significantly decreased at 72h post administration in the dieldrin pretreatment catfish, as compared to controls ($p < 0.05$). When mass balanced, the percentage of the administrated dose among all tissues at 72 h post dose is in Table 2.2. Chronic dieldrin

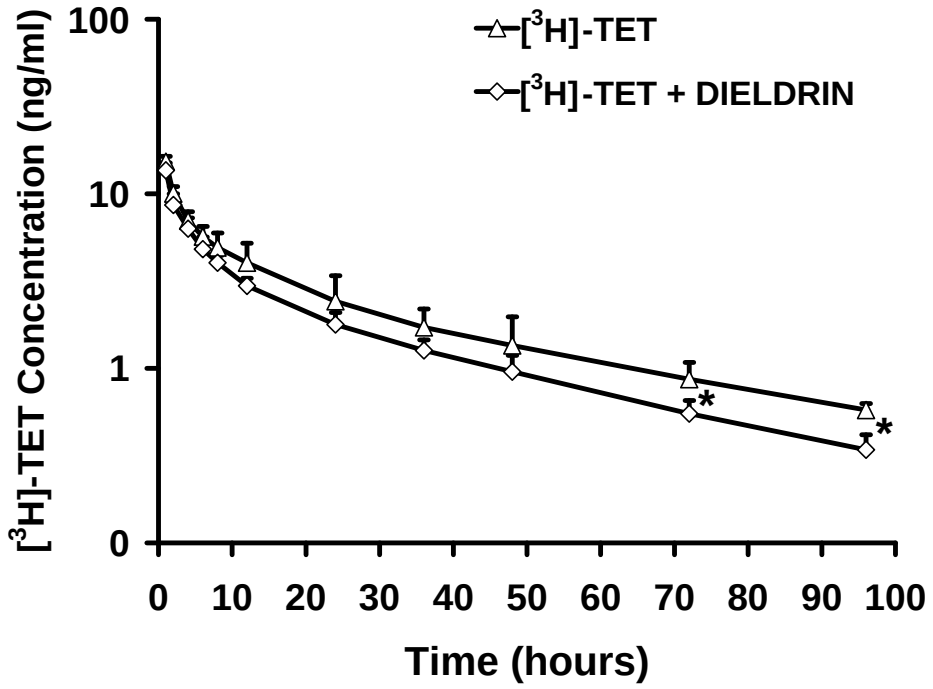


Figure 2.6 - Mean plasma [^{3}H]-Tetracycline (TET) equivalent concentrations following a single intravascular administration of [^{3}H]-TET at 20 µCi/kg body weight (8.31 µg/kg) to catfish pretreated 4 weeks to a diet containing vehicle control (CTR) or dieldrin (0.1 mg/kg body weight per day) (TRT). Data are plotted upon a semilogarithmic scale and each concentration time point represents mean + SD (ng/ml) of five or six measurements (CTR: [^{3}H]-TET; TRT: [^{3}H]-TET + DIELDRIN). * = Significantly different from controls at $p < 0.05$.

Table 2.1 - The values of Pharmacokinetic parameters obtained from two compartment analysis after a single intravascular dose of [^{3}H]-tetracycline (TET) at 20 μCi/kg body weight (8.31 μg/kg) to channel catfish exposed 4 weeks to a diet containing vehicle control or dieldrin (0.1 mg/kg body weight per day) (CTR without dieldrin exposure; TRT with dieldrin exposure).

Parameter	Value	
	CTR	TRT
Alpha (α) (h^{-1})	0.587 ± 0.332	0.505 ± 0.233
Beta (β) (h^{-1})	0.026 ± 0.002	0.031 ± 0.005
A (ng/ml)	4.200 ± 0.667	3.555 ± 0.370
B (ng/ml)	1.214 ± 0.479	0.981 ± 0.171
$t_{1/2(\alpha)}$ (h)	1.474 ± 0.697	1.632 ± 0.730
$t_{1/2(\beta)}$ (h)	26.999 ± 2.494	22.812 ± 3.925
k_{el} (h^{-1})	0.099 ± 0.003	0.116 ± 0.026
k_{12} (h^{-1})	0.355 ± 0.232	0.285 ± 0.160
k_{21} (h^{-1})	0.158 ± 0.103	0.135 ± 0.057
$t_{1/2}$ k_{el} (h)	7.000 ± 0.188	6.214 ± 1.349
AUC (h*ng/ml)	227.27 ± 50.81	*166.52 ± 26.98
Volume (ml/kg)	381 ± 67	445 ± 44
Cmax (ng/ml)	22.465 ± 4.689	18.823 ± 2.013
Clearance (ml/h)	31.64 ± 6.21	*42.12 ± 6.82

† The values of parameters are the mean ± SD of five or six measurements.
† $t_{1/2(\alpha)}$, half-life of [^{3}H]-TET in plasma during distribution phase; $t_{1/2(\beta)}$, half-life of elimination phase; $t_{1/2}$ k_{el} , half-life of elimination; AUC, the area under plasma concentration time curve; Volume, volume of distribution; Clearance, the total plasma clearance. *= Significant difference from controls ($p<0.05$).

Table 2.2 - Tissue concentrations and distribution of [^{3}H]-tetracycline (TET) equivalents at 72h following a single intravascular administration of [^{3}H]-TET at 20 μCi/kg body weight (8.31 μg/kg) to channel catfish exposed 4 weeks to a diet with or without dieldrin (0.1 mg/kg body weight per day) (CTR without dieldrin exposure; TRT with dieldrin exposure).

Tissue/Bile	Concentration ng/kg		% of Dose	
	CTR	TRT	CTR	TRT
Bile	1010.27 ± 883.1	905.94 ± 454.7	9.946 ± 5.741	11.048 ± 4.840
Liver	4.44 ± 1.10	*3.19 ± 0.6	0.673 ± 0.142	0.554 ± 0.113
Spleen	1.30 ± 0.87	1.37 ± 0.64	0.012 ± 0.006	0.013 ± 0.007
Kidney	4.85 ± 0.45	*3.83 ± 0.31	0.325 ± 0.028	*0.265 ± 0.031
Heart	1.75 ± 0.30	1.61 ± 0.42	0.012 ± 0.002	0.012 ± 0.003
Intestine	3.33 ± 0.87	3.76 ± 1.34	0.331 ± 0.165	0.383 ± 0.102
Stomach	1.41 ± 0.28	1.10 ± 0.37	0.145 ± 0.020	0.113 ± 0.034
Fat	1.48 ± 0.05	*1.14 ± 0.17	0.028 ± 0.023	0.017 ± 0.010
Gill	1.14 ± 0.17	1.04 ± 0.25	0.207 ± 0.013	0.195 ± 0.052
Muscle	0.90 ± 0.33	0.66 ± 0.13	3.933 ± 1.389	2.979 ± 0.563
Eggs	0.91 ± 0.22	*0.45 ± 0.08	1.505 ± 0.706	0.611 ± 0.338
Tissue Accumulation			7.171 ± 1.855	*5.1.42 ± 0.803
Total Accumulation			17.117 ± 6.14	16.190 ± 5.44

† Tissue accumulation is the sum of distributions of [^{3}H]-TET equivalents in all tissues, including liver, spleen, kidneys, heart, intestine, stomach, fat, gill, muscle and eggs, whereas total accumulation consists of tissue accumulation and bile distribution.

† Muscle compartment was assumed to be 50% of carcass in catfish (Bosworth et al., 2001).

† Values are expressed as ng [^{3}H]-TET equivalents/kg and data represents mean ± SD. of five or six measurements. * =Significant difference from controls ($p<0.05$).

pretreatment resulted in a trend for increased accumulation of [^{3}H]-TET equivalents in bile as percent of dose, but no statistical difference was detected between controls and treated group due to the high variability among fish. In contrast, the total accumulation of [^{3}H]-TET equivalents as percent of dose in pretreated catfish tissues (5.142 ± 0.803), including liver, spleen, kidneys, heart, intestine, stomach, fat, gill, muscle and eggs, was significantly less (28%) than that in controls (7.171 ± 1.855) ($p < 0.037$). In the kidneys, distribution of [^{3}H]-TET equivalents was significantly reduced 19% by dieldrin pretreatment ($p < 0.008$).

Hepatic Pgp Expression

Pgp expression in hepatic plasma membranes from catfish following 4-week dietary exposure to vehicle control or dieldrin (0.1 mg/kg body weight per day) was measured. A 170 kDa C219 immunoreactive protein was observed in liver membrane preparations in both control and dieldrin treated fish (Fig. 2.7.B). Following dieldrin treatment, the level of staining density of immunoreactive Pgp in hepatic plasma membrane was significantly increased by 23%, as compared to that in controls ($p < 0.037$) (Fig. 2.7.A).

DISCUSSION

The isolated perfused liver Rho-123/tetracycline and tetracycline/verapamil comparisons presented in these studies suggest that tetracycline is transported by Pgp or at least interacts with Pgp substrates. Tetracycline has been shown to be a substrate of Pgp homologues in bacteria and in cultured mammalian cells, however, there have been few comparative studies performed in the whole animal or perfused organs. Investigations performed in bacteria have demonstrated that multidrug resistance (MDR) and gene tranfections of Pgp homologues reduced intracellular accumulation of

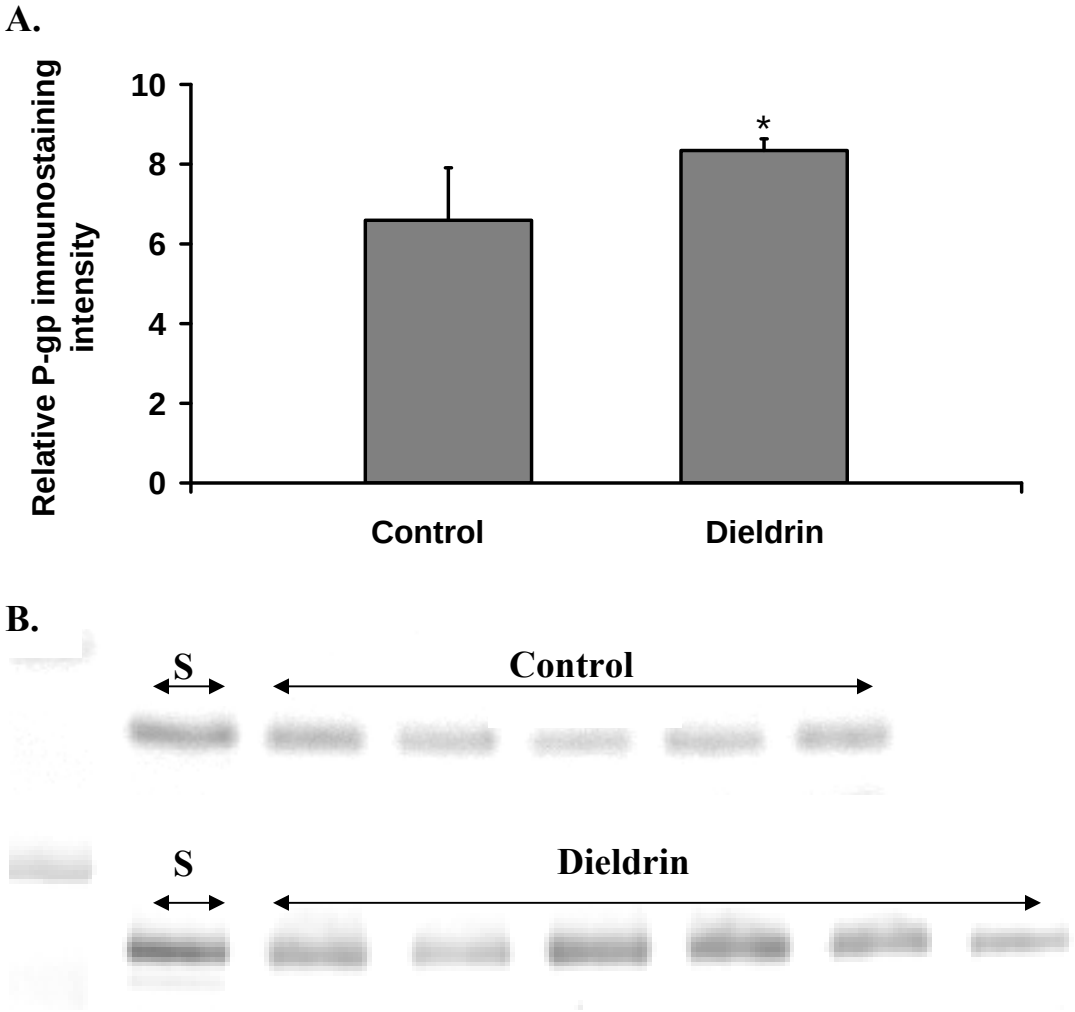


Figure 2.7 – A. Levels of C219 immunoreactive Pgp in the hepatic plasma membrane from catfish following 4-week dietary exposure containing vehicle control or dieldrin (0.1 mg/kg body weight per day). Data are expressed as relative staining intensity of antibody immuoreactivity for Pgp protein following standardization for inter-gel variability. Each column represents mean ± SD of five or six measurements. * = Significantly different from controls at $p < 0.05$. **B.** Western blot of Pgp protein in hepatic plasma membrane proteins in control or dieldrin pretreated fish. S = Pgp protein internal standard.

tetracycline, as compared to non-transfected bacteria (Levy, 1992; George et al., 1996; Chollet et al., 2004; Qin et al., 2004). Furthermore, cyclosporin A and verapamil, both competitive inhibitors of Pgp, completely reversed tetracycline resistance and increased accumulation of tetracycline in two human leukemic cell lines expressing Pgp (Kavallaris et al., 1993). These *in vitro* findings are consistent with the concept that tetracycline is a substrate of Pgp. Many prototypic substrates of Pgp, such as Vinca alkaloids, anthracyclines, epiodophyllotoxins, rhodamine dyes and doxorubicin share a ring structure similar to the four-ringed structure of the amphiphilic tetracycline (Fig. 2.1) (Argast and Beck, 1984; Lampidis et al., 1989; Germann et al., 1996; Sharom, 1997; Mealey et al., 2002). This structure and tetracycline's physical properties appear to enhance partitioning into the cell membrane and the active interaction with Pgp (Bain et al., 1997). The transport mechanism which allows this broad substrate specificity has not been clearly defined. Pgp substrates, however, appear to share several common characteristics, including moderate hydrophobicity, amphiphilicity, low molecular weight, and a complex ring structure (Lampidis et al., 1989; Germann et al., 1996; Bain et al., 1997). Pgp substrates have been shown to gain access to the binding site(s) of the Pgp transporter from the inner leaflet of lipid bilayer of the plasma membranes and interact with Pgp within the membrane rather than in the aqueous phase (Raviv et al., 1990; Higgins and Gottesman, 1992; Sharom, 1997; Romsicki and Sharom, 1999).

Tetracycline (20 μM) resulted in a 45% decrease in transport of Rho-123, a prototypic substrate of Pgp (Fig. 2.2), and verapamil (20 μM), a substrate and competitive inhibitor of Pgp, decreased the amount of [^{3}H]-tetracycline transported into bile by 34% (Fig. 2.4) (Spoelstra et al., 1994; Saeki et al., 1993; Tsuji et al., 1993; Didier et al, 1995; Maki et al., 2003), during the 210 min isolated liver perfusions. Transport

studies suggest that inhibition of Pgp could result either from the competition of substrates at binding sites, from allosterical interactions by simultaneous binding to Pgp at different sites, or from blockage of ATP binding and hydrolysis process (Ford, 1996; Tamai and Safa, 1991; Ramachandra et al., 1998; Senior et al., 1995). Like other classic competitive inhibitors, verapamil has been developed as an inhibitor to identify the potential for drug-drug interactions due to Pgp (Keogh and Kunta, 2006). In the current study, verapamil was employed as an inhibitor to examine the contribution of Pgp to excretion of [^{3}H]-tetracycline into bile. Taking into consideration the limited duration of exposure, the appreciable decrease in [^{3}H]-tetracycline equivalent movement into bile, these results suggest that Pgp transport plays a significant role for biliary excretion of [^{3}H]-tetracycline. These findings are consistent with the concept that tetracycline acts as a substrate, a competitive inhibitor or by altering Rho-123 binding/transport by interacting at another site. While the exact mechanism is unknown, tetracycline appears to interact with Pgp transport.

Interactions of xenobiotics with Pgp are complex. A number of organochlorine compounds, including DDT, DDD and DDE, when dosing occurs at 20 μM have been shown to reduce the efflux transport of the prototypic Pgp substrate Rhodamine B (1 μM) from the gills in the marine mussel *M. galloprovinvialis* (Galgani et al., 1996). Similarly, the current study, when using dieldrin (20μM), another highly hydrophobic organochlorine, observed decreased transport of Rho-123. In this case, transport of Rho-123 (1 μM) into bile from isolated perfused livers was decreased 40% (Fig. 2.3). Other studies, however, have been obtained *in vitro* (using murine melanoma cell lines expressing human MDR1 gene) where the intracellular accumulation of the Pgp substrate, doxorubicin (50 μM), was not affected by DDT, DDE and dieldrin (100 μM)

(Bain and Leblanc, 1996). The inconsistency in response to these organochlorine compounds could be related to the different organchlorine concentrations, concentrations of Pgp substrates used to detect Pgp inhibition, or the duration of exposure.

Several studies have demonstrated that dieldrin inhibits the ATPase system involved with Mg^{2+}, Ca^{2+}, Na^{+}-K^{+} pumps on the plasma membrane in the fish and rabbit (Akera et al., 1971; Desaiah and Koch, 1975; Bandyopadhyay et al., 1982; Mehrotra et al., 1988). As an ATPase inhibitor, the inhibitory action of dieldrin upon Pgp transport of Rho-123 and [^{3}H]-tetracycline in the current study may result from the reduction of ATPase activity. Other studies have reported that ATPase inhibitors, including the flavonoid quercetin and 5,7,3′,4′,5′-pentamethoxyflavone (PMF), inhibited Pgp transport by interfering with the basal and substrate-stimulated ATPase activity (Shapiro and Ling, 1997; Choi et al., 2004). Therefore, dieldrin may alter ATPase system on the plasma membrane (Verma et al., 1978) and hence block the active transport of Pgp substrates through the cell membrane as a noncompetitive inhibitor.

As seen with substrate-metabolizing enzymes, efflux transport activity of Pgp on the cell membrane may be saturated at elevated Pgp substrate concentrations (Lin, 2003). In contrast, the influx of these compounds into the cell by diffusion would not exhibit concentration based limitations other than those already imposed by the physiochemical characteristics of the compound and the membrane. In this situation, as the substrate concentration increases, the impact of Pgp efflux on intracellular accumulation is quantitatively less important (Lin, 2003). To explain these discrepancies, it is plausible that even though Pgp inhibition occurs at lower concentrations, it may not significantly affect the intracellular accumulation of this Pgp substrate at higher concentrations. This phenomenon may contribute to the apparent dichotomy between [^{3}H]-tetracycline

disposition at high dieldrin doses used in the perfusion studies (inhibit movement into bile) and the latter chronic dieldrin exposures which facilitated [^{3}H]-tetracycline elimination.

Alternatively, different findings may result from the use of different species, cell lines, ectera between the current and past studies. Contrasting results were observed with *in vitro* studies in mussel gills examining DDT, DDD and DDE, and polychlorinated biphenyl--Aroclor 1254 as inhibitors for Pgp transport. These compounds acted as inhibitors in one species of mussel (*Mytilus galloprovincialis*) (Galgani et al., 1996), but did not appear to affect Pgp transport activity in another species (*Mytilus californianus*) (Cornwall et al., 1995). A difference in the size of the Pgp-like protein reacting to C219 monoclonal antibody in these two species was observed (Galgani et al., 1996). It remains to be clarified whether such disagreement results from concentration, species or other experimental differences.

The foregoing studies suggest that tetracycline is transported by Pgp and dieldrin may influence Pgp transport activity. Acute high dose dieldrin exposures resulted in marked inhibitory effects on the biliary excretion of [^{3}H]-tetracycline equivalents. Tetracycline equivalent transport was decreased by dieldrin (50%) to a greater extent than observed for verapamil (34%). In addition, a significant decline in transport was achieved earlier during dieldrin treatment than with verapamil. These results suggest that dieldrin acts as a more potent inhibitor of Pgp than verapamil. Inhibition of Pgp by dieldrin slowed down clearance of tetracycline from the liver into bile and prolonged intracellular residence time of tetracycline in the liver. Since biliary excretion is an important pathway for the elimination of tetracyclines out of fish (Plakas et al., 1988), these results would suggest that dieldrin co-exposure would result in decreased transport of tetracycline into

bile, and perhaps decreased clearance from the animal. Unpredictably and in contrast to the inhibitory effects of dieldrin (20 μM) on transport of [^{3}H]-tetracycline into bile, chronic dieldrin pretreatment resulted in significantly lower mean plasma concentrations at 72 h and 96 h post administration (Fig. 2.6), a 27% decrease in the AUC_{0-96h}, and a 17% increase in the plasma clearance of a single and subsequent dose of [^{3}H]-tetracycline, as compared to controls (Table 2.1). These alterations were coincided with a 23% increase in hepatic Pgp expression in dieldrin treated fish (Fig. 2.7), which may contribute to the enhanced clearance and elimination of [^{3}H]-tetracycline equivalents. Regulation of Pgp expression in response to inducers has been extensively studied *in vitro* and *in vivo* (Kliewer et al., 1998; Moore et al., 2000; Staudinger et al., 2001; Mikamo et al., 2003). DDE has been shown to increase expression of Pgp in the gill tissues of the mussel *Mytilus californianus* (Eufemia and Epel, 2000). Induction of Pgp by dieldrin treatment as seen in the current *in vivo* studies with catfish would be consistent with literature findings.

Previous studies showed that chronic dieldrin exposure stimulated biliary excretion of [^{14}C]-dieldrin, [^{3}H]-DMBA and [^{14}C]-BaP in rainbow trout (Gilroy et al., 1993; Donohoe et al., 1998; Barnhill et al., 2003), and an apparent increase of Pgp-related immunoreactivity in liver sections as detected by the C94 monoclonal antibody. The C219 and JSB-1 anti Pgp monoclonals, however, did not show similar differences (Curtis et al., 2000). Differences in sensitivity and specificity of antibodies reacting to the Pgp protein may have contributed to these observed inconsistencies in Pgp-related responses in the rainbow trout liver. The current study showed a significant induction of hepatic C219 reactivity in catfish following comparable dietary dieldrin treatment. The disparity in Pgp-related responses between the current study and the previous Curtis

study (Curtis et al., 2000) may be attributed to species differences in response to the Pgp inducer (Lin, 2003). Cytotoxic drugs, including adriamycin, daunomycin and mitoxantrone, and the polycyclic aromatic hydrocarbon, 3-MC, induced expression of the MDR gene in rat and mouse hepatocytes, with little inductive effect on human cells (Fardel et al., 1996; Chin et al., 1993). The activation of Pgp also occurs at the post-translational level. Studies have shown that phosphorylation of serines and threonines on Pgp protein increased drug resistance activities in mammalian cell lines (Ratnasinghe et al., 1998). The activation of Pgp at the post-translational level is expected to elevate the transport ability of Pgp rather than expression of the MDR gene (quantity of Pgp protein). As an inducer of Pgp, dieldrin may involve different mechanisms to regulate expression of Pgp and activation of Pgp in different species (fish). While these mechanisms are possibilities, it remains to be clarified whether such disagreements result from dosage, detection methods, the length of xenobiotics exposure, experimental conditions, or other experimental differences (Doi et al., 2001).

Mechanisms by which dieldrin exposure altered the pharmacokinetics of tetracycline may include not only induction of Pgp, but also by changes in the biotransformational enzymes, especially CYP450s. Several studies have demonstrated that dieldrin activated PXR and the constitutive androstane receptor (CAR) in mammals to induce CYP3A and CYP2B family enzymes (Coumoul et al., 2002; Wei et al., 2002; Lemaire et al., 2004). However, in rainbow trout, the increased biliary excretion of [^{14}C]-dieldrin, [^{3}H]-DMBA and [^{14}C]-BaP following chronic dieldrin treatment was not correlated to enzyme induction (Gilroy et al., 1993; Donohoe et al., 1998; Barnhill et al., 2003). No significant differences in total hepatic CYP450s, glutathione S-transferase, UDP glucuronosyltransferase and sulfotransferase activities were evident with dieldrin

exposure (Gilroy et al., 1993; Barnhill et al., 2003). Immunoblot analysis also revealed no change in expression of hepatic CYP isozymes (Gilroy et al., 1996). In addition, the biliary metabolite profile of [^{14}C]-BaP was not significantly altered following dieldrin exposure *in vivo* (Barnhill et al., 2003)**.** These findings suggest that induction of metabolizing enzymes by dieldrin treatment is unlikely in fish. On the other side, tetracycline undergoes minimal metabolism and is not biotransformed to a significant extent in animals (Aronson, 1980). Therefore, induction of Pgp may well be the most important factor in alterations of the pharmacokinetics of [^{3}H]-tetracycline in the current study. These studies support the concept that Pgp induction contributed to these alterations.

Following a single intravascular dose of [^{3}H]-tetracycline (20μCi/kg; 8.31μg/kg), tetracycline molar equivalents (parent and metabolites) were widely distributed to most tissues with the highest concentrations in the intestinal, hepatic and renal tissues of both control and dieldrin treated catfish (Table 2.2). These findings are comparable to previous studies with tetracycline (Plakas et al., 1988). Dieldrin has been shown to be readily absorbed and accumulate in liver, kidney, bone marrow and adipose tissues in the body of fish (Jorgenson, 2001). As dieldrin inhibited Pgp transport of [^{3}H]-tetracycline into bile in the isolated perfused liver, dieldrin would be expected to inhibit Pgp transport of [^{3}H]-tetracycline in tissues of mutual distributions. However, concentrations of dieldrin in these tissues may be too low to inhibit Pgp transport activity in the current chronic exposure study. In a previous study, a concentration of only about 1.8 nM (~ 0.7 ug/g tissues) of dieldin was achieved in the liver after dietary exposure to dieldrin at 0.324 mg/kg body weight daily for six weeks in rainbow trout (Barnhill et al., 2003). Although a different species was used in the current study, a lower dosage (0.1 mg/kg body weight

per day) and a shorter exposure period (4 weeks) may have resulted in a lower concentration of dieldrin in the liver of exposed catfish. At such a concentration, dieldrin may not have resulted in significant inhibitory effects on Pgp transport since Pgp inhibition is concentration-dependent (Lin, 2003). Even though Pgp inhibition by dieldrin may have occurred in the chronic dieldrin exposed fish, the inhibition would be much smaller than that observed in the isolated perfused liver. The Pgp inductive effect following chronic dieldrin exposure would mask these inhibitory effects.

Pgp is constitutively located on the bile canalicular membrane of hepatocytes, the brush-border epithelial cells of the intestine, and the luminal side of renal proximal tubules (Cordon-Cardo et al., 1990; Schinkel et al., 1996; Balayssac et al., 2005;). Induction of Pgp may alter tissue distributions and excretion of Pgp substrates (Table 2.2). Consistent with previous findings in the rainbow trout studies, dieldrin pretreatment resulted in greater excretion of [^{3}H]-tetracycline equivalents in bile at 72 h after dosing with a single administration. However, significant differences from the controls were probably hid by large variability among fish. In addition, the inductive effects of Pgp may also occur in the kidneys as well as intestine, leading elevated excretion of [^{3}H]-tetracycline into urine and the intestinal lumen. Compatible to increased excretion of [^{3}H]-tetracycline, the total accumulation of [^{3}H]-tetracycline in pretreated catfish tissues, including liver, spleen, kidneys, heart, intestine, stomach, fat, gill, muscle and eggs, was significantly lower than that in controls, perhaps as a cumulative effect of Pgp induction.

In current study, chronic dieldrin led to a decrease in tissue concentrations and systemic exposure of tetracycline in fish. Drug-drug interactions with Pgp induction are of concern for attenuated therapeutic effects of drugs in clinical treatment (Lin, 2003). In the aquatic environment, fish may be constantly exposed to various contaminants

including dieldrin-like compounds and Pgp induction may occur in response to the contaminant body burdens (Bard, 2000). Few studies exist regarding influence of environmental contaminants on therapeutic effects of drugs in fish or the involvement of Pgp induction. The current study provides information that suggests chronic contaminant exposure may be a determinant factor for therapeutic effects of drugs used in fish.

In summary, this study provides results consistent with tetracycline as a substrate of Pgp and a competitive inhibitor of Rho-123 transport into bile of the isolated perfused liver of catfish. In addition, dieldrin inhibited biliary transport of Rho-123 and tetracycline. In contrast, chronic dieldrin exposure resulted in increased elimination of [^{3}H]-tetracycline, as evidenced by a decrease in AUC, reduced tissue concentrations, and an increase in the whole body clearance. In dieldrin treated fish, induction of Pgp in hepatic plasma membranes may be responsible for alterations of the pharmacokinetics and disposition of [^{3}H]-tetracycline. Cumulatively, these results suggest dieldrin has the potential to inhibit Pgp transport of tetracycline at the high concentrations with an inductive effect upon Pgp expression predominating at the low concentration chronic exposures *in vivo*. Dieldrin co-exposure would result in altered disposition of tetracycline, leading to toxicities and potential misdosing; whereas multidrug resistance may follow induction following chronic dieldrin exposure.

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CHAPTER 3: ENHANCED BIOACCUMULATION OF IVERMECTIN AND BENZO[A]PYRENE IN CATFISH BY EXPOSURE TO THE SURFACTANT C18 LINEAR ALKYLBENZENE SULFONATE

INTRODUCTION

Fish are widely consumed by humans as a healthy low fat, protein rich, micronutrient and omega-3 (n - 3) fatty acid enhanced food (Daviglus et al., 2002). Over the past several decades a large number of studies suggest that fish effectively bioaccumulate and in some cases biomagnify select contaminants from the environment (Bryan, 1979; Clarkson, 1995). Consumption advisories have been issued for eating fish from impacted waters and for specific species of fish occupying higher trophic niches [U.S. Environmental Protection Agency (EPA) 2004]. Compounds such as mercury, polychlorinated biphenyls (PCB), DDT, and polycyclic aromatic hydrocarbons (PAHs) have received much of this attention. Recently the list of compounds which may enter the human food chain via fish has been expanded to include a variety of pharmaceuticals (Hastein et al., 2006). Drugs have entered waterways and fish by aquaculture practices, runoff from concentrated animals feeding operations and municipal sources (Daughton and Ternes, 1999).

The risk of contaminants in the aquatic environment to the fish consuming public is dependent on the uptake or bioavailability of contaminants to the fish, the bioaccumulation of contaminants in the fish to be consumed and the bioavailability of the contaminants from a fish meal to the human consumer. A variety of factors are known to influence bioavailability and bioaccumulation of contaminants in fish (Leblanc, 1995; Ivanciuc et al., 2006). Notably, compound exposure, lipophilicity and the susceptibility of the chemical to biotransformation, play an important role in dictating contaminant body

burdens. Recently, studies in mammals suggest that Phase III transport processes by ABC transporter such as P-glycoprotein (Pgp) and multidrug resistance-related proteins (Mrp) have a significant effect on drug bioavailability from the intestine (Kurata et al ., 2002; Liu and Hu, 2000; Fromm, 2000) and in compound elimination from the body (Shitara, 2006). Likewise, fish have been shown to have ABC "like" efflux transporters in excretory pathways as well as routes of bioavailability such as the intestinal mucosa suggesting similar roles of these "like" transporters in compound disposition and bioavailability.

Contaminants found in the aquatic environment often exist as mixtures with other compounds (Yang et al., 1998). The interactions of components in mixtures have been shown to result in synergistic, antagonistic or additive effects on aquatic organisms (Altenburger et al. 2003). Surfactants, common environmental pollutants and mixture components in aquatic systems (Ahel et al., 1993; Todorov et al., 2002), generally exhibit a low order of direct toxicity to aquatic organisms; however, several studies are suggestive of interactive effects of surfactants upon the bioavailability and disposition of other compounds (Mann, 1962; Solon, et al., 1969; Pärt et al., 1985). Studies examining strategies to inhibit the involvement of ABC transporters in cancer multidrug resistance have examined surfactants as a means to inhibit Pgp and Mrp's contribution to this phenomenon (Kabanov et al., 2002). Initial indications suggest that surfactants may enhance membrane permeability (Drori et al., 1995; Hendrich and Michalak, 2003) or inhibit transporter efflux action directly (Woodcock et al., 1992; Nerurkar et al., 1996; Miller et al., 1999; Regev et al., 1999; Alqawi and Georges, 2003). These activities may increase effective chemotherapeutic levels in cancer cells.

This study addresses experimentally two hypotheses. First, is that the C18 linear alkylbenzene sulfonate (LAS) surfactant at environmental concentrations can inhibit the efflux activity of Pgp "like" transporters in the bile canniculi of the catfish liver. A second hypothesis is that the C18 LAS surfactant at environmental levels, through its action on Pgp and/or membrane permeability, can influence xenobiotic bioavailability or bioaccumulation. The widely used veterinary pharmaceutical ivermectin and the PAH and environmental contaminant benzo[a]pyrene (BaP) were selected as model compounds based on their possible involvement with ABC transporters, environmental relevance and their limited bioaccumulation.

MATERIALS AND METHODS

Chemicals

^{3}H-Ivermectin (IVM) was purchased from American Radiolabeled Chemicals, INC., St Louis, MO. ^{3}H-Benzo[a]pyrene (BaP) was obtained from GE Healthcare UK Limited Amersham Place Little Chalfont Buckinghamshire HP79 NA, UK and BaP from Midwest Research Institute, Kansas City, MO. Methylene blue was obtained from Fisher Scientific Company, Fair Lawn, NJ. Tricane methane sulfonate (MS-222) was provided by Argent Chemical Company, Redmond, WA. Heparin sodium (1000 USP units/ml) was acquired from Elkins-sinn, Inc., Cherry Hill, NJ. Sodium dodecylbenzenesulfonate (C^{18} LAS) (~80%), rhodamine-123 (Rho-123), ivermectin (IVM), sodium tetraborate, chloroform, and all other chemicals used for buffer preparations were supplied by Sigma, St Louis, MO.

Animals

Channel catfish, *Ictalurus punctatus*, were obtained from the Louisiana State University Aquaculture Research Station, Baton Rouge, LA, USA. Animals were grown

and maintained under a 12 h light/dark photoperiod in laboratory tank supplied with flow-through tap water. Water was dechlorinated and maintained at ~18^{o}C. All fish were acclimated for one month prior to use in the experiment.

In Situ Hepatic Rho-123 Disposition Studies

Surgical Procedure

Catfish of both sexes were fasted 24 h prior to surgery. The surgical preparation and perfusion of isolated livers were performed as previously described (Kleinow et al., 2004). Animals were anesthetized with MS-222 at induction and maintenance doses of 106 and 86 mg/l, respectively. The liver, gall bladder and hepatic portal vein were exposed by the ventral midline and transverse incisions posterior to the pectoral girdle. Following collection of residual bile and ligation of common bile duct, the gall bladder was cannulated in the apex with a PE260 tubing for subsequent bile collections. The portal vein and collateral vessels afferent to the liver were isolated, and ligatures were loosely placed around each. The portal vein was cannulated using a Quick-cath 14G cannula filled with heparin anticoagulant. Once ligated in place, oxygenated and heparinized (10 USP/ml) teleost Ringer's solution (117.2 mM NaCl, 4.8 mM KCl, 0.9 mM $CaCl_2$, 1.5 mM $MgSO_4$, 12.3 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, 5 mM glucose, pH 7.4) was continuously perfused into the afferent cannula. Collateral vessels were rapidly tied off, and the efferent hepatic vein was cut at the level of the cardinal sinus to establish flow through the liver. Upon completion of perfusion circuit, the liver was transiently blanched, removed from the animal and placed on a perforated platform of a temperature controlled recirculation perfusion apparatus. Head pressure was maintained at 12 cm to create physiological pressures for media perfusion during the experiment. Livers were pre-perfused with oxygenated teleost Ringer's solution (1L) containing vehicle alone for

control or individual treatments at temperatures dictated by the individual experiments. This process removed remaining blood and preexposed livers to specific treatment conditions.

Isolated Liver Perfusion of Rho-123

To examine the influence of LAS on hepatic disposition of the Pgp prototypic substrate Rho-123, six groups of five fish (1999.5 ± 447.9 g) were prepared for the *in situ* isolated liver perfusions. The six treatments included control, three LAS treatments (1, 5, and 20 μM) maintained at 18 °C, and two additional controls perfused at 23 °C and 28 °C, respectively. The 23 °C and 28 °C experiments served as positive controls for changes in fluidity. Following pre-perfusion, livers were perfused in a recirculating system for 210 min with 250 ml of media, which were thermally and compositionally identical to the pre-perfusion mixture, except containing 1 μM Rho-123. Samples of bile and perfusate were collected at 30 min intervals for 210 min for fluorometric analysis.

Once liver perfusions were completed, the liver was removed and weighed. Two aliquots of liver were snap frozen in liquid nitrogen and stored at -80 °C in the phosphate buffer for extraction of Rho-123 (see below) and in the buffer A for membrane fluidity studies (see below), respectively.

Extraction of Rho-123

To determine the amount of Rho-123 remaining in liver after *in situ* isolated liver perfusions, Rho-123 extraction was performed according to the published methods (Sweatman et al., 1987). The liver tissue for each fish was blotted dry, weighed, and homogenized in 2 volumes of ice-cold phosphate buffer (100 mM Na_2HPO_4 and NaH_2PO_4, pH 8.5). A 3-mL aliquot of the homogenate was transferred into a silicone-coated and capped tube prior to incubation at 4 ^{o}C for 30 min. Samples were extracted 3

times with 7 ml of freshly prepared ethyl acetate:1-butanol (9:1, v/v) and centrifuged each time at 510xg (IEC Centra CL3 Centrifuge) for 3.5 min. Organic phases of Rho-123 extraction were combined in silicone-coated tubes, evaporated to dryness under a stream of nitrogen and reconstituted in 5 ml methanol before fluormetric analysis. The aquatic phases were centrifuged for 12 min at 110xg (Beckman Microfuge 11). The resulting supernatants were transferred into fresh tubes, and spun. The supernatants from the second spin were used for fluorescent assay. Rho-123 concentrations were measured on a fluorescent spectrophotometer described as below except that the standard curve was prepared by diluting stock Rho-123 solutions (2 mg/100ml) with methanol.

Membrane Fluidity

Hepatic Membrane Vesicle Preparations

In order to determine LAS influence on membrane fluidity, hepatic membrane vesicles were prepared and subjected to fluorescent anisotropy measurements according to the method from Kleinow et al. 2006. Hepatic tissues (2 g) were homogenized in 10 volumes of ice-cold buffer A (12 mM Tris, pH 7.4, 300 mM Mannitol, 5 mM EGTA) and centrifuged at 12,000g for 5 min at 4 °C (Rotor type JA 25.50, Beckman). The pellet was discarded while the supernatant was spun at 48,000g for 15 min at 4 °C (Rotor type Ti 70, Beckman). The second pellet was resuspended in buffer and centrifuged again under the same conditions. The pellet from the third spin was suspended in 500 µl of storage buffer (50 mM Tris-HEPES, pH 7.5, 0.1 mM $MgSO_4$, 200 mM KCl, 125 mM D-Mannitol, 5 mM EGTA), which were aliquoted, snap frozen in liquid nitrogen and stored at −80 °C until fluorescence anisotropy analysis. Hepatic membrane protein content was analyzed using folin phenol reagent with bovine serum albumin as a standard (Lowry et al., 1951).

Membrane Fluidity Measurements

Fluidities of hepatic plasma membranes were detected by measuring fluorescence anisotropy (inverse of membrane fluidity) with the fluorescent probe, 1, 6-diphenyl-1,3,5-hexatriene (DPH, Molecular Probes, Eugene, OR) at the corresponding temperature (Houpe et al., 1996). Fluorescence polarizations studies were carried out at λ_{EX} = 362 nm, λ_{EM} = 428 nm and slit width = 2 mm, using a spectrofluorometer (model FL3-22TAU3, Spex Fluorolog-3, Jobin Yvon-Spex, Edison, NJ) equipped with automated polarization accessory and two temperature regulated cell holders. Samples of hepatic membrane vesicles, containing 150 µg protein in 50 µl storage buffer, were incubated with 8 µM DPH in a total volume of 3 ml incubation buffer (10 mM HEPES-Tris, pH 7.5, 200 mM KCl) for 30 min on ice. After transferred into a cuvette, the sample was allowed to equilibrate for 10-15 min in the cell holders set to liver perfusion temperature. The fluorescence anisotropy (r) values were calculated according to the following equation:

$$r = [I_{vv} - (I_{vh} \times G)]/[I_{vv} + (2 \times I_{vh} \times G)]$$

where I_{vv} and I_{vh} are the fluorescence intensities detected with vertical and horizontal polarization of emission, respectively, when the excitation polarization is set in the vertical orientation (Houpe et al., 1996). G factors, ratios of spectrophotometer sensitivity for horizontal and vertical polarized light, were calculated as I_{hv}/I_{hh} where I_{hv} and I_{hh} are the fluorescence intensities detected with vertical and horizontal polarization of emission when the excitation polarization is set in the horizontal orientation (Lakowicz et al., 1979).

Effects of LAS on *In Vivo* Accumulation of ^{3}H-IVM and ^{3}H-BaP

In these experiments, fish were individually housed in 15 L tanks supplied with a constant water flow of 80 ml per min. A peristaltic pump continuously transferred a

stable volume of the freshly prepared LAS solution into a mixed head tank supplied with a constant water flow, in which it was diluted to the appropriate final concentrations. LAS concentrations of test water were determined according to the published methods (Abbott 1962). Concentrated stock solutions (1000 mg/l) of C18 LAS were prepared every third day.

To evaluate the effect of environmental relevant concentrations of LAS on the bioaccumulation of the pharmaceutical drug IVM and the pro-carcinogen BaP, six groups of fish (*n*=3 or 4, 701.1 ±135.4g) were exposed to LAS for 12 days at either 0, 100, or 300 µg/l water. Starting on the 7th day of LAS exposure, catfish were administered 6 daily doses of ^{3}H-IVM (10 µg/day/kg body weight) or ^{3}H-BaP (40 µg/day/kg body weight) by indwelling gavage tube in a slurry of semi synthetic diet.

In order to facilitate multiple gavage administrations of ^{3}H-IVM or ^{3}H-BaP, fish were outfitted in dwelling stomach tubes 24 h prior to the six-day gavage administration. Following anesthetization, fish was weighed and placed with a dorsal recumbent position on operating table. The stomach tube was implanted and anchored to the cartilage rostrum of the nares (Kleinow, 1991). The experimental diet slurry was produced by mixing finely ground semi-synthetic diet with water (1:3). The appropriate amounts of ^{3}H-IVM (0.16 µCi/µg or 354.2 dpm/ng) in methanol or ^{3}H-BaP (0.095 µCi/µg or 213.5 dpm/ng) in toluene were then evenly dispersed over the slurry diet, which were thoroughly blended, evaporated under nitrogen and stored at −4°C until gavage administration. In addition, BaP diet was shielded from light and stored under nitrogen.

Following six-day gavage administration, catfish were fasted for 24 h and euthanized with MS-222 (250 mg/l). Blood samples (~ 2 ml) were first collected from the caudal vein. Bile, gonads, abdominal lipids, liver, spleen, kidney, stomach, intestine,

muscle, gill and brain were then harvested and weighed for total mass balance and aliqots radioassayed for ^{3}H-IVM or^{3}H-BaP in the animals. In a similar fashion and purpose, intestines were cut open, inverted, contents rinsed out with 0.9% saline; blotted and dried before sample collected and analyzed. The volume of blood in fish was assumed to be 5% of total body weight while the mass of the muscle tissue was regarded as carcass in the mass balance determinations.

Fluorescent Assay

Fluorescence of Rho-123 in bile and perfusate samples from isolated perfused livers was measured at excitation and emission settings of 507 and 529 nm wavelengths, respectively, and 20 nm slit width, using a Hitachi F-2000 Fluorescent Spectrophotometer. Bile samples (20 µl) or those less than 20 µl added zero time-point bile up to a volume of 20 µl, were diluted with teleost Ringer's solution to a final volume of 600 µl. Each perfusate sample (20 µl) was combined with zero time bile (20 µl) and teleost Ringer's solution to a final volume of 600 µl. Concentrations of Rho-123 was calculated in dilutions by interpolation upon a standard curve of Rho-123 concentrations formulated for each fish, using their individual zero time bile to minimize effects of different bile salt concentrations and compositions on fluorescence background. If a sample had reading outside of the standard curve range, it was diluted again up to a final volume of 600 µl, using teleost Ringer's solution mixed with zero time bile (30:1, v/v).

Radioassay

Tissues samples (~ 100 mg) and blood aliquots (100 µl) were digested at 50°C in 0.5 ml of tissue solubilizer TS-2 (Research Products International Corp., Mount Prospect, IL) for 24 h. Following cooling, the samples were neutralized with glacial acetic acid (18 µL) and added to 4.5 ml of scintillant (Ultima Gold, Packard, Downers Grove, IL). Bile

samples (100 µl) were not digested prior to adding 4.5 ml of scintillant. All scintillation samples were dark adapted overnight before counting for 30min each with a Tri-Carb Liquid Scintillation Analyzers (Model 1900 TR). Quench correction counting efficiency and background were performed for all samples.

Methylene Blue Active Substances Analysis for Measurement of Concentrations of LAS

Concentrations of anionic surfactant LAS in the water were determined by the methylene blue technique (Abbott, 1962). 100 ml of water sample freshly collected were treated with alkalified methylene blue solutions. The anionic surfactant LAS reacts with the methylene blue active substance to form a chloroform soluble complex. The complex is extracted and dissolved in the chloroform phase by rapidly shaking the separating funnel for 1 min. The chloroform extract then passes through an acidified methylene blue solution to wash out inorganic anions associated with methylene blue substances, which have a low solubility in chloroform phase. The final chloroform extracts were measured by a spectrophotometer (U-2000, Hitachi) at a wavelength of 650 nm. Quantification was based on a four-point standard curve which was established from the results by treating 100 ml of water samples containing suitable amounts of LAS (0, 10, 20 and 40 µg).

Statistical Analysis

All data were processed and graphed with Microsoft Excel 2003 (Redmond, WA). Data analysis was performed with the statistical software SigmaStat for Windows (Version 1.0, Jandal Corporation, San Rafael, CA). One-way ANOVA was employed to examine for significant differences at $P < 0.05$.

RESULTS

Effects of LAS on Biliary Excretion of Rho-123

To investigate the effect of LAS on transport activity of Pgp, accumulation of the prototypic Pgp substrate, Rho-123, in bile was examined, using *in situ* prepared, isolated perfused livers. As seen in Figure 3.1, the addition of LAS (1, 5, 20 μM) decreased movement of Rho-123 into bile in a concentration-dependent fashion. During the 210-min liver perfusions the cumulative amounts of Rho-123 (nM/kg body weight) in bile were significantly decreased by 18.6 (12.35 ± 1.91), 38.1 (9.39 ± 1.40) and 66.7% (5.06 ± 1.79) with increasing LAS concentrations at 1, 5 and 20 μM, respectively ($p < 0.05$).

The effect of acute temperature change on biliary accumulation of Rho-123 was also evaluated. Temperature treatments of 23 ^{o}C and 28 ^{o}C as compared to controls (18 ^{o}C) resulted in dramatic decreases in biliary excretion of Rho-123 by 90.4 (1.46 ± 0.41) and 93.7% (0.97 ± 0.45), respectively, during the 210-min perfusions ($p < 0.0001$).

Effects of LAS on Hepatobiliary Disposition of Rho-123

The distribution of Rho-123 in liver as parent and metabolites, bile and perfusing media, is shown with increasing LAS concentrations (Table 3.1). LAS at 1, 5 and 20 μM resulted in significant increases of Rho-123 retained in liver by 14.4 (65.39 ± 3.55 μg), 20.0 (68.63 ± 3.67 μg) and 25.7% (71.85 ± 3.98 μg), respectively, as compared with control (57.16 ± 6.34 μg) ($p < 0.05$). This increased hepatic Rho-123 retention was exhibited primarily as significant increases in the organic parent fraction. In contrast, metabolites as aqueous Rho-123 in the liver was significantly decreased by LAS treatments at 1 and 20 μM, suggesting decreased metabolism of Rho-123 in the liver (Table 3.1). The increase in hepatic Rho-123 content was accompanied by decreased movement of Rho-123 into bile for each of the LAS treatments (Table 3.1). Acute

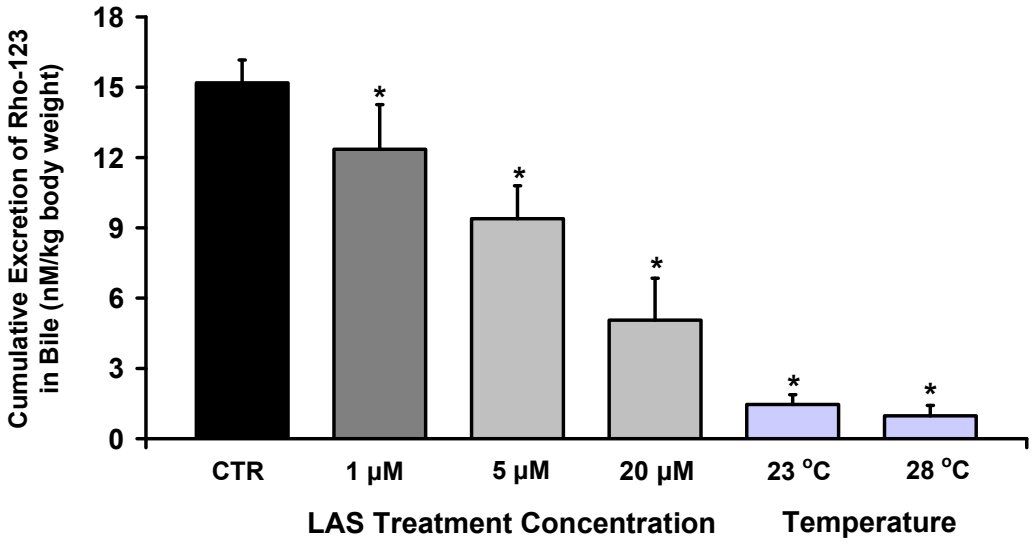

Figure 3.1 - Excretion of Rho-123 into bile from isolated perfused catfish livers exposed 210 min to 1 µM Rho-123 with vehicle control or LAS (1 µM, 5 µM or 20 µM) at 18 °C, or vehicle alone treated at 23 °C or 28 °C. Each column represents mean ± SD (nM Rho-123 /kg body weight) (n = 5). * = Significantly different from controls at $p < 0.05$.

Table 3.1 - Effects of LAS on hepatobiliary disposition of Rho-123 at the end of 210-min isolated liver perfusion of 1 μM Rho-123, coupled with vehicle or LAS at 1, 5 or 20 μM at 18˚C, or with temperature treatments at 23˚C or 28˚C, respectively.

	Bile Excretion	Liver Extraction		Media	Total
	Total	Aqueous	Organic	Total	
	μg/210 min	μg/whole liver weight		μg/250 ml	μg
Control	14.31 ± 2.33	0.79 ± 0.29	57.16 ± 6.34	6.30 ± 1.58	78.56 ± 7.82
1 μM LAS	*7.99 ± 1.45	* 0.34 ± 0.08	*65.39 ± 3.55	8.22 ± 1.14	81.94 ± 2.41
5 μM LAS	*7.30 ± 2.22	0.57 ± 0.23	*68.63 ± 3.67	5.26 ± 0.37	81.75 ± 4.53
20 μM LAS	*3.93 ± 0.66	* 0.39 ± 0.12	*71.85 ± 3.98	5.59 ± 1.35	81.75 ± 2.82
Temp 23˚C	*0.95 ± 0.24	* 0.31 ± 0.05	*67.54 ± 4.93	*11.82 ± 1.64	80.63 ± 5.99
Temp 28˚C	*0.67 ± 0.33	* 0.37 ± 0.18	*69.94 ± 3.94	*11.40 ± 1.35	82.38 ± 3.91

† Data represents mean ± SD. of three or four measurements.
*=Significantly different from controls ($p<0.05$).

temperature increases of 5 and 10 ^{o}C (23^{o}C and 28^{o}C) resulted in increased retention of Rho-123 in the whole liver by 18.2 (67.54 ± 4.93 μg) and 22.4% (69.94 ± 3.94 μg), respectively. These increases in hepatic Rho-123 retention were a result of significant increases of parent Rho-123 content (as detected in the organic fraction) and significant decreases in metabolites (the aqueous fraction) as compared to the control ($p < 0.05$). In addition, acute temperature treatments dramatically decreased Rho-123 equivalents found in bile and significantly increased Rho-123 retained in the media by 87.6 (11.82 ± 1.64 μg) and 81.0% (11.40 ± 1.35 μg), as compared with the control (6.30 ± 1.58 μg) ($p < 0.01$) (Table 3.1).

Effects of LAS on Membrane Fluidity

In an effort to elucidate the mechanism by which LAS reduces Rho-123 movement into bile of the isolated liver perfusions, a DPH fluorescence polarization study was performed. LAS treatments at 1, 5 and 20 μM resulted in significant decreases of anisotropies (inverse of membrane fluidity) in the corresponding hepatic membranes by 29.7 (0.102 ± 0.004), 32.4 (0.098 ± 0.005) and 38.6% (0.089 ± 0.003), respectively, as compared with control (0.145 ± 0.005) ($p < 0.001$) (Fig. 3.2). The most prominent decrease in anisotropy or increase in membrane fluidity was evident between control and the 1 μM LAS treatment, while fluidity continued to increase with LAS concentration, the changes were much smaller than the initial 1 μM treatment. Acute temperature increases of 5 and 10 ^{o}C (23 ^{o}C and 28 ^{o}C) also resulted in significant decreases in fluorescence anisotropy by 42.8 (0.083 ± 0.002) and 49.7% (0.073 ± 0.001), respectively (Fig. 3.2). These findings suggest membrane fluidity was altered with LAS exposure and acute increases in temperatures.

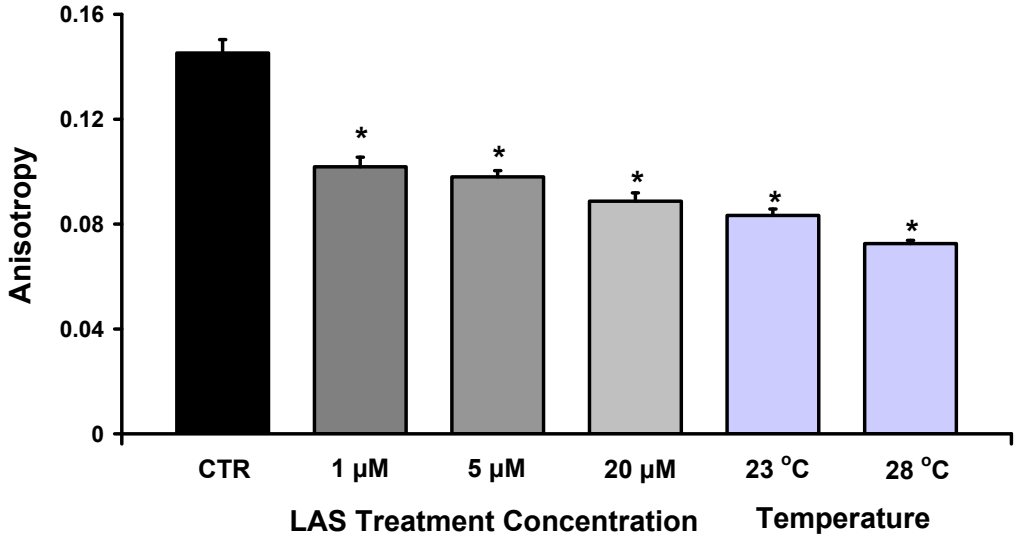

Figure 3.2 - Anisotropy of hepatic membrane vesicles prepared from isolated perfused livers, following exposure 210 min to 1 µM Rho-123 with vehicle control or LAS (1 µM, 5 µM or 20 µM) at 18°C, or vehicle alone maintained at 23°C or 28°C. Each column represents mean ± SD (n = 5). * = Significantly different from controls at $p < 0.05$.

Influence of LAS on *In Vivo* Accumulation and Disposition of ^{3}H-IVM

^{3}H-IVM molar equivalent concentrations were significantly increased in blood and major individual organs including liver, kidneys, brain, gills, intestine, stomach, muscle, and eggs, with increasing LAS treatments (Table 3.2). LAS treatments of 100 and 300 µg/l increased the dose remaining in fish at 24 hours after six daily consecutive dosings by 39.0 and 77.9% for ^{3}H-IVM, respectively (Fig. 3.3). When considering the organ weight or volume, muscle, eggs and blood contributed the most to the composite increase with the muscle contributing 24.8 and 53.0% of the observed increase for the 100 and 300 µg/l LAS dose, respectively. Concentrations of ^{3}H-IVM equivalents increased 1.6- and 2.2-fold in blood, 1.3- and 1.6-fold in liver, 1.5- and 2.2-fold in kidney, 1.4- and 1.5-fold in intestine, 1.7- and 3.2-fold in brain, 1.4- and 1.8-fold in muscle with LAS exposure at the 100 and 300 µg/l, respectively. As presented in Fig. 3.4, LAS treatments at 100 and 300 µg/l resulted in significant decreases of 26.7 and 51.0% in the ratios of bile/blood concentrations and 20.7 and 28.0% in the ratios of liver/blood concentrations, respectively. The decreasing bile/blood ratios with either LAS treatment were the result of significant increases in blood ^{3}H-IVM in face of no significant change in movement into bile (Table 3.2). The liver exhibited a modest increase in ^{3}H-IVM concentrations with LAS treatments. These ^{3}H-IVM increases in the liver were less than those observed for blood resulting in decreasing liver/blood concentration ratios (Table 3.2).

Influence of LAS on *In Vivo* Accumulation and Disposition of ^{3}H-BaP

LAS treatments lead to the increasing levels of ^{3}H-BaP molar equivalents in blood, bile and tissues such as liver, kidney, heart, eggs and stomach (Table 3.3). The

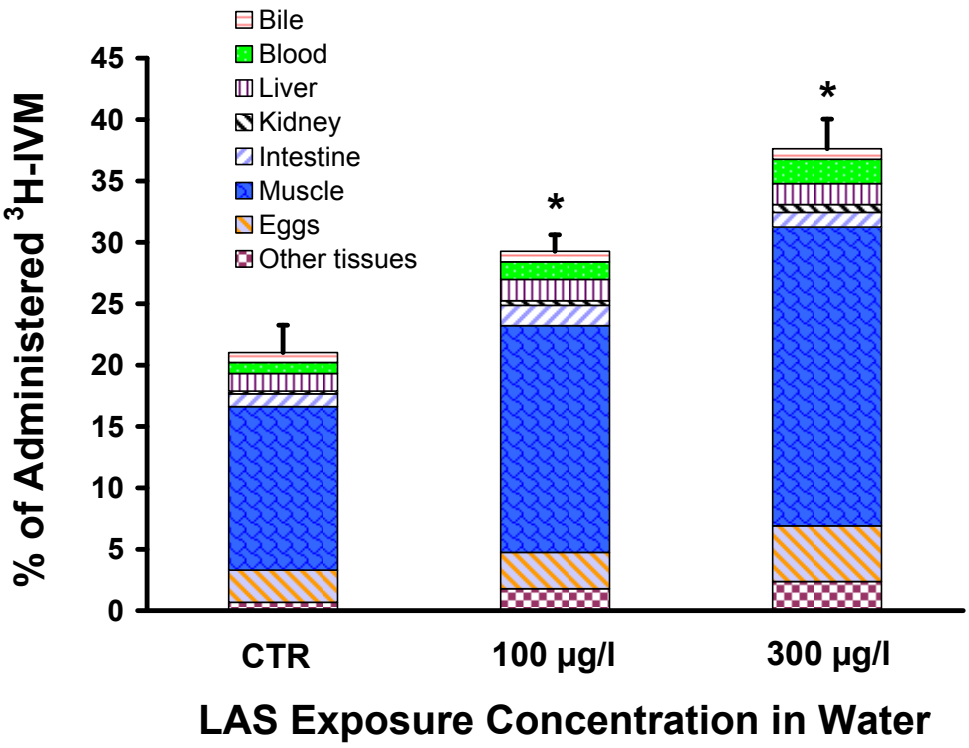

Figure 3.3 - Tissue distribution of ^{3}H-IVM equivalents as per cent of dose following daily dietary administration of ^{3}H-IVM at 10 µg/kg body weight to catfish for six days starting on 7th day during a twelve-day exposure to water alone (as control: CTR) or LAS at 100 or 300 µg/l. Each column represents mean ± SD (n = 3 or 4). * = Significantly different from controls at $p < 0.05$.

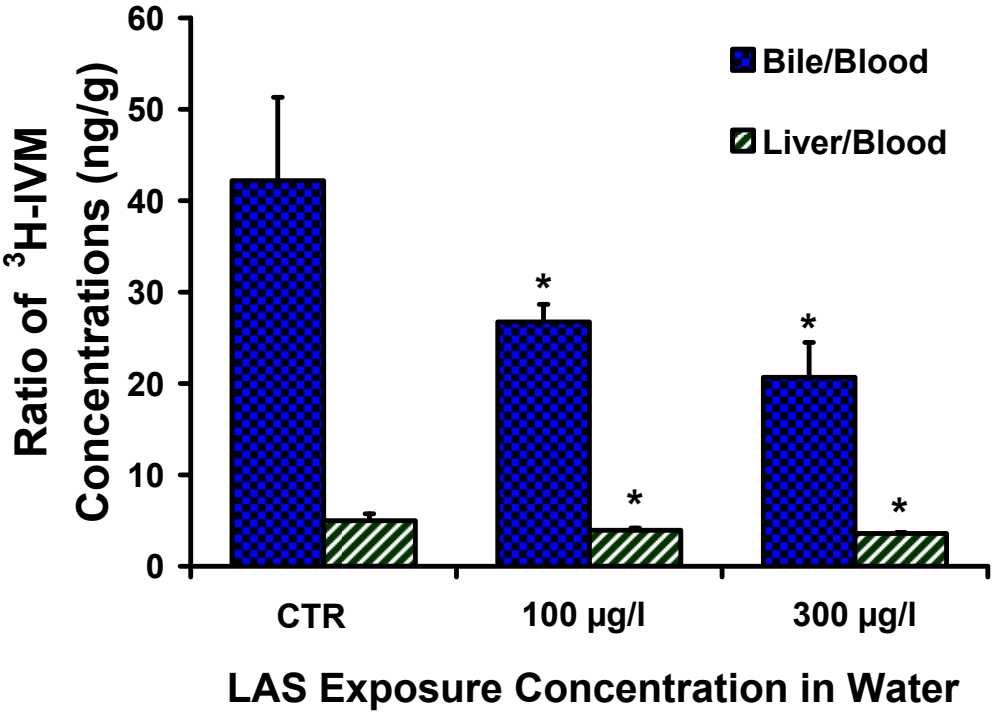

Figure 3.4 - Bile/blood and liver/blood ^{3}H-IVM concentration ratios in catfish following daily dietary administration of ^{3}H-IVM at 10 µg/kg body weight to catfish for six days starting on 7th day during a twelve-day treatment with water alone (as control: CTR) or LAS at 100 or 300 µg/l. Each column represents mean ± SD (n = 3 or 4). * = Significantly different from controls at $p < 0.05$.

Table 3.2 - Tissue concentrations of ^{3}H-IVM equivalents following daily dietary administrations of ^{3}H-IVM at 10 µg/kg body weight to catfish for six days starting on 7th day during a twelve-day exposure to water alone (as control: CTR) or LAS at 100 or 300 µg/l water.

	^{3}H-IVM Equivalent Concentrations (ng/g)		
Tissues	**CTR**	**LAS 100 µg/L**	**LAS 300 µg/L**
Blood	10.72 ± 1.28	* 17.23 ± 1.44	* 23.81 ± 3.45
Bile	444.84 ± 59.51	459.29 ± 36.74	491.81 ± 108.85
Liver	52.74 ± 5.63	* 67.71 ± 2.59	* 85.75 ± 13.97
Spleen	10.48 ± 2.92	13.43 ± 0.9	* 22.26 ± 2.13
Kidney	28.46 ± 3.54	* 42.93 ± 3.04	* 61.39 ± 8.91
Heart	19.17 ± 6.12	20.82 ± 2.92	* 35.56 ± 8.28
Lipids	54.17 ± 12.73	55.94 ± 13.01	61.02 ± 20.07
Brain	9.83 ± 1.17	* 17.10 ± 4.25	* 31.29 ± 2.37
Gill	15.91 ± 1.16	* 21.66 ± 1.54	* 33.58 ± 6.25
Intestine	43.8 ± 4.80	* 61.87 ± 4.36	* 66.41 ± 3.63
Stomach	25.84 ± 2.91	* 34.96 ± 3.39	* 40.32 ± 7.49
Muscle	9.62 ± 0.84	* 13.68 ± 0.47	* 17.61 ± 0.63
Eggs	18.13 ± 2.46	* 25.31 ± 1.24	* 36.51 ± 2.91

† Values are expressed as ng ^{3}H-IVM equivalents/g and data represents mean ± SD. of three or four measurements.
* =Significantly different from controls ($p<0.05$).

doses recovered from the whole fish at 24 hours after consecutive dosings were increased by 50 and 157% with LAS exposure at 100 and 300 μg/l, respectively (Fig. 3.5). This composite increase mostly came from contributions of intestine, muscle and liver. With exposure to LAS at 100 and 300 μg/l, concentrations of ^{3}H-BaP equivalents increased 1.6- and 3.8-fold in liver, 1.7- and 4.3-fold in kidney, 1.3- and 1.7-fold in intestine, 2.0- and 3.9-fold in brain, 1.5- and 3.0-fold in muscle, 1.9- and 3.3-fold in eggs, respectively. Bile concentrations increased significantly 1.7- to 1.9-fold for the 100 and 300 μg/l LAS concentrations whereas liver concentrations increased 1.6- to 3.8-fold (Table 3.3). Blood exhibited concurrent concentration increases (Table 3.3). Bile/blood concentration ratios were significantly decreased by 39.2 % only with LAS exposure at 300 μg/l. No significant change was noted for liver/blood ^{3}H-BaP concentration ratios at either the 100 or 300 μg/l LAS concentration (Fig. 3.6).

DISCUSSION

Surfactants are among the most common contaminants found in domestic and industrial wastewater and frequently occur with other contaminants as mixtures in the aquatic environment (Lewis, 1992; Yang et al., 1998). As one of the most widely used classes of surfactants, LAS has been found worldwide in a variety of aquatic environments at different concentrations. LAS has been detected in sewage effluents and in surface waters in the UK (1090 μg/l and 416 μg/l) (Holt et al., 1998; Fox et al., 2000) as well as in the effluents of wastewater treatment plants in the USA (4 to 94 μg/l) (Trehy et al., 1996). LAS has also been reported at concentrations of 20 to 1000 μg/l in outlets to estuaries and near-shore marine waters in the North Sea (Berna et al., 1991; Stalmans et al., 1991) and in Mississippi River water (0.1 to 2.8 μg/l) (Tabor and Barber, 1996). Generally LAS levels in sediment have been reported to be much higher than in the water

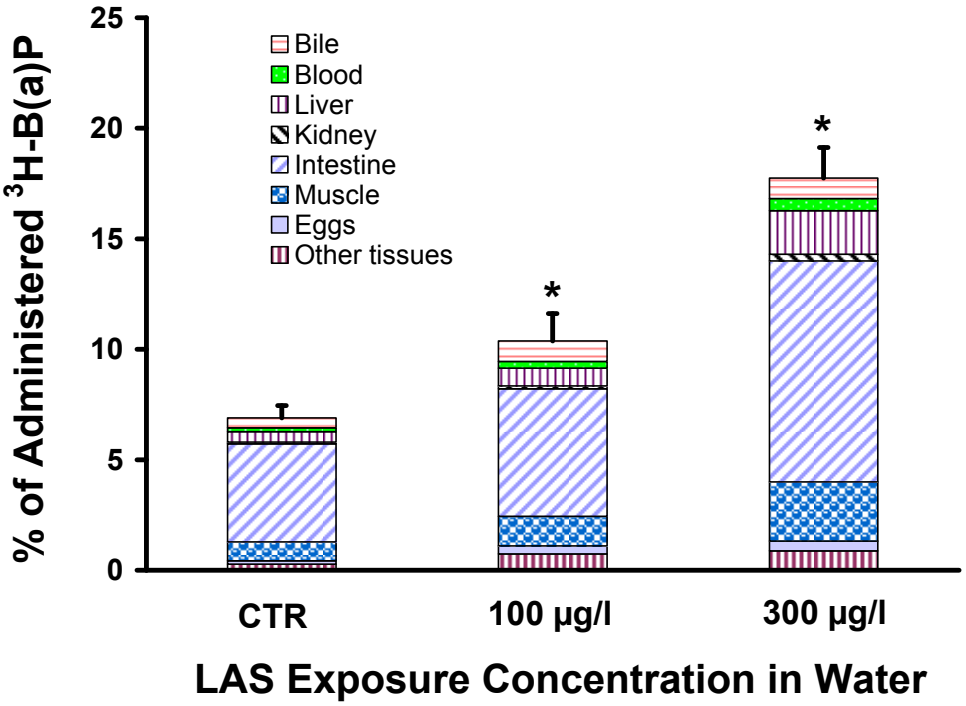

Figure 3.5 - Tissue distribution of ^{3}H-BaP equivalents as per cent of total dose following daily dietary administration of ^{3}H-BaP at 40 µg/kg body weight to catfish for six days starting on 7th day during a twelve-day exposure to water alone (as control: CTR) or LAS at 100 or 300 µg/l. Each column represents mean ± SD (n = 3 or 4). * = Significantly different from controls at $p < 0.05$.

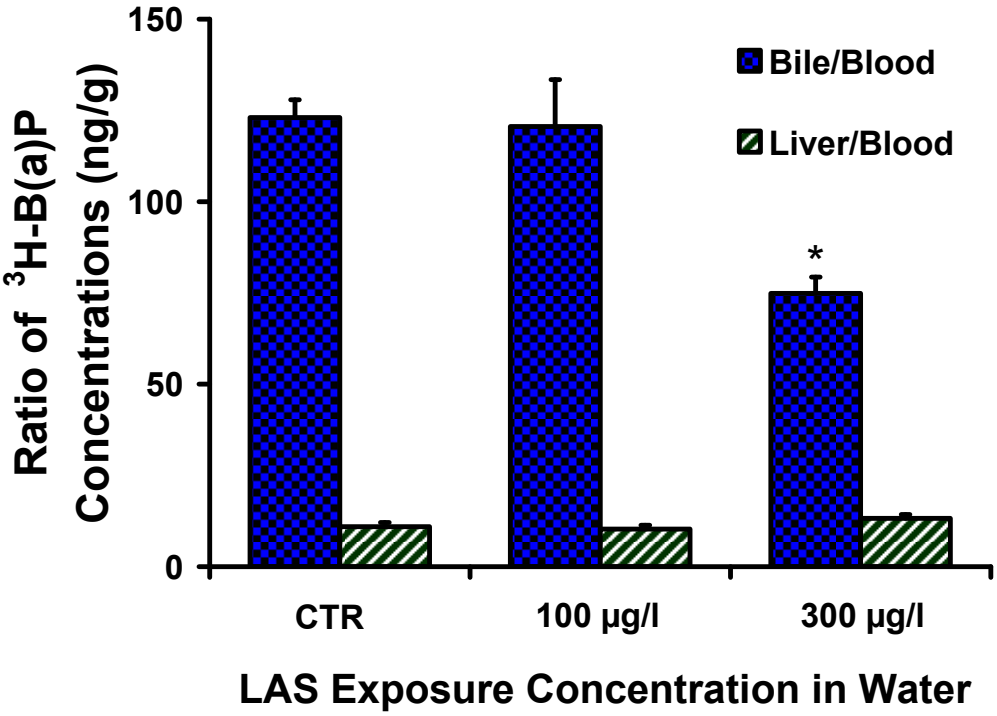

Figure 3.6 - Bile/blood and liver/blood ^{3}H-BaP concentration ratios following daily dietary administration of ^{3}H-BaP at 40 µg/kg body weight to catfish for six days starting on 7th day during a twelve-day exposure to water alone (as control: CTR) or LAS at 100 or 300 µg/l. Each column represents mean ± SD (n = 3 or 4). * = Significantly different from controls at $p < 0.05$.

Table 3.3 - Tissue concentrations of ^{3}H-BaP equivalents in catfish following daily dietary administrations of ^{3}H-BaP at 40 µg/kg body weight for six days starting on 7th day during a twelve-day exposure to water alone (as control: CTR) or LAS at 100 or 300 µg/l.

	^{3}H-BaP Equivalent Concentrations (ng/g)		
Tissues	**CTR**	**LAS 100 µg/l**	**LAS 300 µg/l**
Blood	8.44 ± 0.98	* 14.75 ± 1.91	* 26.34 ± 1.33
Bile	1040.41 ± 155.96	* 1779.63 ± 291.68	* 1970.91 ± 147.24
Liver	92.25 ± 4.33	* 150.86 ± 10.92	* 349.68 ± 29.11
Spleen	7.94 ± 2.04	11.98 ± 3.50	* 18.51 ± 1.66
Kidney	38.03 ± 2.01	* 64.69 ± 14.88	* 162.71 ± 38.37
Heart	8.58 ± 0.68	* 16.10 ± 4.61	* 18.43 ± 1.55
Lipids	9.87 ± 2.53	14.75 ± 3.10	* 61.48 ± 12.24
Brain	5.10 ± 1.12	* 10.02 ± 2.08	* 20.02 ± 4.81
Gill	14.35 ± 1.79	30.98 ± 11.22	* 36.04 ± 8.55
Intestine	965.60 ± 62.97	1228.17 ± 186.37	* 1667.35 ± 363.2
Stomach	69.84 ± 33.06	* 203.28 ± 27.94	* 222.31 ± 73.98
Muscle	2.66 ± 0.46	4.07 ± 1.08	* 8.05 ± 0.80
Eggs	3.68 ± 0.44	* 7.11 ± 1.97	* 12.16 ± 0.29

† Values are expressed as ng ^{3}H-BaP equivalents/g and data represents mean ± SD. of three or four measurements.
* =Significantly different from controls ($p<0.05$).

column due to limited degradation under anaerobic conditions (Ying, 2006). A range of 37 to 500,000 μg LAS /kg sediment (dry weight) have been reported (Bester et al., 2001; International Program on Chemical Safety, 1996; Jensen, 1999).

Several early studies have suggested that surfactants enhance the bioavailability and alter the disposition of other compounds in fish (Mann, 1962; Solon, et al., 1969; Pärt et al., 1985). More up to date, investigations in the cancer treatment field have indicated that surfactants have the potential to override the transporter mediated multidrug resistance phenomenon exhibited by cancer cells (Orlowski et al., 1998; Bogman et al., 2003; Regev et al., 1999; Drori et al., 1995; Hendrich and Michalak, 2003). The efflux action of such transporters in the intestine is now known to be an important modulator of bioavailability for a variety of orally administered drugs (Chan et al., 2004). In addition, the excipient actions of surfactants have been shown to increase drug bioavailability from the intestinal tract (Rege et al., 2002; Hugger et al., 2003). Collectively, these effects of surfactants have been reported to occur as a result of specific transporter inhibition (Rege et al., 2002; Orlowski et al., 1998; Bogman et al., 2003), membrane fluidization (Regev et al., 1999; Hugger et al., 2003), compound solubilization (Strickley, 2004) and cellular reentrance of effluxed compounds due to general increases in bidirectional membrane permeability (Drori et al., 1995; Hendrich and Michalak, 2003). The current study demonstrates that LAS inhibits the movement of Rho-123 into bile in the *in situ* prepared isolated liver preparation and increases at environmentally relevant concentrations the *in vivo* bioaccumulation of dietary ^{3}H-IVM and ^{3}H-BaP molar equivalents (parent and metabolites) in catfish. These latter data suggest that LAS at low concentrations, for which little overt toxicity is evident, may alter the exposure and bioaccumulation of other potentially hazardous chemicals to which fish may be co-exposed. Such mixture

interactions may augment exposure risk to not only the fish themselves, but also humans consuming those contaminated fish that may have higher than expected body burdens.

LAS treatment resulted in a concentration-dependent inhibition of Rho-123 movement into bile of the *in situ* perfused liver (Fig. 3.1) and increased retention of Rho-123 in liver tissues (Table 3.1). This increased compound retention in hepatic tissues was associated primarily with increases in parent compound and a significant, but small decline in formed metabolites. This latter finding is indicative of a limited, but measurable interaction of LAS with some facet of the biotransformation process. The nature of the interaction is unknown, but is likely to be associated with a membrane linked enzymatic system such as Cytochrome P450. Other studies have shown the effects of surfactants on biotransformation systems (Johnson et al., 2002; Batrakova et al., 2003 and 2004). Importantly, the increases in parent compound retention in the liver while not stochiometrically related to the decrease in metabolites formed appeared to be primarily related to total declines in compound content in the bile. Since the total amount of Rho-123 used and observed in the perfusion media were similar for all liver perfusion treatments, hepatic levels of Rho-123 increased with LAS treatment as would be expected with characteristic surfactant properties, and bile Rho-123 amounts dropped these results are most likely due to LAS effects upon permeability or transport function within the hepatocyte-biliary vectorial pathway rather than the amount of Rho-123 accessing the hepatocytes from the perfusion media.

Membrane permeability and Pgp transport function are interdependent and for all practical purposes inseparable. The functional vectorial transport of substrates by Pgp has been clearly shown to be dependent on the passive permeability of the membrane to the transported substrate (Eytan et al., 1996 and 1997). Compounds considered to be non-

transported modulators of Pgp activity have shown greater permeability and inward diffusability (Scala et al., 1997; Mülder et al., 1995). For these compounds, if transported by Pgp, movement is unrecognizable due to the membrane's inability to maintain the gradient and prevent compound back-diffusion (Barnes et al., 1996; Fert'e, 2000). Under normal physiological conditions, Rho-123, a cationic Pgp substrate, demonstrates restricted membrane movement and vectorial transport (Yumoto et al., 2001; Speelmans et al., 1994; Heywang et al., 1998; James et al., 1977). The decrease in Rho-123 movement into bile with LAS exposure may due to altered membrane permeability resulting from LAS-membrane interactions, by direct inhibition of Pgp transport or via alteration of the membrane environment around Pgp, therefore influencing transporter function. One of these alternatives or a combination at the apical hepatocyte bile interface may result in increasing accumulation of Rho-123 in liver and decreased biliary excretion.

Surfactants, as amphipathetic molecules, whose structures imitate that of glycerophospholipids, are able to insert themselves between lipophilic tails of membrane bilayers, resulting in disorder of the lipid-packing arrangement of membranes (Fert'e, 2000). The net result is the membrane becomes fluidized, facilitating the passive diffusion of compounds (Lo, 2003), and increasing permeability (Drori et al., 1995; Hendrich and Michalak, 2003). In the present Rho-123 perfusion experiment, LAS exposure resulted in increased hepatic membrane fluidity. The fluorescence anisotropy measurements demonstrated a 29.7% decrease at the 1 μM LAS concentration and modest additional changes in membrane fluidity (inversely related to anisotropy) for the higher 5 and 20 μM LAS concentrations (Fig. 3.2). These changes in membrane fluidity with the 1 mM LAS treatment are likely to contribute to the increases in Rho-123

permeability. Membrane fluidity changes, especially with the higher LAS concentrations, were not stoichiometrically consistent with the concentration-dependent effects on Rho-123 amounts transferred to the bile. The inconsistency here may be due to significant LAS dose dependent changes in Rho-123 permeability occurring over a small range of altered membrane fluidities once a critical point in gel /liquid phase transitions have been attained (Block et al., 1976). Currently, there is little evidence in the literature to support this concept. A second possibility is that the dose dependent decrease in Rho-123 movement into bile with LAS is not merely the result of changes in membrane permeability as associated with increases in fluidity. From this perspective it is likely that Pgp as the efflux transporter of Rho-123 may be at least partially inhibited by LAS. It has been postulated that Pgp-interacting compounds exhibit characteristic surface-active properties such as the capability to partition into a membrane and modify the surface tension (Schinkel et al., 1996; Seelig, 1998). Clearly surfactants have these abilities. In fact a number of studies have suggested that amphipathic surfactants including Tween 80, Triton X-100 and Solutol HS-15 may be substrates or modulators of Pgp (Coon et al., 1991; Zordan-Nudo et al., 1993). A likely conclusion for the present study is that LAS is competing with Rho-123 for transport. Although an attractive explanation, characterization of similar interactions for other classes of better-studied amphipathic lipophilic Pgp substrates and modulators have shown an array of relations including competitive, noncompetitive and mixed interactions. This mixed profile provides a great deal of uncertainty regarding their connection via a common transport site (Ayesh et al., 1996). To add to the uncertainty both substrates and modulators stimulate Pgp ATP utilization and displace photoligand binding on Pgp (Dayan et al., 1997; Beck and Qian, 1992; Liu and Sharom, 1996). A commonly cited hypothesis for these disparate results

suggests that Pgp has multiple sites, some independent of others, for compounds to interact with Pgp along its hydrophobic transmembrane domain (Ayesh et al., 1996; Boer et al., 1996). The actual mechanisms and interrelationships have yet to be defined.

In the present study, acute temperature changes were used as a LAS free means for membrane fluidization (Kleinow et al., 2006) and as a comparison for the effect of LAS induced fluidity on transport of Rho-123 into bile. Acute increases of the perfusion media and isolated liver preparation temperature [5 and 10 ^{o}C (actual temperatures of 23 ^{o}C and 28^{o}C respectively)] resulted in 42.8 and 49.7% decreases in fluorescence anisotropy (increases in fluidity) (Fig. 3.2). This increase in fluidity almost completely abolished transfer of Rho-123 into bile (Fig. 3.1). One interesting observation is that the temperature increases resulted in hepatic Rho-123 levels in the same range as the LAS treatments, however, caused 5.9-11.9 fold lower Rho-123 levels in the bile. These results suggest that the hepatocyte to bile component of the pathway is preferentially affected and that greater fluidity may produce further declines in Rho-123 transport into the bile. Such findings are consistent with previous studies, which have found that acute incremental elevations in temperature increased membrane fluidity (Robertson and Hazel et al., 1999) along with intestinal membrane Rho-123 permeability and movement (Kleinow et al., 2006).

It is useful to recognize that the anisotropy and hence fluidity measurements as reported here are composite readings associated with the whole isolated hepatocyte plasma membrane. As with other species, differences in membrane composition and fluidity exist for regional domains of the plasma membrane (Schachter and Shinitzky, 1977) and with different cell types (Shinitzky and Inbar, 1976; Schulthess and Hauser, 1995; Crockett and Hazel, 1995). Specific domains, which are more influential in

regionalized Rho-123 movement are possible. Larger changes in membrane fluidity for specific domains with surfactants have been reported for fish (Zehmer and Hazel, 2003).

Regardless of the mechanism of interaction it is of interest to note that even with the declines in bile levels of Rho-123 with LAS treatment, the bile concentrations when normalized on a volume to a weight basis with the liver and media, were much higher than both the liver and Rho-123 perfusion media concentrations suggesting that an active process was still operative (Table 3.1). Together with the thermal results these studies indicate that LAS and possibly other surfactants have the potential for greater fluidity effects on transport and highlight a potential synergy with acute temperature change.

In the current study, LAS treatment increased the organ retention of all three compounds examined. For Rho-123 this was evaluated only in the liver while for the *in vivo* ^{3}H-BaP and ^{3}H-IVM experiments this finding was evident for all organs in the mass balance assessments. The increases in the compound concentration followed a dose response relationship with LAS exposure. This effect was generally greater for ^{3}H-BaP than ^{3}H-IVM or Rho-123 at comparable LAS concentrations. In the liver, increases in molar equivalent residues with LAS treatment over controls represented approximately 14.4% for Rho-123, 18.1-20.0% (Concentration: 28.4-62.6%) for ^{3}H-IVM and 71.6-321.0% (concentration: 63.5-279.1%) for ^{3}H-BaP. A similar phenomenon was observed for other organs in the *in vivo* studies for ^{3}H-IVM and ^{3}H-BaP. LAS increased ^{3}H-IVM and ^{3}H-BaP whole body molar equivalents 39.0% and 77.9% and 50 and 157%, respectively, for the 100 and 300 μg/l exposures. After considering organ weight and fluid volumes, muscle, eggs, and blood contributed disproportionably more to retention of ^{3}H-IVM equivalents. For ^{3}H-BaP equivalents, similar findings were evident for the intestine, muscle, and liver. These single point mass balance studies suggest that LAS

exposure at environmentally relevant concentrations result in altered residue amounts due to changes in disposition ultimately affecting toxicokinetics. What is particularly significant to note is that this effect was evident for all of these compounds of varying chemical characteristics, but the effects were greatest for a lipophilic compound seldom associated with Pgp transport.

Different allocation patterns for Rho-123, ^{3}H-IVM and ^{3}H-BaP equivalents among the blood, liver and bile components of the hepatobiliary vectorial pathway suggest that LAS interactions may differ qualitatively or quantitatively with different compounds. LAS treatment decreased Rho-123 derived concentrations in bile, increased Rho-123 levels in the liver and maintained concentrations in media at near constant levels. Due to the chemical and transport characteristics of this compound these findings are likely to be due to the effects of LAS on Pgp transport function and / or permeability as described above. Increases in liver levels are likely to be related to reduction of Rho-123 transport to the bile. On the contrary, upon LAS exposure, ^{3}H-IVM molar equivalents exhibited little change in bile levels and significant increases in both the blood and liver ^{3}H-IVM molar equivalent concentrations. The high bile to blood ratio of ^{3}H-IVM equivalents for the control is indicative of an active concentrating process. What is of interest is that the bile to blood ratio declined with exposure to both the 100 and 300 LAS µg/l concentrations. This was a result of a very small increase in ^{3}H-IVM equivalents in the bile in face of significant increases in blood concentrations. A similar, but less definitive finding (due to a moderate increase in liver IVM) was evident for liver / blood ratios. This suggests that LAS influences ^{3}H-IVM movement at the apical hepatocyte membrane and possibly the basal membrane. In addition, the significantly elevated blood levels suggest that LAS increased intestinal bioavailability, possibly

through excipient action or inhibition of Pgp in the intestine. LAS treatments lead to increasing levels of ^{3}H-BaP molar equivalents in blood and major organs including liver, kidney, heart, eggs and stomach. A similar high bile to blood ratio was evident for both controls and the 100 µg/l LAS exposure suggesting that an active secretion of ^{3}H-BaP equivalents into bile was evident. This could be contrasted with much lower liver/blood ratios. Such results strongly suggest while some compound equivalent preference occurred for the liver over blood it was much less pronounced than similar comparisons for bile. These results suggest that the major active concentration process was likely to occur on the apical canalicular interface. Although liver, bile and blood concentration increased with the increased 300 µg/l LAS exposure concentration (Fig. 3.3) the relative increase in bile concentrations were much smaller than the nearly proportional ^{3}H-BaP equivalent concentration increases in liver and blood for control, 100 µg/l LAS and 300 µg/l LAS exposures. The net result was a significant decline in the bile/blood ratio at 300 µg/l LAS. A number of features may be operative. The increases in ^{3}H-BaP equivalents for all components (bile, liver, blood) of the vectorial pathway at 100 µg/l LAS as well as maintenance of bile/blood and bile/blood ratios as seen for controls suggest that the 100 µg/l LAS exposure increased intestinal bioavailability, or the total ^{3}H-BaP equivalent amount in the pathway. At the 300 µg/l LAS concentration, the bile concentration increases modestly (89.4%) in comparison to blood (212.1%) and liver (279.1%). The net result is a significant decline in bile/blood ratios. What may cause this is unknown, but it is likely the higher LAS exposure not only facilitated greater intestinal permeability, but also curtailed effective biliary transport. The latter may be evident through direct transporter inhibition or an increase in apical permeability allowing compounds to back

diffuse to the hepatocyte. Both processes result in accumulation of ^{3}H-BaP equivalents in liver, other organs and blood.

At the intestine greater compound solubility, greater mucosal permeability or inhibition of retrograde ABC transporter efflux action, all potential mechanisms of the surfactant LAS, would contribute to greater xenobiotic availability following a downhill gradient from the intestinal lumen to the blood. On the elimination side enhanced permeability of the bile canniculi or proximal tubular membrane or inhibition of efflux transport action to the bile or urine would have the opposite effect. Less compound equivalents would be available for elimination as transporter inhibition would reduce the compound concentrating effect in bile or urine and increased permeability would facilitate a down hill reentrance of compounds back into the tissues. The net result of interactions at either one or both locations would be the same, accumulation of xenobiotics or their metabolites in the fish. The significance of these effects is dependent on a number of potential determinants including chemical characteristics, surfactant interactions with the membranes or transporters as well as the operative concentration gradients both at the intestine and elimination portals. The latter are likely to change with exposure history and even with the course of the elimination and digestive processes. The results presented for each Rho-123, ^{3}H-IVM or ^{3}H-BaP are consistent with varying levels of interplay between the effects of LAS on membrane permeability and transporter action at both the site of compound uptake (*in vivo*-intestinal bioavailability) and elimination. Compound molecular weight, charge, lipid solubility and perhaps the transporter involved in compound movement are likely compound specific features altering permeability or transport. For Rho-123 studies that increased intestinal bioavailability was not available for interaction because of the *in situ* experimental design. This

precludes direct comparison with *in vivo* studies, but also isolates the effect of LAS largely to the hepatocyte-bile vectorial pathway of elimination.

Since membranes are amphipathic with both polar interfacial areas and a hydrophobic core, compounds transversing the plasma membrane or accessing Pgp in the inner leaflet must partition in the polar interfacial region of the membrane bilayer, partition and diffuse through the hydrophobic membrane core and desorb from the opposite interfacial region of the membrane. This process does not appear to be determined solely by compound lipophicity, but appears to be influenced by other compound-membrane interactions in the various regions of the membrane such as electrostatic interactions, charge delocalization or screening, affinity for anionic lipids, dielectric constant and surface potential of membrane interface (Speelmans et al., 1994; James et al., 1977; Madden and Redelmeier, 1994; Webb et al., 1995; Mankhetkorn and Garnier-Suillerot, 1998; Fernández and Fromherz, 1977; Flewelling and Hubbell, 1986). Of the compounds examined, Rho-123 (mw: 469 g/mol), a moderately lipophilic cation, and IVM (mw: 875 g/mol), a moderately lipophilic neutral compound, have been shown to be substrates of Pgp (Yumoto et al., 2001; Schinkel et al., 1994). BaP (mw: 252 g/mol), a more lipophilic neutral compound, is readily metabolized to a variety of anionic conjugates with characteristics consistent with Mrp substrates (Srivastava et al., 2002). For Pgp substrates which are lipophlic cationic compounds, such as Rho-123, high affinity for polar interfacial sites resulting from electrostatic interactions oppose movement to the opposite membrane leaflet. However, once compounds are in the lipophilic core the charge of the cation is delocalized or screened by lipophilic moieties allowing movement through the core. This initial restriction on the passive permeation process allows Rho-123 to be transported out of the cell of reducing accumulation. For

weak lipophilic bases the protonated form has high affinity for anionic lipids and the charge impedes diffusion. Neutral forms favored at lower pKa diffuses move rapidly. Proportions of the charged and uncharged forms at the membrane surface depend on pH and ionic compositions of aqueous phase and the dielectric constant and surface potential of membrane interface. In contrast lipophlic anions such as BaP conjugates diffuse more rapidly than similar featured cations, due to dipolar potential, have lower affinity for membranes and accumulate independent of Pgp activity.

Compounds that exhibit some degree of lipophilicity and capability to cross membranes also display energetically unfavorable interactions with water in the aquatic environment. Water promotes partitioning of these types of compounds into organic sediments and living organisms including primary producers and consumers at the assorted trophic levels. Research has indicated that a primary means that these contaminants enter fish is via dietary transfer and for certain contaminants biomagnification along the aquatic food chain (Dietz et al., 2000). Factors that influence the efficiency and the amount of contaminants passed through food include the type and length of the food chain (Rasmussen et al., 1990; Broman et al., 1992; Cabana and Rasmussen, 1994), existing body burdens (Doi et al., 2000; Barber et al., 1991), the lipophilicity and size of the compound, exposure concentrations, bioavailability, biotransformation (Ivanciuc et al., 2006) and physical features such as temperature (Gewurtz et al., 2006). Fish represent a significant portal by which contaminants enter into the human diet. On a global and per capita basis fish are consumed in increasingly greater quantities (Hastein et al., 2006). As a high protein foodstuff consumption of fish has been publicized as providing healthful effects in preventing cardiovascular disease (Mori and Woodman, 2006; Breslow 2006), strokes (Bouzan et al., 2005), macular

degeneration (Seddon et al., 2006), dementia (Huang et al., 2005) as well as a variety of other disorders. For certain classes of contaminants such as organochlorines and polyaromatic hydrocarbons, consumption of fish has been estimated to account for as much as 65-90% of the existing human body burden (Fürst et al., 1992; Svensson et al., 1995; Hattemer-Frey and Travis, 1991). Consistent with these findings correlations have been made between consumption patterns of contaminated fish and health effects including developmental and educational deficits in children (Mendola et al., 2005; Vartiainen et al., 1998; Faroon et al., 2001).

Risk evaluation of chemicals in the environment must take in account the compound's toxicity, persistence and bioaccumulation. For many environmental chemicals complete information is rare especially that regarding bioaccumulation in fish. In 2004, the UN established a directive requiring that information regarding compound bioaccumulation in fish must be available for each of the chemicals in production. The goal of this process is to identify compounds, which pose undue risk because of their bioaccumulation properties. Existing partitioning techniques using physicochemical characteristics such as lipophilicity, have identified compounds with the potential to bioaccumulate (Voutsas et al., 2002), however, these methods do not identify compounds for which bioaccumulation is significantly reduced by biotransformation. Due to the thousands of compounds yet unevaluated and the potential restrictions placed upon targeted compounds, efforts are underway to develop *in vitro* and *in silico* techniques to accurately and cost-effectively predict bioaccumulation in light of a compound's potential for biotransformation. Conversely, factors that enhance bioaccumulation have not received much attention probably due to the complexity and unknown importance of the issue.

The abundant and widespread use of surfactants presents the opportunity for potential interactions with the mechanisms of disposition and toxicity for a multitude of other compounds in the environment. Degradation of surfactants discharged from municipal or industrial sources, is dependent on the time maintained under aerobic conditions (Kimerle et al., 1977; Kimerle, 1989; Karsa and Porter, 1995). This degradation process is significantly reduced for shorter intervals and under anaerobic conditions (Federle and Schwab, 1992; Prats-a et al., 2000). Anaerobic sediments often act as repository for elevated surfactant concentrations and many other contaminants (Westall et al., 1999; Federle and Schwab, 1992), support a variety of food chain organisms and provide a food chain exposure route from the contained contaminant mixture to the aforementioned organisms (Reynoldson, 1987; Casellato et al., 1992). Surfactants also enter the environment by actions associated with remediation of hazardous substances. Surfactants may be used in remediation of contaminated soils to facilitate compound recovery (Burchfield et al., 1994, Baran et al., 1998) as well as for aquatic based oil spills to disperse coalesced oil (Edwards et al., 2003; Harvey et al., 1990). Such procedures can potentially bring significant amounts of surfactants, toxic compounds and components of the aquatic and human food chain together (Lewis, 1992). The current studies suggest that through alteration of permeability and transporter function, LAS may enhance bioavailability, alter distribution and /or limit dispositional processes so to increase bioaccumulation of mixture components. The possibility exists that surfactants will not only increase the levels of contaminants in fish, but also by so doing increase the subsequent exposure of humans.

In conclusion, these results suggest that LAS may influence the bioaccumulation of xenobiotics from environmental mixtures. LAS at environmentally relevant exposure

concentrations enhanced both oral bioavailability and decreased compound disposition over the time frame and sampling regimes examined. At this time it is unknown if the effects observed are representative, however, these results do occur for several compounds with different characteristics. Responses were not confined to known Pgp substrates. While these compounds all exhibit some degree of lipophilicity, it is uncertain what compound properties promote or conceivably enhance the effects observed. Initial indications suggest that variation in findings between compounds may result from differing contributions of altered permeability and transporter function both at the site of absorption and elimination. However, the general response elicited for all compounds suggests that basic mechanisms, perhaps at the membrane may be involved. From the animal perspective, it is also of interest that waterborne LAS exposure influences uptake and disposition of dietary administered compounds. At the environmental level it is unknown if such a response is more pronounced for certain organisms such as those associated with the benthos or if the response will be seen or even magnified at each level of the food chain. Full-scale bioaccumulation studies will be necessary to better evaluate the effects of LAS on this process. These investigations will define the environmental and toxicological relevancy of these mixture interactions.

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CHAPTER 4: SUMMARY AND CONCLUSIONS

Fish in impacted environments are exposed a great number of contaminants as well as pharmaceutical agents as runoff from agriculture and used in aquaculture. The extent of systemic toxicity and biochemical effects of these compounds to fish and the fish consuming public is dependent on the uptake, bioavailability and bioaccumulation of these chemicals in fish. Therefore, understanding factors that influence bioavailability and bioaccumulation of these chemicals from the environment becomes essential in the determination of the effects of these compounds to fish and human health. Membrane permeability is a key determinant of absorption, distribution and elimination of chemicals in the body (Ayrton and Morgan, 2001). Recently, studies in mammals suggest the active membrane efflux mechanism of the ABC transporters P-glycoprotein (Pgp) and multidrug resistance related protein (Mrp) may limit absorption and facilitate elimination of drugs (Kurata et al ., 2002; Liu and Hu, 2000; Fromm, 2000). The studies presented here investigated the effect of the environmental contaminants, dieldrin, a persistant organochlorine pesticide, and linear alkylbenzene sulfonates (LAS), a surfactant, on Pgp transport and membrane permeability as related to the retention of two pharmaceutical drugs tetracycline and ivermectin (IVM) and the PAH and carcinogen benzo[a]pyrene (BaP). These compounds were selected based on indications of possible involvement with the mechanisms examined and environmental relevance.

Initial *in situ* experiments using isolated perfused liver preparations demonstrated that the antibiotic drug tetracycline decreased movement of the Pgp prototypic substrate Rhodamine-123 (Rho-123) into bile. This finding was consistent with tetracycline as a substrate as well as a competitive inhibitor of Pgp transport. Further, verapamil, a Pgp prototypic competitive inhibitor, resulted in a significant decrease in biliary excretion of

^{3}H-tetracycline, providing further evidence that Pgp transport plays a role in the hepatic disposition and bioavailability of tetracycline. Biliary excretion is a key pathway for elimination of tetracycline from the body (Plakas et al., 1988). Finally, the marked inhibitory effects of dieldrin on biliary excretion of Rho-123 and ^{3}H-tetracycline were demonstrated. Dieldrin at 20 μM inhibited ^{3}H-tetracycline transport to a greater extent than verapamil. These results suggested that dieldrin acted as a more potent inhibitor of Pgp than verapamil.

In contrast to the inhibitory effects of dieldrin on transport of ^{3}H-tetracycline into bile in isolated perfused livers, a chronic low-dose pretreatment of dieldrin (0.1 mg/kg body weight per day for 4 weeks) resulted in an increase in the plasma clearance as well as a decrease in tissue distributions and AUC_{0-96h} for administration of a subsequent single intra-aorta dose of ^{3}H-tetracycline in catfish. These alterations were correlated reasonably well with an increase of Pgp expression in the liver tissues in dieldrin pretreated fish, which facilitated the clearance and elimination of ^{3}H-tetracycline. These findings indicated that Pgp induction here may well be the most important factor for these alterations. Besides Pgp induction, induced metabolized enzymes may be involved to alter disposition and pharmacokinetics of tetracycline in fish. However, previous studies have shown that dieldrin-induced biotransformation enzymes were not observed in fish (Vodicnik et al., 1981) and tetracycline has been shown to undergo minimal metabolism in animals (Aronson, 1980). Therefore, these studies support the concept that Pgp induction contributed to these alterations of the *in vivo* disposition and pharmacokinetics of ^{3}H-tetracycline.

Additional *in situ* studies examined the effect of the surfactant C18 LAS (1, 5, and 20 μM), including environmentally relevant concentrations, on the hepatobiliary

disposition of Rho-123 in isolated perfused livers. These studies demonstrated a concentration-dependent inhibition of Rho-123 movement into bile and increased retention of Rho-123 in liver tissues. The increased hepatic retention of Rho-123 was associated primarily with an increase in parent compound and a small decline in formed metabolites. The latter finding suggested that a limited, but measurable inhibition of biotransformation may occur. The increases in the parent compound in liver tissues appear to be related to the declines in compound content in bile. LAS treatment at the 1 μM concentration resulted in a 29.7% decrease in fluorescence anisotropy (increased membrane fluidity) in the corresponding hepatic membranes, and modest additional changes at higher LAS treatments, which were not stoichiometrically related to changes in Rho-123 movement into bile. The inconsistency suggested that effects of LAS on Rho-123 movement into bile are not merely the result of changes in membrane permeability as associated with increases in fluidity. It is likely that Pgp may be at least partially inhibited by LAS treatments.

Sequential *in vivo* studies demonstrated that LAS at environmentally relevant concentrations (100 and 300 μg/L) resulted in elevated ^{3}H-ivermectin (IVM) and ^{3}H-benzopyrene (BaP) molar equivalents remaining in catfish in a dose-response fashion. Concentrations of ^{3}H-IVM and ^{3}H-BaP equivalents in blood and major organs following dietary administration increased with increasing LAS concentrations in the water. After considering organ weight and fluid volumes, muscle, eggs and blood contributed disproportionably more to increased retention of ^{3}H-IVM equivalents in catfish, while for ^{3}H-BaP equivalents, similar findings were evident for intestine, muscle and liver. In addition, different allocation patterns for ^{3}H-IVM and ^{3}H-BaP equivalents among the blood, liver and bile components of the hepatobiliary vectorial pathway were observed,

suggesting that LAS interactions may differ qualitatively or quantitatively with different compounds. These results were consistent with varying levels of interplay between the effects of LAS on membrane permeability and transporter action at both sites of compound uptake in intestine and elimination such as the bile. Compound molecular weight, charge, lipid solubility and perhaps the transporter involved in compound movement are likely compound specific features altering permeability or transport.

In summary, these studies have shown that ABC transporters and/or membrane permeability play a critical role in disposition and bioaccumulation of xenobiotics in fish. Environmental contaminants may influence bioaccumulation and bioavailability of other compounds in environmental mixtures through different interactions with transporters and/or the plasma membrane. At the high concentrations, dieldrin appears to be a potent Pgp inhibitor, which decreased biliary elimination of Pgp substrates Rho-123 and ^{3}H-tetracycline. In contrast, inductive effects of Pgp predominated at the low dose of dieldrin with chronic exposure, which led to increased plasma clearance and decreased tissue distributions of ^{3}H-tetracycline. Studies with surfactants suggest that LAS at environmentally relevant concentrations altered membrane permeability and the efflux activity of Pgp in the isolated perfused liver and enhanced the oral bioaccumulation and curtailed compound elimination in catfish. These effects occurred for a variety of compounds with different characteristics. The importance of these later studies is that apparently innocuous compounds such as surfactants may enhance the bioaccumulation of more hazardous environmental contaminants in fish and hence in the human diet.

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VITA

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