MOLECULAR MECHANISM OF POLYBROMINATED DIPHENYL ETHER DISPOSITION IN THE LIVER

by

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Submitted to the graduate degree program in the Department of Pharmacology, Toxicology and Therapeutics and the Graduate Faculty of the University of Kansas Medical Center in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Dedication

I would like to dedicate the work herein to my wife.

Acknowledgements

This work was supported by NIH Grants RR021940, DK081343. I would like to give my thanks to the taxpayers of the United States whose tax revenues made my dissertation research possible.

I would like to thank my mentor, Dr. Grace L. Guo. I take great pride in being your first graduate student and have immensely enjoyed the five years working in your lab. You are a great mentor and I have enjoyed working with you and watching your growth as a mentor and researcher. As your student, I have always been impressed by your great ideas and your high standards in science. I appreciate your willingness to aid in my advancement not only as a scientist, but as a person as well. Thank you for all your time, your patience, your encouragement and your guidance.

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I also want to thank Stacie's family: Judy, John W., John P., Michelle, Kylie, and Tanner. You are my family and thank you for your love and prayers.

Last but not least, to my wife Stacie: Thank you, love. You are my best friend and have had unwavering support for me. For this I am forever grateful to you. I love you.

Abstract

Polybrominated diphenyl ethers (PBDEs) were introduced in the late 1970's as additive flame retardants incorporated into textiles, electronics, plastics and furniture. Although 2,2',3,3',4,4',5,5',6,6'-decabromodiphenyl ether (BDE209) is the only congener currently on the market, 2,2`,4,4`-tetrabromodiphenyl ether (BDE47), 2,2`,4,4`,5-pentabromodiphenyl ether (BDE99), and 2,2`,4,4`,5,5`-hexabromodiphenyl ether (BDE153) are the predominant congeners detected in human and wildlife samples. Upon exposure, PBDEs enter the liver where they are biotransformed to potentially toxic metabolites. Although the human liver burden of PBDEs is not clear, the presence of PBDEs in human liver is particularly alarming because it has been demonstrated in rodents that hydroxylated metabolites may play a pivotal role in PBDE-mediated toxicity. The mechanism by which PBDEs enter the liver was not known. However, due to their large molecular weights (MWs ~485 to 1000 Da), they were not likely to enter hepatocytes by simple diffusion. Organic anion transporting polypeptides (OATPs: human; Oatps: rodents) are responsible for hepatic uptake of a variety of amphipathic compounds of MWs larger than 350 Da. Therefore, I tested the hypothesis that OATPs/Oatps expressed in human and mouse hepatocytes are responsible for the uptake of PBDE congeners 47, 99, and 153 by using Chinese hamster ovary (CHO) cell lines expressing OATP1B1, OATP1B3, or OATP2B1 and Human Embryonic Kidney 293 (HEK293) cells transiently expressing Oatp1a1, Oatp1a4, Oatp1b2, or Oatp2b1. Direct uptake studies illustrated that

PBDE congeners are substrates of human and mouse hepatic OATPs/Oatps, except for Oatp1a1. Detailed kinetic analysis revealed that OATP1B1, OATP1B3, Oatp1a4, and Oatp1b2 transport BDE47 with the highest affinity followed by BDE99 and BDE153. However, both OATP2B1 and Oatp2b1 transported all three congeners with similar affinities. The importance of hepatic Oatps for the accumulation of BDE47 in liver was confirmed using Oatp1a4- and Oatp1b2-null mice. These results clearly suggest that uptake of PBDEs via these OATPs/Oatps are responsible for liver-specific accumulation of PBDEs. In mouse liver, PBDEs induce drug metabolizing enzymes, namely cytochrome P450s (Cyps). However, the molecular mechanisms underlying this induction was unknown. Cyp2b10 and 3a11 are target genes of the xenobiotic nuclear receptors, the constitutive androstane receptor (CAR) and pregnane X receptor (PXR), both of which are responsible for mediating induction of Cyp2b10 and Cyp3a11, respectively. I hypothesized that PBDE congeners are CAR and/or PXR activators. Using reporter-gene luciferase assays I showed that BDE47, BDE99 and BDE209 activate human and mouse CAR and PXR in a concentration-dependent manner. Furthermore, induction of Cyp2b10 and Cyp3a11 was markedly suppressed in CAR- and PXR-null mice, respectively, indicating that PBDE congeners activate these receptors *in vivo*. BDE47 and BDE99, the primary congeners detected in humans in the United States, are capable of inducing Cyp2b and Cyp3a enzymes in rodents. However, it is not clear which Cyp isoform, if any, is preferentially induced upon exposure to BDE47 or BDE99. Induction of mouse hepatic Cyp2b10 and 3a11 by PBDEs

showed distinct dose-responses, with Cyp2b10 being induced at lower doses and Cyp3a11 at much higher doses, indicating PBDEs are more likely to induce hepatic enzymes at doses that humans are exposured to. Currently, daily exposure of PBDEs is estimated to be 0.003mg/kg for adults. This study shows that effects of PBDEs are seen in animal models at concentrations within ~10-fold of the high end of the human population. Together, the results from the current dissertational study demonstrate that PBDEs are substrates of OATPs/Oatps and activators of CAR and PXR. This study not only provides a molecular basis for understanding PBDE disposition and toxicity in the liver but also cautions PBDE exposure may result in broader impact on liver physiology and toxicology.

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List of Abbreviations

AF-1	**Activation Functions 1**
AF-2	**Activation Functions 2**
AhR	**aryl hydrocarbon receptor**
BDE209	**2,2',3,3',4,4',5,5',6,6'-decabromodiphenyl ether**
BDE99	**2,2`,4,4`,5-pentabromodiphenyl ether**
BDE153	**2,2`,4,4`,5,5`-hexabromodiphenyl ether**
BDE47	**2,2',4,4'-tetrabromodiphenyl ether**
bDNA	**branched DNA**
CAR	**constitutive androstane receptor**
CHO	**Chinese hamster ovary**
CYPs	**cytochrome P450 enzymes**
DBD	**DNA binding domain**
FXR	**farnesoid X receptor**
LBD	**ligand binding domain**
LXR	**liver X receptor**
HEK293	**human embryonic kidney 293**
ip	**intraperitoneal**
K_m	**Michaelis-Menten constant**
mRNA	**messenger RNA**
MWs	**molecular weights**
NRs	**nuclear receptors**
OATPs/Oatps	**organic anion transporting polypeptides**

OH-PBDEs	**hydroxylated polybrominated diphenyl ethers**
PB	**phenobarbital**
PBB	**polybrominated biphenyls**
PBDEs	**polybrominated diphenyl ethers**
PCBs	**polychlorinated biphenyls**
PCN	**pregnenolone-16α-carbonitrile**
PPAR	**peroxisome proliferator-activated receptor**
PXR	**pregnane X receptor**
PKC	**protein kinase C**
RXR	**retinoid X receptor**
SXR	**steroid X receptor**
TCPOBOP	**(1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene**
TM	**transmembrane**
TTR	**transthyretin**
T_4	**thyroxin**
T_3	**triiodothyronine**
V_{max}	**maximal transport rate**
WT	**wild type**

Chapter 1

Background and Significance

I - Introduction to polybrominated diphenyl ethers

During the twentieth century, manufacturers began to replace traditional materials such as wood, metal, and wool with petroleum-derived products such as plastics and polyurethane foam. The new materials are more flammable and, once alight, combust more rapidly, allowing people less time to escape. Fires are a leading cause of death among children in the U.S. Each year, more than 600 children ages 14 and under die, and nearly 47,000 are injured in fires (http://www.usa.safekids.org). Strict U.S. fire safety regulations may be a reason that flame-retardants are used more here than in other countries. PBDEs are part of a class of brominated flame retardants, and because they are persistent and bioaccumulate, they have emerged as a major environmental pollutant. PBDEs decompose at a temperature that is approximately 50°C below that of the material that they are serving to protect by liberating bromine atoms (www.ebfrip.org). These bromine atoms are very effective reducing agents and free radical inhibitors and thus attenuate the fire or the spreading of fire by diminishing the oxygen supply.

PBDEs are flame-retardants used as additives in polymers incorporated into textiles, electronics, plastics, and furniture. The frequent use of PBDEs is

attributed to their low manufacturing cost as well as their high degree of resistance to degradation by environmental and biological systems (Darnerud et al., 2001). BDE47 is the predominant congener detected in human and wildlife samples, followed by BDE99 and BDE153. BDE-209 is the predominant

congener in the only available commercial mixture available today (Figure 1-1) (Darnerud et al., 2001; Lorber, 2008).

A. Manufacturing

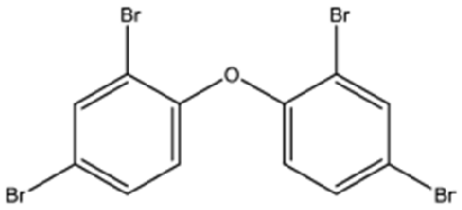
PBDEs are manufactured by the bromination of diphenyl oxide resulting in a mixture of various tetra-, penta-, hexa-, hepta-, octa- and deca- congeners in varying percentages (Darnerud et al., 2001) (Table1-1). PBDEs have been marketed in three primary formulations, the penta formulation, commercially known as DE-71 and Bromkal 70–5DE, the octa formulation F DE-79, and the deca formulation F DE-83R or Saytex 102E. The formulations differ in their composition of BDE congeners. The penta formulation is dominated by penta congeners (50–62% by weight) with secondary contributions by tetra (24–38%) and hexa congeners (4–12%). The octa formulation is predominantly hepta (45%) and octa congeners (33%), with secondary contributions from hexa (12%) and nona (10%) congeners. The deca formulation is composed of essentially all BDE 209 (97–99%, with 1–3% other, mainly nona, congeners), which is the congener with all 10 bromine positions occupied.

Table 1-1. PBDE congener profile in 3 commercial mixtures

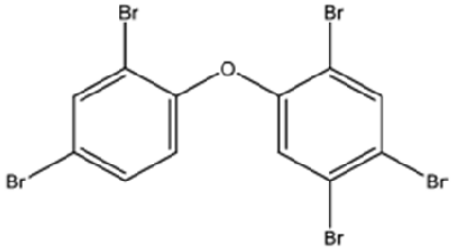
Congener Percentage								
	BDE28	BDE47	BDE99	BDE153	BDE183	BDE187	BDE206	BDE209
Penta-PBDE	<1	24-38	50-60	4-8				
Octa-PBDE				10-12	43-44	31-35	10-11	<1
Deca-PBDE							<3	97-98

Note: PBDEs have been marketed in three primary formulations, the penta formulation, commercially known as DE-71 and Bromkal 70–5DE, the octa formulation (DE-79), and the deca formulation (DE-83R or Saytex 102E).

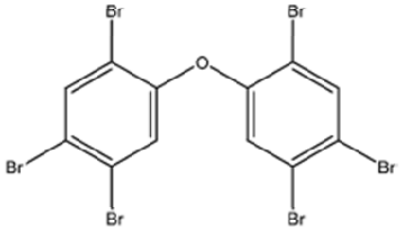
Figure 1-1



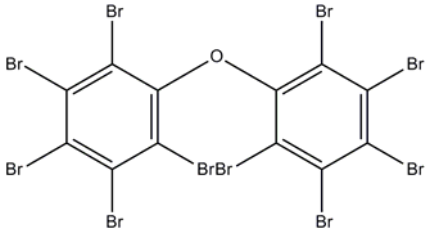
2,2',4,4'-tetrabromodiphenyl ether
(BDE47)



2,2',4,4',5-pentabromodiphenyl ether
(BDE99)



2,2',4,4',5,5'-hexabromodiphenyl ether
(BDE153)



2,2',3,3',4,4',5,5',6,6'-decabromodiphenyl ether
(BDE209)

Figure 1-1 Chemical structures of BDE47, BDE99, BDE153, and BDE209.

B. Exposure

The penta- and octa-PBDE mixtures are being voluntarily phased out in the United States. However, a large number of products containing these flame retardants are still in use. This means their release into the environment will continue throughout product lifecycle, possibly for several more decades. In humans, PBDEs are detected primarily in adipose, liver, kidney and blood. Human exposure to PBDEs is chronic and likely is through multiple routes of exposure.

Overall, European and Asian PBDE body burdens are approximately 2 orders of magnitudes less than levels detected in the United States (Petreas et al., 2003; Schecter et al., 2003; Hites et al., 2004; Johnson-Restrepo et al., 2005; Schecter et al., 2005b; Lorber, 2008). The main sources of PBDE exposure are the diet and the indoor environment. However, occupational exposure has also been documented. Among foods, fish, meats, and dairy products contain the highest concentrations of PBDEs. In the U.S., fish has the highest content of PBDEs, followed by meat and dairy products; however, given the food consumption patterns in this country, meat is estimated to be the major source of PBDEs from the diet (Schecter et al., 2003; Schecter et al., 2004; Schecter et al., 2005a; Schecter et al., 2006; Gomara et al., 2007). In Europe, fish is a major source of dietary exposure to PBDEs. Independent of the specific food source, exposure to PBDEs through the diet is only slightly higher in the U.S. than in Europe

(Schecter et al., 2006; Gomara et al., 2007). Thus, diet alone cannot fully explain the higher levels of PBDEs found in human tissues of children and adults in North America.

Several studies have indicated that a major source of exposure to PBDEs is by house dust. Household cats, exposed to PBDEs partially through the diet and through house dust, have been found to have serum levels of PBDEs that were 20-100 fold higher than the mean levels in U.S. adults, and have been suggested to serve as “sentinels” for indoor exposure to PBDEs (Dye et al., 2007). For toddlers in particular, dust has been estimated to be the primary route of exposure. A recent study indicated that children are exposed to 3-4 fold higher PBDE levels than adults, and that house dust accounts for >80% of exposure (Allen et al., 2007). Moreover, the highest serum levels of PBDEs are found in infants and toddlers, as a result of exposure through maternal milk and house dust (Fischer et al., 2006). In contrast to polychlorinated biphenyls (PCBs), whose concentration increases with age due to accumulation in adipose tissue, PBDE levels do not appear to increase with age and have been detected primarily in adipose, liver, kidney and blood (Darnerud et al., 2001; Sjodin et al., 2004; Schecter et al., 2005b; Sjodin et al., 2008b; Doucet et al., 2009).

Although PBDE toxicity in humans is not clear, the presence of PBDEs in human tissue is of concern because of potential toxic effects including endocrine

disruption, neurotoxicity, and carcinogenicity identified in rodents (Darnerud et al., 2001; Costa et al., 2008).

C. General toxicology and adverse health effects

Until recently, PBDEs were considered obscure members of the persistent organic pollutants family, including PCBs, polychlorinated dibenzodioxins and furans, and organochlorine pesticides (McDonald, 2002). There is an acceptable body of information on the general toxicology of PBDEs, but for several emerging end-points of toxicity, information is still limited. With chronic exposure, target organs for toxicity include liver, kidney and the thyroid gland. The toxicity of PBDEs, in general, decreases with increasing bromination. For example, in subchronic toxicity studies in rats, no observed effect levels are usually in the g/kg/day range for BDE209, but less than 10mg/kg/day for BDE99 (Darnerud et al., 2001; Darnerud, 2003).

Toxicokinetic studies in adult animals have indicated that absorption, metabolism and excretion of PBDEs are congener-, species- and gender-dependent (Darnerud et al., 2001; Hakk and Letcher, 2003). For example, lower brominated congeners are metabolized to mono- and di-hydroxylated metabolites (e.g. 6-OHBDE47), which may have toxicological relevance, and appear to bioaccumulate in serum, whereas BDE209 may be metabolized in the small intestine and liver to lower brominated congeners (Hakk and Letcher, 2003;

Sandholm et al., 2003; Athanasiadou et al., 2008; Doucet et al., 2009; Qiu et al., 2009). With respect to BDE47, male mice have a higher rate of urinary excretion compared to female mice. Young animals have a reduced ability to excrete PBDEs, which may contribute to a higher body burden (Staskal et al., 2005; Staskal et al., 2006b).

PBDEs have been shown to be involved in various adverse effects such as endocrine disruption, alteration of neurological functions, and increased incidence of liver tumors. In addition, exposure to PBDEs results in alterations of thyroid function. PBDEs have also been reported to decrease levels of total and free thyroxine (T_4) in adolescent and adult animals and following developmental exposure (Fowles et al., 1994; Hallgren et al., 2001; Zhou et al., 2001). One study demonstrated that decreased T_4 was associated with induction of uridine diphosphate glucuronyltransferase, a key phase II metabolizing enzyme involved in conjugation of T_4 leading to reduced serum levels of both free and total thyroxine.

Additionally, hydoxylated PBDEs (OH-PBDEs), because they are structurally similar to thyroid hormones, can compete for thyroid hormone receptors, and thus alter T_4 regulation of metabolism. OH-PBDEs, such as 4´-OH-1, 3, 3´, 5-BDE47 and 4´-OH-1, 3, 3´, 5, 5´-BDE99, can compete with triiodothyronine (T_3) and T_4, respectively, as well as disrupt the transport of thyroid hormone by displacing it from the thyroxine transport protein transthyretin (Xie et al.) (Meerts

et al., 2000; Zhou et al., 2001; Darnerud et al., 2007). Displacement of T_4 from TTR may lead to increased glucuronidation and a consequent lower level of T_4 (Hamers et al., 2006; Hamers et al., 2008).

A current potential for adverse health effects of PBDEs relates to their developmental neurotoxicity. Such concern is supported by the fact that infant and toddlers have the highest body burden of PBDEs, due to exposure via maternal milk and house dust (Costa and Giordano, 2007), and that a number of animal studies have provided indications of long lasting behavioral alterations, particularly in the domains of motor activity and cognitive functions, upon pre- and postnatal exposures to PBDEs. A series of studies have shown that exposure of neonatal mice and rats to various PBDEs (BDE47, BDE99, BDE153, BDE209) as a single oral dose, in most cases on PND 10, causes long-lasting changes in spontaneous behavior, mostly characterized as hyperactivity (decreased habituation), and disrupts performance in learning and memory tests (Eriksson et al., 2002; Viberg et al., 2003a; Viberg et al., 2003b; Viberg et al., 2006). In contrast with the large database on body burden (levels of PBDEs in serum, adipose tissue, breast milk), there is almost no information on possible developmental adverse effects in humans from PBDE exposure. In a study in Scandinavia, milk PBDE levels were associated with an increased incidence of cryptorchidism in newborn boys (Main et al., 2007).

Studies in rats have shown that BDE209 produced thyroid follicular cell hyperplasia as well as slightly increased incidences of follicular cell adenomas and carcinomas (NTP, 1986). There have been very few studies of thyroid hormone levels in humans exposed to PBDEs. One study found workers exposed to PBDEs and polybrominated biphenyls (PBBs) during manufacturing had a statistically significant increase in hypothyroidism (Darnerud, 2003). However, it cannot be determined if this was the result of exposure to PBDE, PBB, or the combination of both. PBBs also have been associated with decreased thyroid hormone levels in animal studies (Darnerud, 2003).

Only BDE209 has been tested for carcinogenicity in animals (NTP, 1986). Dosing of rats with BDE209 caused statistically significant increases in hepatocellular carcinomas (2/50, 8/50, and 15/49 for control, low dose, and high dose, respectively) and marginal increases in thyroid follicular cell carcinomas (2/50, 10/50, and 19/50 for control, low dose, and high dose, respectively) in male mice (NTP, 1986). Furthermore, dose-related increases in benign liver and pancreatic adenomas were discovered in the treated rats. Because of these studies, the International Agency for Research on Cancer has classified BDE209 as having limited evidence for a carcinogenic effect in animals, but stopped short of classifying the substance as carcinogenic in humans (IARC, 1991).

II - The roles of transporters in xenobiotic metabolism

A. Introduction to membrane transporters

The cell membrane functions as a semi-permeable barrier, controlling both intercellular and exchange of nutrients, ions, metabolites, signaling molecules and xenobiotics. However, membranes are not impenetrable walls. Nutrients must enter the cell and waste products must leave in order for the cell to survive. Thus, it is imperative that membranes be selectively permeable. Initial evidence showed that BDE47 accumulated in the liver before redistributing to other organs (Staskal et al., 2005). It is known that passive diffusion is not a probable event for chemicals with large MWs. Given that OATP substrates are, in general, amphipathic molecules with molecular weights (MWs) of more than 350 (Hagenbuch and Gui, 2008), hepatic OATPs/Oatps family members are promising candidate transport systems for hepatic uptake of BDE47 (MW: 485.5), BDE99 (MW: 564.7), and BDE153 (MW: 643.6).

B. Introduction to OATP/Oatps

The first Oatp cDNA to be cloned was Oatp1a1 from rat liver in 1994 (Jacquemin et al., 1994). Since then, numerous genes encoding OATPs/Oatps have been cloned from various tissues and species (Hagenbuch and Gui, 2008). These genes are classified within the solute carrier families *SLCO* (humans: *SLCO*; rodents: *Slco)* (Hagenbuch and Meier, 2003; Hagenbuch and Meier, 2004). OATPs/Oatps are a group of membrane transporters responsible for the sodium-independent transmembrane transport of a wide array of amphipathic substrates

(Hagenbuch and Gui, 2008; Kalliokoski and Niemi, 2009). OATPs/Oatps form a superfamily classified into 6 families. Oatps/OATPs within the same family share >40% amino acid sequence identities. Individual subfamilies contain amino acid homologies greater than 60% (Hagenbuch and Meier, 2003; Hagenbuch and Meier, 2004; Hagenbuch and Gui, 2008). A modified tree containing human and mouse members of the OATP1B and 2B family is shown in Figure 1-2.

As a superfamily, OATPs are detected in the liver, kidney, brain, and intestine, implying a critical role in drug disposition (Hagenbuch and Meier, 2003; Kalliokoski and Niemi, 2009). However, the focus of this dissertation will be on OATPs/Oatps expressed in human and murine hepatocytes. The OATP1A sub-family has a single human member OATP1A2 (*SLCO1A2)* and two mouse members, Oatp1a1 (*Slco1a1*) and Oatp1a4 (*Slco1a4*). In contrast to its murine orthologues, OATP1A2 expression in the liver is restricted to the epithelial cells of the bile duct (Lee et al., 2005). OATP1B1 (*SLCO1B1*) and OATP1B3 (*SLCO1B3*), two human members in the OATP1B subfamily, as well as Oatp1b2 (*Slco1b2*), the murine ortholog of both human OATP1B1 and OATP1B3, are expressed at the basolateral membrane of hepatocytes (Abe et al., 1999; Hsiang et al., 1999; Konig et al., 2000a; Konig et al., 2000b). The OATP2B subfamily has a single human and mouse member, OATP2B1 (*SLCO2B1*) and Oatp2b1 (*Slco2b1*), respectively.

Figure 1-2

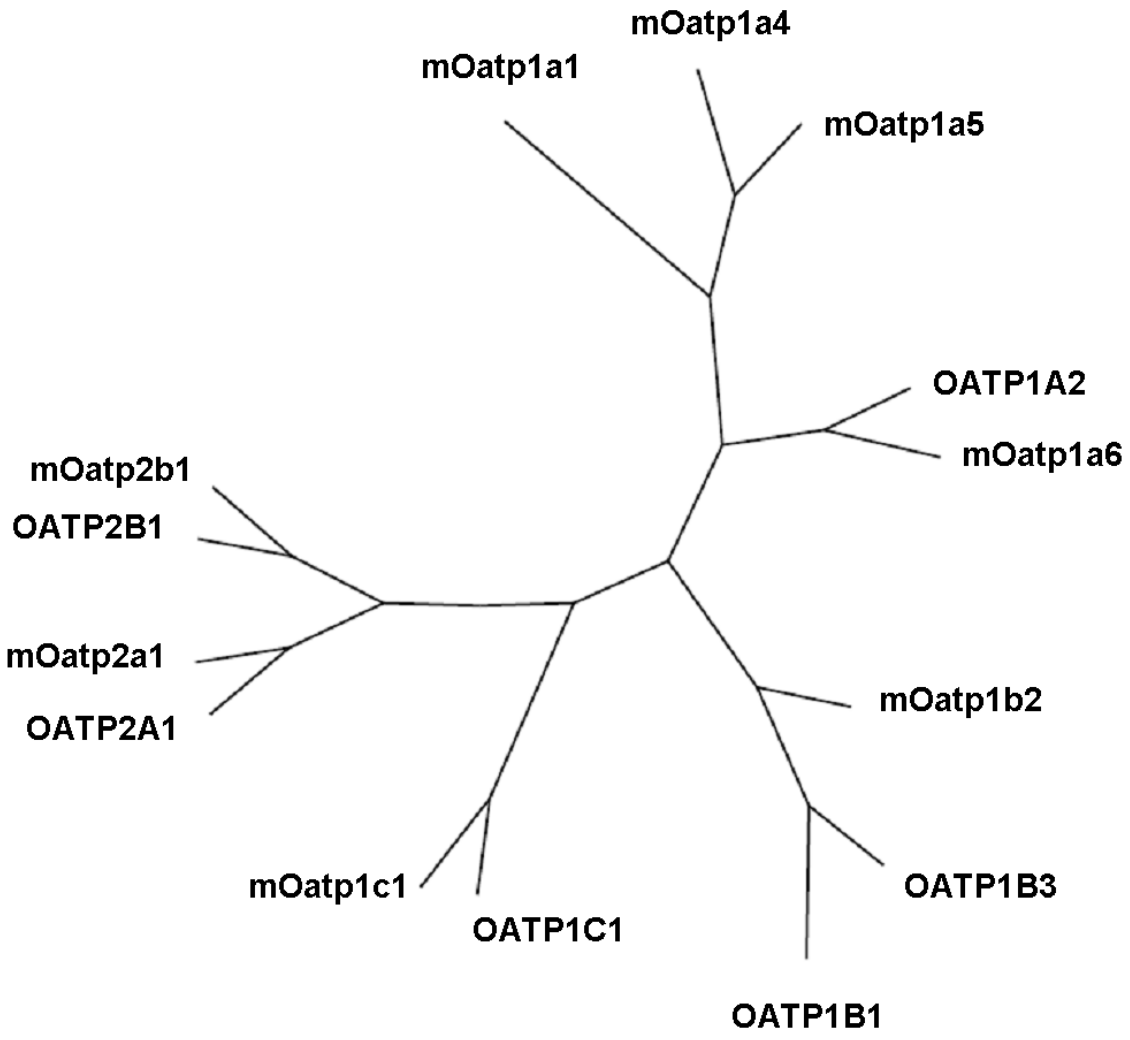

mOatp1a4
mOatp1a1
mOatp1a5
OATP1A2
mOatp1a6
mOatp2b1
OATP2B1
mOatp2a1
OATP2A1
mOatp1b2
mOatp1c1
OATP1C1
OATP1B3
OATP1B1

Figure 1-2 Phylogenetic tree of the OATP1 and OATP2 families. OATPs/Oatps with more than 40% amino acid sequence identity are classified in the same family (e.g. OATP1 and OATP2). Members with more than 60% amino acid sequence identity are classified in the same sub-family (e.g. OATP1A and OATP1B). Human OATPs are given in all capitals; mouse is indicated with a small m preceding the protein symbol Oatp.

Compared to the liver-specific expression pattern of OATP1B1 and OATP1B3, studies have demonstrated OATP2B1 and Oatp2b1 are ubiquitously expressed in tissues including the liver, heart, placenta, brain, and the small intestine (Kullak-Ublick et al., 2001; St-Pierre et al., 2002; Kobayashi et al., 2003a; Bronger et al., 2005; Cheng et al., 2005; Grube et al., 2006; Grube et al., 2007).

C. Structure of OATPs/Oatps

All OATP/Oatp family members contain 12 transmembrane (TM) domains based solely upon hydropathy plots (Hagenbuch et al., 2000; van Montfoort et al., 2002; Hagenbuch and Meier, 2004; Meyer Zu Schwabedissen et al., 2009). It is important to mention that this TM structure has not been proven experimentally for any of the OATPs/Oatps. However, it has been demonstrated, by epitope-specific immunofluorescence analysis, that rat Oatp1a1 has a 12 TM topology. Common structural features among all Oatps/OATPs include large extracellular domain situated between TMs 9 and 10 containing many conserved cysteine residues resembling the zinc finger domains of DNA binding proteins. Furthermore, it has been shown experimentally for OATP2B1 that these residues are imperative for proper membrane localization and function (Hanggi et al., 2006). Membrane targeting and functional activity of rat Oatp1a1 has been shown to be dependent upon the extent of *N*-glycosylation and thus provides a means of co- and post-translational regulation (Lee et al., 2003). Furthermore, activation of rat Oatp1a1 and Oatp1a4 by PKC has been shown to be another

post-translational modulator of transporter activity (Guo and Klaassen, 2001). General features of OATP/Oatps are shown in Figure 1-3.

Figure 1-3

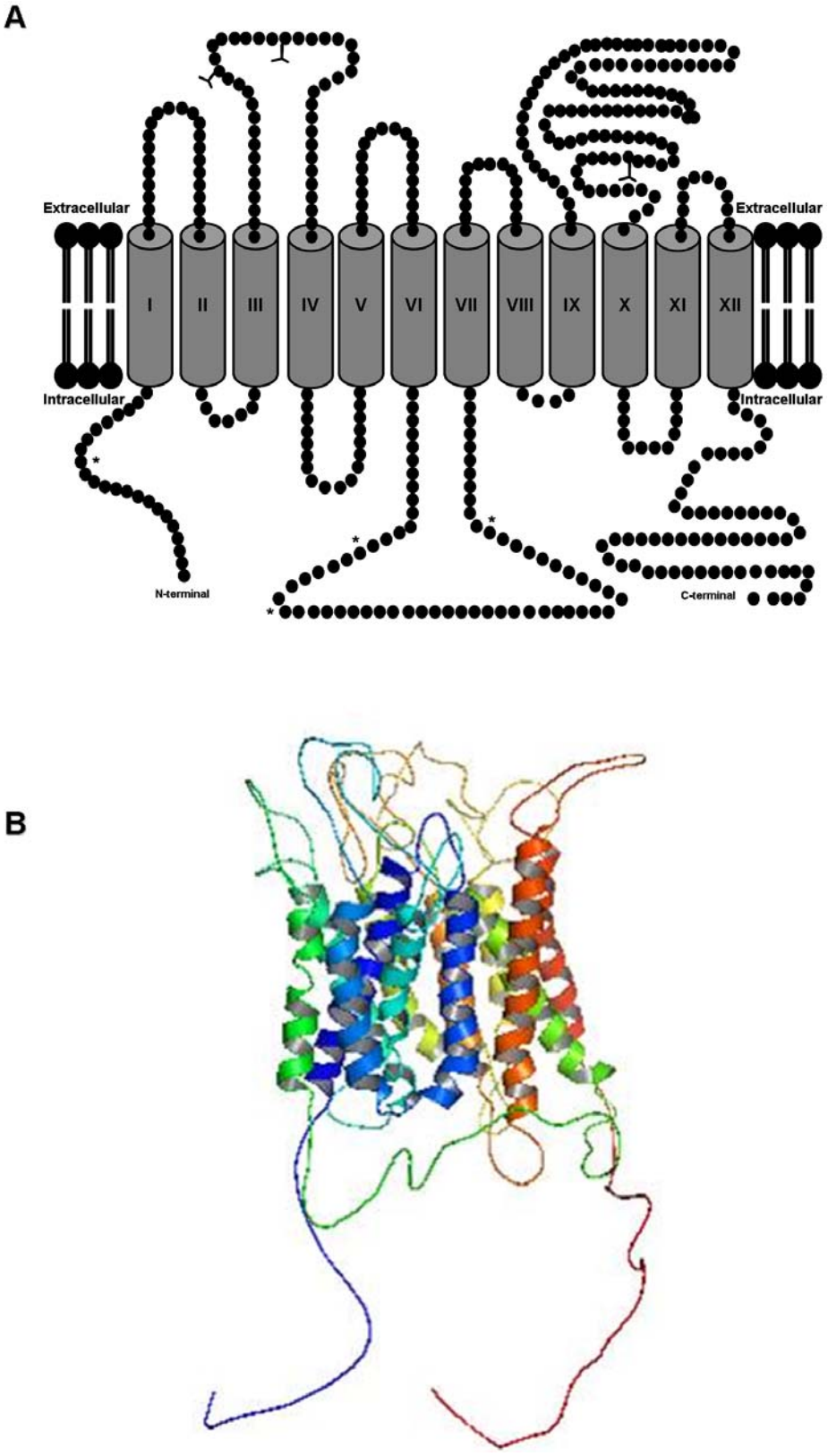

A
Extracellular
Extracellular
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
Intracellular
Intracellular
N-terminal
C-terminal
B

Figure 1-3 *In silico* structural analysis of mouse Oatp1b2. (A) Predicted topological twelve TM domain model of mouse Oatp1b2 as predicted by TMPred (http://www.ch.embnet.org/software/TMPRED_form.html). Three *N*-glycosylation sites are indicated (Y) in extracellular loops E2 and E5 whereas potential PKC sites are indicated by (*). (B) 3D model predicted by Insight II modeling software (Accelrys, Inc., San Diego, CA).

The mechanism of OATP/Oatp-mediated transport is still unknown. OATP/Oatp-mediated transport is independent of sodium gradient (Kullak-Ublick et al., 1995; Noe et al., 1997). It has been demonstrated that rat Oatp1a1 may function as an exchanger, specifically, for intracellular glutathione (Li et al., 1998; Li et al., 2000). Furthermore, Oatp-mediated taurocholate transport was shown to be accompanied by HCO_3^- exchange (Shi et al., 1995; Satlin et al., 1997). OATP2B1 has been shown to be more active and transport a broader range of substrates at low pH (Kobayashi et al., 2003a; Nozawa et al., 2004). Moreover, a low extracellular pH leads to a decreased K_m value (increased affinity) with no marked effect on V_{max} (transport capacity) for all Oatps/OATPs investigated. This effect was shown to be dependent upon a conserved histidine in the third transmembrane domain (Leuthold et al., 2009).

D. Functional characterization of human and mouse hepatic OATPs/Oatps

In general, most substrates of OATP/Oatps are amphipathic organic anions with a molecular weight of more than 350. OATP/Oatp substrates usually are bound to albumin under normal physiological conditions.

In liver, OATP1A2 is not expressed in hepatocytes, but rather in the epithelial cells of the bile duct (Lee et al., 2005). In general, Oatp1a1 and Oatp1a4 demonstrate overlapping substrate specificity, and transport substrates inlcuding

taurocholic acid, sulphobromopthalein (BSP), and estrone-3-sulfate (Hagenbuch et al., 2000; van Montfoort et al., 2002; Meyer Zu Schwabedissen et al., 2009). However, unique substrates, such as the cardiac glycoside digoxin, have been shown to be transported specifically by Oatp1a4 (Hagenbuch et al., 2000; van Montfoort et al., 2002).

OATP1B1 and OATP1B3 are expressed exclusively at the basolateral membrane of hepatocytes (Abe et al., 1999; Hsiang et al., 1999; Konig et al., 2000a; Konig et al., 2000b). OATP1B1 and OATP1B3 transport endogenous compounds such as bilirubin and its metabolites (Briz et al., 2003a; Briz et al., 2003b), taurocholate (Abe et al., 1999; Hsiang et al., 1999; Kullak-Ublick et al., 2001; Briz et al., 2006) and T_4 (Abe et al., 1999; Abe et al., 2001; Kullak-Ublick et al., 2001). Additionally, OATP1B1 and OATP1B3 also mediate the hepatic uptake of a large number of drugs (Hagenbuch and Gui, 2008).

Oatp1b2 is the ortholog of both human OATP1B1 and OATP1B3, (Cattori et al., 2000; Choudhuri et al., 2000; Ogura et al., 2000; Meyer Zu Schwabedissen et al., 2009). Oatp1b2 has been shown to transport various statins as well as taurocholate (Meyer Zu Schwabedissen et al., 2009). Recently, the development of Oatp1b2-null mice were used to determine the *in vivo* contribution of hepatic Oatps to drug substrates identified using *in vitro* systems (Lu et al., 2008; Zaher et al., 2008).

OATP2B1 has relatively narrow substrate specificity at pH 7.4. OATP2B1 transports BSP (Kullak-Ublick et al., 2001), estrone-3-sulfate and dehydroepiandrosterone-3-sulfate (Pizzagalli et al., 2003). However, at lower pH, additional substrates, such as taurocholate and statins, have been reported (Nozawa et al., 2004). Therefore, the pH of the micro environment might be important for OATP2B1 transport activity. Oatp2b1 is the mouse orthologue of human OATP2B1. To our knowledge, no functional studies of Oatp2b1 exist.

III - Introduction to hepatic xenobiotic-sensing nuclear receptors, CAR and PXR

A. Introduction to nuclear receptors

Nuclear receptors (NRs) define the largest superfamily of ligand-dependent transcription factors and are involved in a wide variety of biological functions, including cell proliferation, differentiation, development and homeostasis (Giguere, 1999). Since the initial discovery of NRs in the 1970s, several structurally similar receptors were discovered thus giving rise to a new class of NRs, termed the orphan nuclear receptors (Giguere, 1999). Initially, orphan nuclear receptors lacked physiological ligands or activators. However, recent work has identified the biological role of several of these orphans. The liver X receptor (LXR), peroxisome proliferator-activated receptor (Main et al.), and farnesoid X receptor (FXR) have been identified as sensors for cholesterol, fatty acids, and bile acids, respectively, serving to coordinately regulate lipid

homeostasis (Chawla et al., 2001). Together, CAR and PXR function as xenobiotic sensors responsible for directing the induction of drug metabolic pathways to ensure rapid clearance of potentially toxic compounds. This dissertation will focus on CAR and PXR so subsequent discussions will introduce and clarify the roles of CAR and PXR in mediating chemical metabolism and disposition.

The classical function of NRs is to transcriptionally regulate expression of target genes by the recruitment of coactivators or co-repressors. Ligand binding to these receptors recruits coactivators (activation) or co-repressors (repression), thereby regulating the coordinate expression of their target genes (McKenna et al., 1999; McKenna and O'Malley, 2002). Because the expression of a large number of genes is regulated by NRs, ligands that activate these receptors can have profound effects on the organism. Regulation of gene expression at the transcriptional level by nuclear receptors has an important role in both cellular developmental processes, energy homeostasis as well as the body's defense systems including the multiple phase I and phase II drug-metabolizing enzymes as well as transporter systems.

Table 1-2. Nuclear receptor superfamily classification

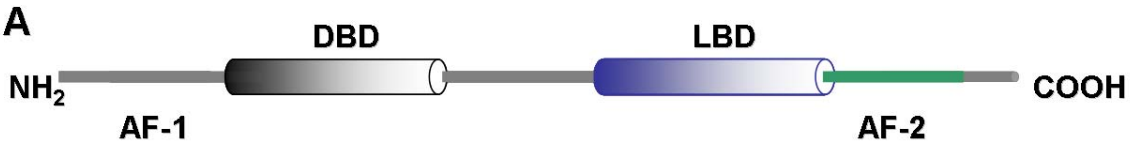
	Group I	Group II	Group III	Group IV
Nuclear Receptor	Steroid hormone receptors	Adopted orphan nuclear receptors	Orphan nuclear receptors	Others
Representatives	ER, GR, PR, TR, VDR	PPARs, RXR, LXR, FXR, PXR, CAR	SHP, LRH-1, HNF4	AhR, Nrf2
Ligands	Steroid hormones	Dietary lipids	Unknown	Xenobioic or oxidative stress
Binding partner	Self	Non-specific, RXRα	none	specific

Note: ER = estrogen receptor; GR = glucocorticoid receptor; PR = progesterone receptor; TR = thyroid hormone receptor; VDR = vitamin D receptor; PPAR = peroxisome proliferator-activated receptors; RXR = retinoid X receptor; LXR = liver X receptor; FXR = farnesoid X receptor; PXR = pregnane X receptor; CAR = constitutive androstane receptor; SHP = small heterodimer partner; LRH-1 = liver receptor homolog-1; HNF4 = hepatocyte nuclear factor 4; AhR = aryl hydrocarbon receptor; Nrf2 = nuclear factor erythroid 2-related factor 2

B. Structure of nuclear receptors

In general, NRs have a highly conserved DNA-binding domain (DBD) and a ligand-binding domain (LBD), and are classified into four groups, based mainly upon their dimerization and ligand properties (Table 1-3; Chawla et al., 2001). The putative modular structure, as shown in Figure 1-4, has various regions corresponding to functional domains responsible for NR function (Aranda and Pascual, 2001). A prototypical NR consists of a variable NH_2-terminal region, a conserved DBD, a linker region and a conserved LBD followed by a COOH-terminal region. Many NRs have at least two activation domains, the ligand-independent activation function (AF)-1, which generally resides in the NH_2-terminal region, and the ligand-dependent AF-2, which is localized in the COOH-terminal LBD. The DBD is the most conserved region and is responsible for recognition of response elements on target genes. Ligand binding induces significant conformational changes in the folding of the LBD, and leads to the recruitment of coactivator proteins, such as steroid receptor co-activators responsible for the transactivation of the target genes (Naar et al., 2001; Urnov and Wolffe, 2001; Urnov et al., 2001).

Figure 1-4



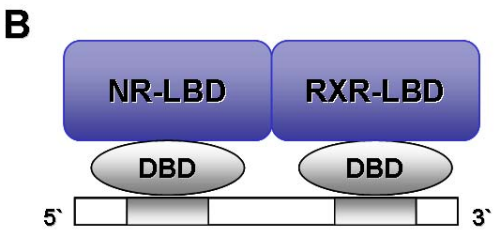


Figure 1-4 Structure of NRs and their functional domains. (A) Basic structure of NRs. AF-1 and DBD are at the amino-terminal, and AF-2 and LBD are at the carboxy-terminal. (B) NRs can bind to and regulate the expression of their target sequences as heterodimers with RXR. AF = activation function; DBD = DNA-binding domain; LBD = ligand-binding domain; RXR = retinoid X receptor

C. Introduction to CAR and PXR

CAR is a member of the NR1I subfamily. CAR is present at low levels in heart, muscle, kidney and lung, and is expressed at much higher levels in the liver and small intestine. After activation by ligands/activators and dimerization with RXRα, CAR usually binds to a direct repeat separated by 4 nucleotides (DR4) response element in the promoter region of its target genes (Honkakoski et al., 1998; Ueda et al., 2002). CAR can be activated by a wide variety of xenobiotics and regulates the expression of genes involved in phase I and II metabolism of drugs, steroid hormones, thyroid hormones and bilirubin.

Unlike most nuclear receptors, which are activated after ligand binding, CAR is constitutively active and sequestered in the cytoplasm in the absence of ligand. This was shown by cytosolic staining of CAR in livers of untreated mice whereas PB treatment resulted in nuclear staining of CAR (Kawamoto et al., 1999). One such inhibitor, okadaic acid, blocks the PB induction of rodent CYP2B genes [(Sidhu and Omiecinski, 1997), and treatment with OA was shown to prevent nuclear accumulation of CAR (Honkakoski et al. 1998). Thus, dephosphorylation of CAR constitutes a crucial signal for its release from the cytosolic complex upon activator treatment.

There exist definite species differences between human and mice regarding the mechanism of CAR activation. At this point, the conclusions regarding the role of CAR in the regulation of expression of CYP2B or other genes are based almost

entirely on rodent CAR, primarily mCAR (Honkakoski et al., 1998; Kemper, 1998). Human CAR exhibits some common characteristics with its rodent counterparts, such as undergoing nuclear translocation after PB treatment and binding to the PBREM. However, there are distinct differences between the substrate specificities of rodent and human CAR. For example, TCPOBOP and (6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde*O*-(3,4-dichlorobenzyl)oxime) (CITCO) are the only compounds shown to specifically bind to mouse CAR and human CAR, respectively. Overall, current evidence suggests that there are clear species-specific differences in CYP2B induction and CAR activation, which may hamper extrapolation of animal data to humans.

Based upon sequence homology with other nuclear receptors PXR was originally identified in 1997. It was named according to ligands which include various natural and synthetic pregnanes (Kliewer et al., 1998). PXR is highly expressed in liver and the gastrointestinal tract (Bertilsson et al., 1998; Blumberg et al., 1998; Kliewer et al., 1998; Lehmann et al., 1998). As a member of the group-II NR family, PXR can be activated by several chemicals with dexamethasone, rifampicin, spironolactone, and pregnenolone-16α-carbonitrile (PCN) being among the best characterized. It can also bind some specific bile acids such as litocholic, 3-ketolitocholic, cholic and deoxycholic acids.

The human counterpart for PXR (NR1I) is the steroid and xenobiotic receptor (SXR) (Blumberg et al., 1998). Following ligand binding and dimerization with

RXRα, PXR binds to a direct repeat separated by 3 or 4 nucleotides in the enhancer region of its target genes (Goodwin et al., 1999). Recently, a novel PXR motif was identified by which binding to spacers of a periodicity of 5 bp, forming a novel DR-(5n+4) pattern for PXR binding (Cui et al., 2010). SXR usually binds to an ER6 (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998). The species difference between PXR and SXR has been elegantly studied using humanized PXR mice (Xie et al., 2000a; Cheung et al., 2005). For example, PCN strongly activates rodent PXR, whereas its binding affinity to human SXR is very low. Whereas rifampicin binds to and activates human SXR, it only weakly binds to rodent PXR (Blumberg et al., 1998; Lehmann et al., 1998).

D. Role of CAR and PXR in hepatic xenobiotic metabolism and disposition

CAR and PXR are important regulators of several steroid and xenobiotic metabolizing enzymes and transporters in the liver and thus are important regulators of adaptation to chemical stress (Figure 1-5). The detoxification proteins induced are responsible for the metabolism, deactivation and transport of bile acids, thyroid and steroid hormones, numerous environmental chemicals, and several drugs (Kretschmer and Baldwin, 2005).

Detoxification genes induced by PXR and CAR include several Cyps, crucial in the oxidative metabolism of a wide range of endobiotics and xenobiotics. The Cyps are phase I enzymes that can oxidize or reduce various substrates, often

Figure 1-5

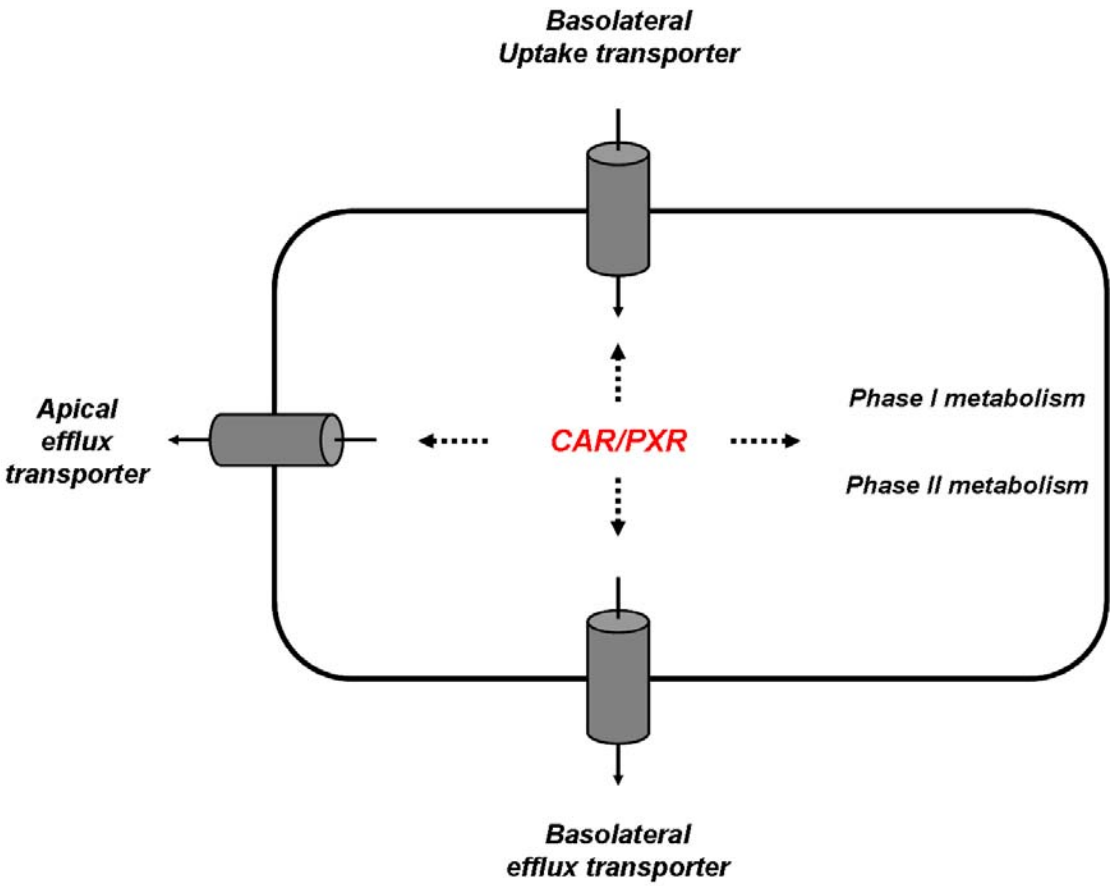

Basolateral
Uptake transporter
Apical
efflux
transporter
CAR/PXR
Phase I metabolism
Phase II metabolism
Basolateral
efflux transporter

Figure 1-5 Function of CAR and PXR in hepatic metabolism and disposition. CAR and PXR mediate hepatic disposition through the induction of phase I and II enzymes involved in detoxification, and transporters responsible for the uptake and efflux toxicants.

making more polar, water-soluble metabolites. Frequently these metabolites then can be conjugated by phase II enzymes and removed more rapidly. Induction of CYP3A and CYP2B has been studied and was found to be a biomarker of CAR and PXR activation. PXR and CAR also regulate the expression of a number of phase II enzymes and transporters. Phase II enzymes are normally involved in detoxification by functioning in conjugation reactions where an endogenous hydrophilic moiety is added to the substrate producing a more polar metabolite. Typical conjugation reactions involve glucuronidation, sulfation, acetylation, and conjugation to glutathione (Krishna and Klotz, 1994). Finally, transport proteins in the removal of these polar compounds are components of efflux transporters. Elimination of the toxicants from hepatocytes may be accomplished by secretion to the blood at the sinusoidal membrane or secretion to the bile at the cannalicular membrane (Borst et al., 2000; Litman et al., 2001).

CAR and PXR cross-talk by sharing response elements in the promoter or enhancer region of their target genes (Heery et al., 1997; Xie et al., 2000b; Smirlis et al., 2001; Wei et al., 2002; Xie et al., 2003). However, the affinity for CAR to bind to DR4 is stronger than to DR3, and the affinity for PXR to bind to DR3 is much stronger than to DR4. The result of CAR and PXR cross-talk is that they induce a distinct, but largely overlapping set of genes. Overall, because CAR and PXR are activated by some of the same ligands and induce specific but overlapping sets of genes, they provide the cell with two overlapping and semi-redundant mechanisms for recognizing and eliminating toxicants.

IV - Specific aims of this dissertation

PBDEs are not covalently bound to products, but instead are chemical additives and as a result they are subject to leaching out into the environment. Consequently, PBDEs have been detected in sediment, wildlife and potentially more pertinent to human health risks, food, indoor air and house dust. Upon exposure PBDEs enter the liver where they are biotransformed to potentially toxic metabolites; however, the mechanism(s) for such toxicities remains unclear. In addition, we know very little of PBDE toxicokinetics.

T*he goal of my PhD dissertation is to elucidate the molecular mechanism responsible for the hepatic transport and induction of drug metabolizing enzymes by which PBDEs may be used to predict and clarify the toxic effects of PBDEs in humans.* The preliminary data showed PBDEs were highly accumulated in the liver even though they are compounds with large molecular weight, inhibited OATP1B1- and 1B3-mediated transport, and induced Cyp2b10 and 3a11 gene expression in rodents, target genes of xenobiotic NRs, CAR and PXR. Based upon these compelling data, **I hypothesize that PBDEs enter hepatocytes by OATP/Oatp-mediated uptake, activate the xenobiotic NRs, and induce enzymes and transporters critical for endobiotic and xenobiotic metabolism.** This hypothesis was tested via the following specific aims.

Specific Aim 1: Identify the hepatic OATP/Oatp family responsible for the uptake of PBDEs.

The working hypothesis for this aim ***was that members of the human and mouse OATP1A, OATP1B and OATP2B subfamilies transport PBDE congeners*.** I suspect that this is the mechanism responsible for liver-specific accumulation of PBDEs. To investigate the mechanism of PBDE uptake by human and mouse livers, OATP/Oatp-mediated uptake of PBDEs was characterized.

Specific Aim 2: Clarify the mechanism by which PBDEs induce Phase I metabolizing enzymes Cyp3a11 and Cyp2b10.

The working ***hypothesis was that PBDEs are activators for CAR and PXR*.** I tested this hypothesis using *in vitro* and *in vivo* models. Activation of human and mouse CAR and PXR will be determined using reporter-gene luciferase assays as well as using mice deficient in CAR or PXR. Mice were treated in a dose-dependent manner with BDE47 or BDE99 in order to clarify which which Cyp isoform, if any, is preferentially induced.

The presence of PBDEs in human liver is particularly alarming because more severe adverse effects have been documented for the OH-PBDEs relative to the PBDEs. Rodent studies have identified PBDEs as inducers of phase I enzymes,

however, prior to this dissertation work, it was not known how PBDEs enter the liver and the mechanism responsible for enzyme induction. Results from this study aids in a better evaluation of the possible risks for human beings by providing the molecular basis for PBDE toxicokinetics.. In addition, our study, once accomplished, will help us to understand the species difference of transporters and NRs in interaction with PBDEs between mice and human. Therefore, the results from the proposed studies may help to predict human toxicity with PBDE exposure.

Chapter 2

Materials and Methods

I - Chemicals and reagents

Radiolabeled [^{14}C]BDE47 (36.5 mCi/mmol) was a gift from Dr Kevin Crofton (U.S. Environmental Protection Agency, National Health and Environmental Effects Laboratory). Radiolabeled [^{14}C]BDE99 (36.5 mCi/mmol) and [^{14}C]BDE153 (27.8 mCi/mmol) were gifts from Dr Mike Sanders (National Toxicology Program at the National Institute of Environmental Health Sciences). BDE47, BDE99, and BDE153 were obtained from Cerilliant (Round Rock, TX). [^{3}H]Estradiol-17-β-glucuronide (47.1 Ci/mmol), [^{3}H]estrone-3-sulfate (57.1 Ci/mmol) and [^{32}P] were purchased from Perkin-Elmer (Waltham, MA). Unlabeled estradiol-17-β-glucuronide, estrone-3-sulfate, and cell culture reagents were from Sigma-Aldrich (St Louis, MO). Turbofect transfection reagent was purchased from Fermentas (Glen Burnie, MD). Reverse transcriptase PCR (RT-PCR) and Dual-Glo Stop and Glo luciferase kits were purchased from Promega (Madison, WI). 5-α-Androstan-3-α-ol was purchased from Steraloids (Newport, RI).

II - Functional studies of PBDE transport in WT and OATP-expressing CHO cells

Wild-type CHO and OATP1B1- or OATP1B3-expressing cells were plated at 40,000 cells per well on 24-well plates, and 48 hr later medium was replaced with medium containing 5mM sodium butyrate to induce nonspecific gene expression.

After an additional 24 hr in culture, the cells were used for uptake experiments. Vector-transfected and OATP2B1-expressing Flp-In-CHO cells were plated at 40,000 cells per well on 24-well plates, and 48 hr later the cells were used for uptake experiments. Cells were washed two times with 37°C prewarmed uptake buffer (116.4mM NaCl, 5.3mM KCl,1mMNaH2PO4, 0.8mM MgSO4, 5.5mM D-glucose, and 20mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH adjusted to 7.4 with Trizma base), and for the determination of initial linear rate conditions, uptake was started by adding 200 µL of uptake buffer containing 0.3 µCi/mL of the radiolabeled substrate. Uptake was stopped at various time points by removing the uptake solution and washing the cells four times with ice-cold uptake buffer. To analyze the kinetics of OATP-mediated PBDE transport, wild-type, control, or OATP-expressing cells were incubated with [^{14}C]BDE47, [^{14}C]BDE99, or [^{14}C]BDE153 at increasing substrate concentrations for a previously determined time within the initial linear uptake portion. After stopping, the cells were solubilized with 400 µL of 1% Triton X-100 and 300 µL were used for liquid scintillation counting. Protein concentration was determined using the BCA assay with BSA as a standard from the remaining samples. Uptake rates were calculated based on net uptake (in nanomoles per milligram of total protein per minute) after subtracting the values obtained with wild-type or pcDNA5/FRT-transfected CHO cells (in nanomoles per milligram of total protein per minute).

Estradiol-17-β-glucuronide, a model substrate of OATP1B1 and OATP1B3, and estrone-3-sulfate, a model substrate of OATP2B1, were used to test for PBDE

inhibition. Cells were incubated in the absence or presence of BDE47, BDE99, or BDE153 at concentrations of 100pM, 100nM, or 100μM for 20 s (OATP1B1 and OATP1B3) or 30 s (OATP2B1). Values obtained with wild-type or vector-transfected CHO cells were subtracted from values obtained with OATP-expressing CHO cells and is given as percent of the control.

III - Cloning of the mouse Oatp expression plasmid constructs

The initial cloning of mouse Oatp1a1 and Oatp1a4 have been reported previously (Hagenbuch et al., 2000; van Montfoort et al., 2002). These two cDNAs were subcloned into the mammalian expression vector pExpress-1 using unique restriction endonucleases. To clone the mouse Oatp1b2 and Oatp2b1, RNA was isolated from C57BL/6 mouse liver using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. cDNA was synthesized from mouse liver RNA using the RT-PCR kit (Promega, Madison, WI). Oatp1b2 and Oatp2b1 coding sequences were amplified using the Phusion High-Fidelity DNA Polymerase with gene-specific primers (Finnzymes, Inc., Woburn, MA). After gel purification, the amplicons were inserted into the mammalian expression vector pcDNA5/FRT and sequenced on both strands.

IV - Functional studies with Oatp1a1, Oatp1a4, Oatp1b2, and Oatp2b1 in HEK293 cells

HEK293 cells were seeded in poly-D-lysine-coated 24-well plates and were transiently transfected with Oatp1a1, Oatp1a4, Oatp1b2, Oatp2b1 or empty

vector (pExpress-1 for Oatp1a1 and Oatp1a4; pcDNA5FRT for Oatp1b2 and Oatp2b1) with TurboFect according to the manufacturer's protocol. Transport assays were performed 48 hr post-transfection. Uptake assays (time- and concentration-dependent) were performed as described above.

V - Animals and treatments

WT mice with a C57BL/6 genetic background were obtained from Jackson Laboratories (Bar Harbor, ME). Oatp1a4-null mice were originally obtained from Bristol Meyers Squibb (Princeton, NJ) whereas the generation of the Oatp1b2-null mouse has been previously described (Lu et al., 2008). The PXR-null mice were originally obtained from Dr Steve Kliewer (University of Texas Southwestern Medical Center) and backcrossed five generations to C57BL/6 background (Guo et al., 2004). The CAR-null mice were obtained from Tularik (San Francisco, CA). All mice were housed in a pathogen-free animal facility under a standard 12-hr light:dark cycle with access to regular rodent chow and autoclaved tap water ad libitum. All protocols and procedures were approved by the University of Kansas Medical Center Animal Care and Use Committee. All the chemicals were first dissolved in acetone, followed by addition of corn oil. The remaining acetone was evaporated by speed vacuum centrifugation. All the chemicals are soluble in acetone, except for BDE209, which was used as a suspension.

Four groups of female WT, Oatp1a4- or Oatp1b2-null mice (10-12 weeks old, n= 3–4 per group) were given a single dose (1mg/kg) of BDE47 or corn oil (vehicle control) via oral gavage (p.o.). Corn oil was then added to the vials by weight, followed by the addition of [^{14}C]BDE47 directly to the dosing solution from the stock solution. After indicated time points (0, 1, 3, and 8 hrs), mice were euthanized by CO_2 asphyxiation followed by cervical dislocation. The gender, dose, and time post treatment for liver collection was selected based on a previous kinetic study on BDE47 (Staskal et al., 2005).

Twelve groups of male C57BL/6 mice (10 weeks old, n= 3–4 per group) were treated with PBDE congeners and the appropriate controls. Corn oil was used as the vehicle, and PCB126 was used as the positive control for activation of AhR and induction of Cyp1a1/2; PCB153 and TCPOBOP for activation of CAR and induction of Cyp2b10; and PCN for the activation of PXR and induction of Cyp3a11 (Table 5-1 on page 132). Male WT, CAR- or or PXR-null mice(10-12 week old, n = 3 per group) were treated via ip injection with corn oil, PCN (50 mg/kg/day), TCPOBOP (3 mg/kg/day), PB (50 mg/kg) or PBDEs 47 (49 mg/kg), 99 (57 mg/kg), and 209 (96 mg/kg) for four consecutive days.

For the dose-response study of BDE47 and BDE99, WT male mice (10-12 week old, n = 6 per group) were administered corn oil, BDE47 (0.015, 0.049, 0.15, 0.49, 1.5, 4.9, 15 or 49mg/kg/day) or BDE99 (0.017, 0.056, 0.17, 0.56, 1.7, 5.6, 17 or 57mg/kg/day) as an i.p. dose for four consecutive days.

For all studies, the livers were removed, immediately frozen in liquid nitrogen and stored at -80°C.

VI - Analysis of hepatic BDE47 content

The sample preparation method for liquid scintillation analysis was determined using a modified assay based on a previously published method (L'Annunziata, 1989). Briefly, approximately 50 mg of liver was added to an 18-mL glass vial containing 1 mL Solvable (Perkin Elmer, Waltham, MA.) and was incubated overnight at 55°C to ensure all the tissue was solubilized. The samples were then brought to room temperature followed by the addition of 200 µL 30% hydrogen peroxide (H_2O_2) in two 100-µL portions with gentle swirling between additions. After the reaction had subsided, the samples were heated again to 55°C for ~1 hr to complete bleaching and to eliminate the excess of hydrogen peroxide. Samples were cooled to room temperature, 200 µL aliquots were added to 4 mL Ultima Gold scintillation cocktail (Perkin Elmer, Waltham, MA). Radioactivity was quantified using a MicroBeta TriLux liquid scintillation counter (Perkin Elmer, Waltham, MA).

VII - Messenger RNA (mRNA) analysis

The levels of mRNA were determined by three independent methods: Northern blot analysis (Cyp3a11, 2b10, 1a1/2, and 18s), branched DNA (bDNA) assay

(Cyp3a11, 2b10, 1a1/2, and 18s), and quantitative PCR (qPCR; Cyp3a11, 2b10, and 18s) Specifically, total RNA was isolated from the frozen liver using Trizol following the manufacturer's instructions (Invitrogen Inc., Carlsbad, CA). Total RNA concentrations were determined by UV spectrophotometry. For Northern blot analysis, total RNA was analyzed by electrophoresis in a 1% agarose gel containing 0.22M formaldehyde. The sequences for cDNA probes (Cyp3a11, 2b10, 1a1/2, and 18s) are available upon request. The probes were ^{32}P-labeled using a random primer labeling kit from Roche (Indianapolis, IN). The details for Northern blot analysis and the bDNA can be found in a previous report (Hartley and Klaassen, 2000; Guo et al., 2003). The Northern blot signal was quantified by using ImageQuant software (GE Healthcare, Piscataway, NJ). The details for qPCR can be found in a previous study (Maran et al., 2009).

VIII - Reporter-gene assays

Luciferase reporter assays were used to test activation of human and mouse CAR and PXR by PBDEs. For human and mouse CAR, CV-1 cells were seeded onto 24-well plates and transiently transfected with Turbofect and a cohort of plasmids, including a mammalian expression vector containing cDNA of human (pSG5-hCAR) or mouse (pSG5-mCAR) CAR, a pGL4-TK luciferase vector with three copies of PB-response element (PBRE, AGGTCAnnnAGGTCA), and pCMV-renilla luciferase vector. For mouse PXR activation, HepG2 cells were seeded onto 24-well plates and transiently transfected with lipofectamine 2000 (Invitrogen) and a cohort of plasmids, including a mammalian expression vector

containing the cDNA of mouse PXR (pSG5-mPXR), a pGL4-TK luciferase vector with three copies of PXR-response element (DR3, AGGTCAnnnAGGTCA), and pCMV-renilla luciferase vector. For human SXR, a HepG2 cell line (DPX2) with stable transfection of human SXR and SXR-response element cloned in a luciferase vector was obtained from Puracyp Inc. (Carlsbad, CA). A similar HepG2 cell line, termed DRE, with stable transfection of human AhR and AhR-response element was also obtained from Puracyp, Inc.

Five hrs after the transfection, the transfection medium was removed and PB, known to activate human and mouse CAR was used as the positive control (in 0.1% ethanol final concentration). PCN and rifampicin were used as the positive controls for mouse and human PXR activation, respectively. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was used as a marker for AhR activation. BDE47, BDE99 or BDE209 (in 0.1% DMSO final concentration) at 100mM as stocks were further diluted in the cell-culture medium to the desired concentrations in the presence of 1μM 5-α-Androstan-3-α-ol (Tzameli et al., 2003).

Twenty-four hrs later, firefly luciferase and renilla luciferase activities were quantified using a Dual Luciferase Kit from Promega (Madison, WI) with a Synergy-HT plate reader (Bio-Tek Instruments, Inc., Winooski, VT). Transfection efficiency was normalized by renilla luciferase activity except for activation of SXR and AhR for which protein concentration was used to normalize the

luciferase signals. The reason for using total protein concentration to normalize the response is that all stably transfected cells are derived from a single colony; thus, the transfection efficiency is the same for every cell. However, cell numbers may be different in each well due to human error, which can be normalized by measuring total protein concentration to ensure even cell numbers in each well. Relative luciferase activity was calculated based on luciferase activity in positive control or PBDE-treated cells after subtracting the values obtained with ethanol/DMSO-treated cells. The data were presented as the average of six wells, and the experiments were repeated at least twice.

IX - Western-blot analysis to determine hepatic protein levels

Fifty micrograms of liver protein was separated were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis at 110V and then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) for 1 hr at 100V. The membrane was blocked for 1 hr with 5% non-fat dry milk in 1X TBS at room temperature, followed by overnight incubation of anti-Cyp2b10 or anti-Cyp3a11 (1:1000 dilution) at 4°C. After washing with 1X TBS 3 times for 10 min each, the membrane was incubated with horseradish peroxidase-conjugated antibody (goat anti-mouse IgG for Cyp2b10; goat anti-rabbit IgG for Cyp3a11) for 1 hr at room temperature in 2.5% milk in 1X TBS (1:10,000 dilution). Following extensive washing with 1X TBS (3 times for 10 minutes each), the secondary antibody was

detected using the ECL kit (Midwest Scientific, St. Louis, MO). Protein loading was normalized using an antibody againt the β-actin gene (1:1,000). The Western blotsignal was quantified by using ImageQuant software (GE Healthcare).

X - Statistical analysis

Statistical analysis of inhibition studies between control and experimental groups were performed by one-way ANOVA followed by the Bonferroni t-test. To determine whether uptake in OATP-expressing cells was different from that of control cells, the unpaired Student's t-test was used. Statistical difference among multiple groups was analyzed by one-way ANOVA analysis, followed by Student-Newman-Keuls test. The p value for statistical significance was set to < 0.05. Data with error bars represent the means ± SE. All statistical analysis was performed using SigmaStat 3.5 (Systat Software, Inc., San Jose, CA), while kinetic parameters were calculated using the nonlinear regression analysis module from SigmaPlot (Version 9.01; Systat Software, Inc., Point Richmond, CA).

Chapter 3

Mechanism of Polybrominated Diphenyl Ether Uptake into the Liver: PBDE Congeners are Substrates of Human Hepatic OATP Transporters

I - Introduction

PBDEs are flame-retardants used as additives in polymers incorporated into textiles, electronics, plastics, and furniture (Figure 1-1). The frequent use of PBDEs is attributed to their low manufacturing cost as well as their high degree of resistance to degradation by environmental and biological systems (Darnerud et al., 2001). BDE47 is the predominant congener detected in human and wildlife samples, followed by BDE99 and BDE153 (Lorber, 2008). The concern of rising PBDE body burdens is so significant that the penta and octa mixtures have been banned in Europe and currently are being voluntarily phased out in the United States (Birnbaum and Cohen Hubal, 2006).

Human exposure to PBDEs is chronic and likely PBDE exposure routes are multiple. Recent data suggest that diet and inhalation are the predominant routes of exposure (Schecter et al., 2004; Schecter et al., 2005a; Schecter et al., 2006). When compared to Europe and Japan, body burden of PBDEs in North America are one to two orders of magnitude higher and furthermore PBDE levels have doubled every 4–6 years (Petreas et al., 2003; Hites, 2004; Schecter et al.,

2005b; Schecter et al., 2007). The presence of PBDEs in human tissue is of great concern because of potential toxicological end points including carcinogenicity, neurotoxicity, reproductive toxicity, and endocrine toxicity, which have been shown in animals (Darnerud et al., 2001). PBDEs have been detected in plasma and breast milk as well as liver, kidney, and adipose (Hites, 2004). In mice, after administration, BDE47 initially accumulates in the liver, followed by redistribution to other tissues, including the kidney and the adipose tissue (Staskal et al., 2005). Although the human liver burden of PBDEs is not clarified, the presence of PBDEs in human liver is particularly alarming since it has been suggested that hydroxylated metabolites may play a pivotal role in PBDE-mediated toxicity (Meerts et al., 2000; Darnerud et al., 2001; Meerts et al., 2001; Zhou et al., 2001; Hamers et al., 2008).

An important function of the liver is to remove a variety of chemicals from the portal blood. Hepatic uptake is a prerequisite for biotransformation and subsequent elimination of various endogenous and exogenous compounds. Uptake transporters involved in this process include OATPs that are a group of membrane transporters responsible for the transport of a wide array of amphipathic substrates (Hagenbuch and Gui, 2008; Kalliokoski and Niemi, 2009). As a family, OATPs are expressed in the liver, kidney, brain, and intestines, implying a critical role in drug disposition (Hagenbuch and Meier, 2003; Hagenbuch and Meier, 2004). OATP1B1 (*SLCO1B1*) and OATP1B3 (*SLCO1B3*) are two human members in the OATP1B subfamily expressed at the basolateral

membrane of hepatocytes (Abe et al., 1999; Hsiang et al., 1999; Konig et al., 2000a; Konig et al., 2000b). OATP2B1 (*SLCO2B1*) is the sole human member of the OATP2B family. Compared to the liver-specific expression pattern of OATP1B1 and OATP1B3, studies have demonstrated OATP2B1 protein to be expressed in the liver, heart, placenta, brain, and the small intestine (Kullak-Ublick et al., 2001; St-Pierre et al., 2002; Kobayashi et al., 2003b; Bronger et al., 2005; Grube et al., 2006). Given that OATP substrates are, in general, amphipathic molecules with MWs of more than 350 (Hagenbuch and Gui, 2008), hepatic OATP family members are promising candidate transport systems for hepatic uptake of BDE47 (MW: 485.5), BDE99 (MW: 564.7), and BDE153 (MW: 643.6).

In the current study, we evaluated the substrate specificity of OATP1B1, OATP1B3, and OATP2B1 for PBDE transport in order to test the hypothesis that the mechanism of hepatic PBDE uptake is mediated by OATPs.

II - Results

3.1 Concentration-dependent Inhibition of OATP1B1-, OATP1B3- and OATP2B1-mediated Uptake by PBDE Congeners

In order to investigate whether PBDE congeners can interact with OATP-mediated transport, we determined uptake of the model substrates estradiol-17 β-glucuronide for OATP1B1 and OATP1B3 and estrone-3-sulfate for OATP2B1, in the absence or presence of BDE47, BDE99, or BDE153. All three PBDE

congeners inhibited OATP1B1- and OATP1B3-mediated uptake of estradiol-17 β-glucuronide in a concentration-dependent manner (Figure 3-1A, B). For OATP1B1, BDE47 exhibited the greatest effect at all the tested concentrations (100pM, 100nM, 100μM), while BDE99 and BDE153 showed comparable levels of inhibition at all tested concentrations. Inhibition of OATP1B3-mediated uptake of estradiol-17β-glucuronide was comparable for all three tested PBDE congeners with BDE153 exhibiting slightly greater effect. OATP2B1-mediated estrone-3-sulfate uptake was inhibited by BDE47, BDE99, and BDE153 in a similar manner but only at the 100nM and 100μM concentrations (Figure 3-1C).

Figure 3-1

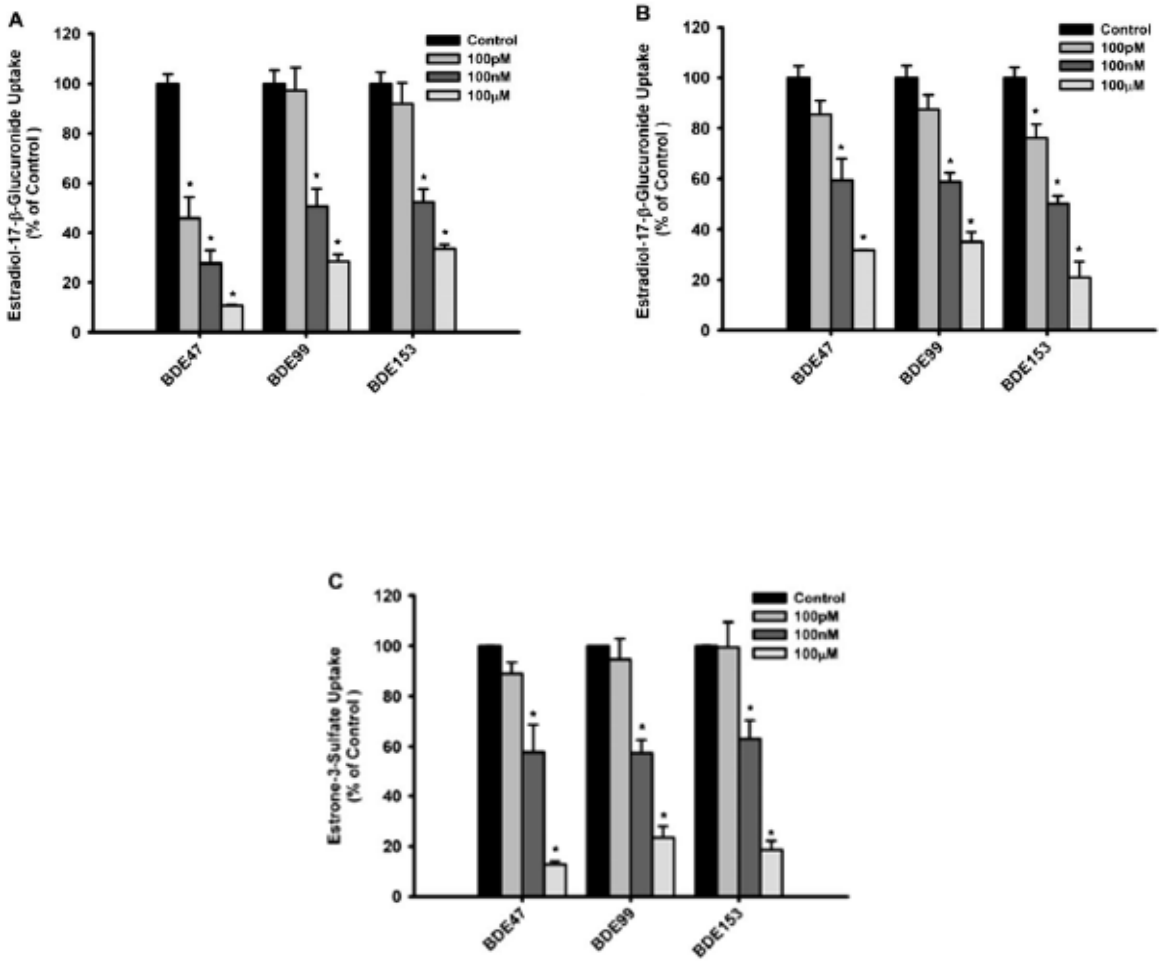

A
Estradiol-17-β-Glucuronide Uptake
(% of Control)
120
100
80
60
40
20
0
Control
100pM
100nM
100μM
BDE47
BDE99
BDE153
B
Estradiol-17-β-Glucuronide Uptake
(% of Control)
Control
100pM
100nM
100μM
BDE47
BDE99
BDE153
C
Estrone-3-Sulfate Uptake
(% of Control)
Control
100pM
100nM
100μM
BDE47
BDE99
BDE153

Figure 3-1 Effect of PBDE congeners on OATP-mediated uptake of known substrates in stably transfected CHO cells. In the absence or presence of BDE47, BDE99, or BDE153 at the indicated concentrations, uptake of 1μM [3H]estradiol-17β-glucuronide was measured at 37°C for 20 s with (A) OATP1B1- or (B) OATP1B3-expressing and WT CHO cells, while uptake of 1μM [3H]estrone-3-sulfate was measured at 37°C for 30 s with (C) OATP2B1- or pcDNA5/FRT-expressing CHO cells. Values obtained with WT or vector-transfected CHO cells were subtracted from values obtained with OATP-expressing CHO cells and are given as percent of the control. Means ± SE of triplicate determinations are given. Differences were considered significant at $p < 0.05$.

3.2 Time-dependent BDE47, BDE99, and BDE153 uptake by OATP1B1, OATP1B3, and OATP2B1

To test whether these PBDE congeners indeed are OATP substrates, uptake of [^{14}C]BDE47, [^{14}C]BDE99, and [^{14}C]BDE153 by OATP1B1, OATP1B3, and OATP2B1 was measured in a time-dependent manner. BDE47 (Figure 3-2A, B), BDE99 (Figure 3-3A, B), and BDE153 (Figure 3-4A, B) were clearly transported to a greater extent into OATP1B1- and OATP1B3-expressing cells when compared to the WT control cells. Similarly, uptake of BDE47 (Figure 3-2C), BDE99 (Figure 3-3C), and BDE153 (Figure 3-4C) into OATP2B1-expressing cells was significantly higher than uptake into the vector-transfected control cells. Furthermore, for all three congeners uptake was linear for at least 1 min. Thus, subsequent concentration-dependent kinetic studies were performed at 30 s.

Figure 3-2

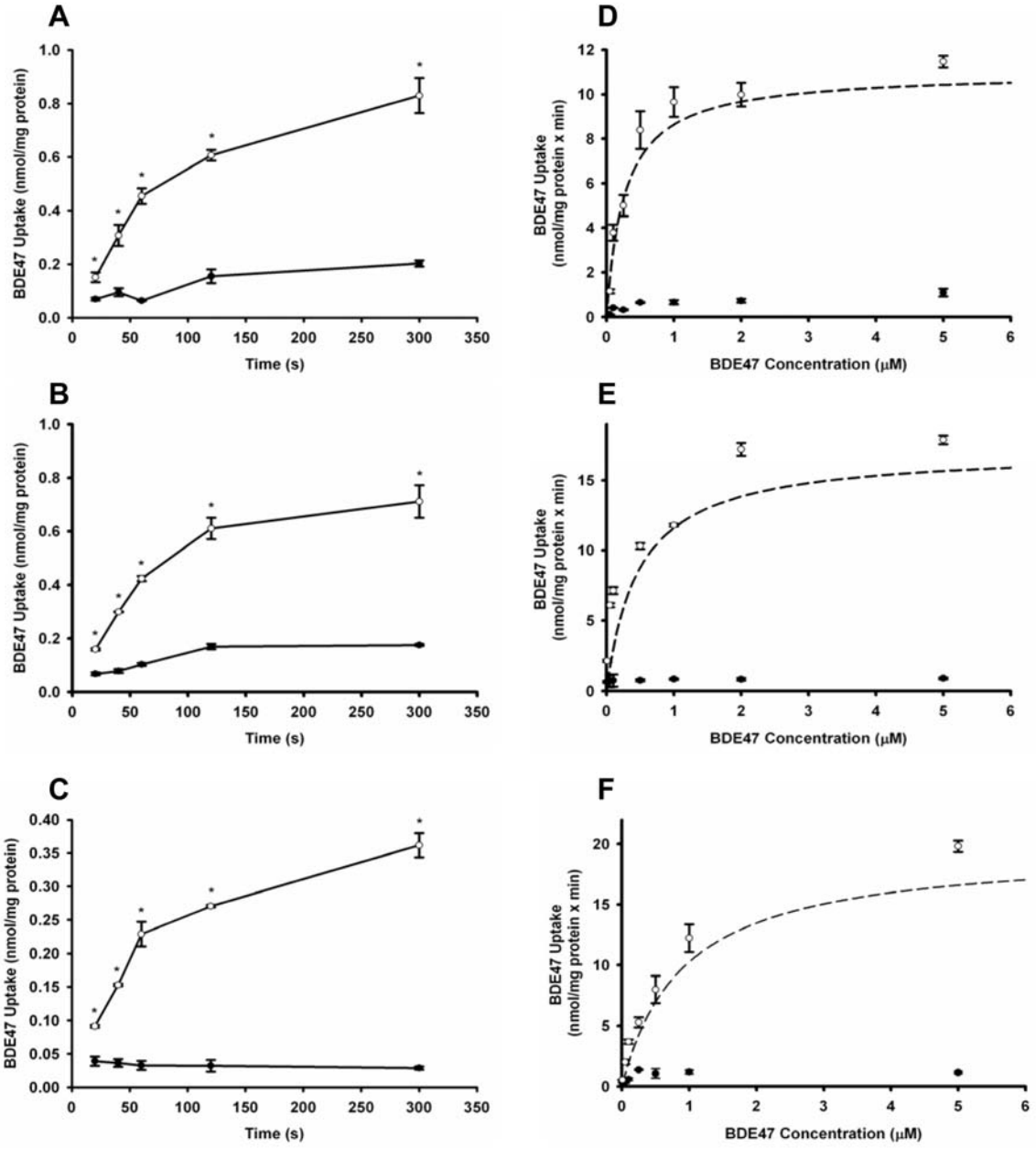

A
BDE47 Uptake (nmol/mg protein)
Time (s)
B
BDE47 Uptake (nmol/mg protein)
Time (s)
C
BDE47 Uptake (nmol/mg protein)
Time (s)
D
BDE47 Uptake (nmol/mg protein x min)
BDE47 Concentration (μM)
E
BDE47 Uptake (nmol/mg protein x min)
BDE47 Concentration (μM)
F
BDE47 Uptake (nmol/mg protein x min)
BDE47 Concentration (μM)

Figure 3-2 Time- and concentration-dependent uptake of BDE47 by OATP1B1, OATP1B3, and OATP2B1. (A) OATP1B1-, (B) OATP1B3-, or (C) OATP2B1-mediated uptake of [^{14}C]BDE47 at 37°C at the indicated time points. Filled circles (●) represent control (WT CHO; pcDNA5/FRT CHO) uptake, while open circles (○) represent OATP1B1, OATP1B3, or OATP2B1 uptake. Determination of kinetic parameters was performed in CHO cells expressing OATP1B1, OATP1B3, or OATP2B1. Uptake of increasing concentrations of [^{14}C]BDE47 was measured at 37°C for 30 s. After subtracting the values of the control (d) from OATP-expressing cells, net (D) OATP1B1-, (E) OATP1B3-, or (F) OATP2B1-mediated uptake data were fitted by nonlinear regression analysis to the Michaelis-Menten equation and plotted as a dashed line (- - -). Means ± SE of triplicate determinations are given. The unpaired Student's t-test was performed to determine statistical significance. Differences were considered significant at $p < 0.05$.

3.3 Determination of Kinetic Parameters for OATP1B1-, OATP1B3- and OATP2B1-mediated PBDE Congener Uptake

To further characterize OATP-mediated PBDE transport, we performed kinetic experiments. Uptake of all three PBDE congeners by all three OATPs was saturable. The kinetic parameters are summarized in Table 3-1. BDE47 exhibited the highest affinity for both OATP1B1 (0.31 ± 0.02μM) and OATP1B3 (0.34 ± 0.02 μM), respectively (Figure 3-2D, E). This was followed by BDE99 (0.80 ± 0.07μM for OATP1B1; 0.72 ± 0.12μM for OATP1B3) (Figure 3-3D, E). BDE153 (1.9 ± 0.17μM for OATP1B1; 1.7 ± 0.48μM for OATP1B3) demonstrated the least affinity (Figure 3-4D, E). OATP1B1 transported BDE153 with a 10-fold lower capacity than BDE47 and BDE99, while the maximal transport rates were comparable for OATP1B3-mediated transport of all three BDE congeners. Overall transport efficiency, characterized by V_{max}/K_m, followed a similar trend for both OATP1B1 and OATP1B3, with BDE47 being transported with the greatest efficiency followed by BDE99 and then BDE153.

Figure 3-3

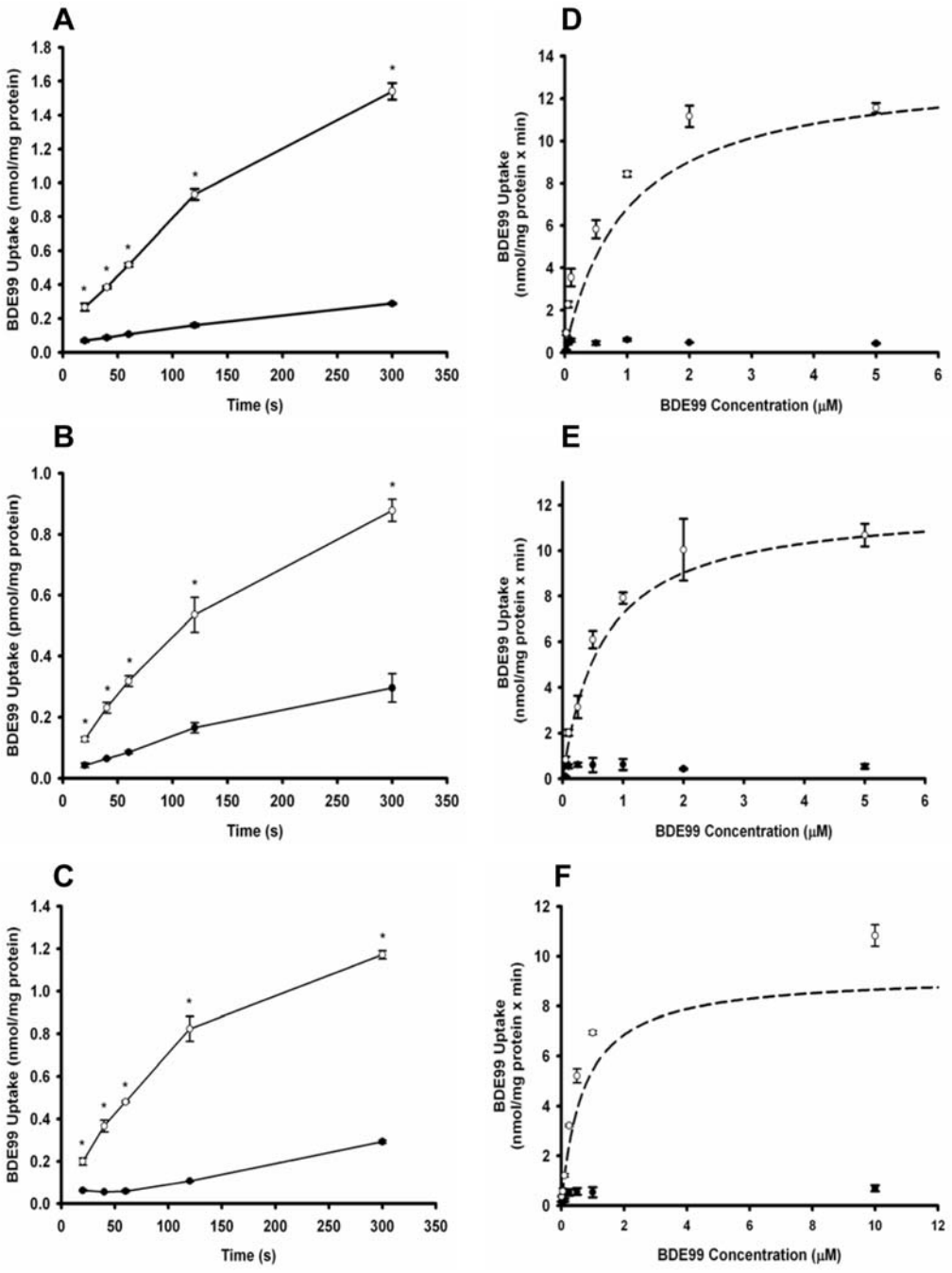

A
BDE99 Uptake (nmol/mg protein)
Time (s)
D
BDE99 Uptake (nmol/mg protein x min)
BDE99 Concentration (μM)
B
BDE99 Uptake (pmol/mg protein)
Time (s)
E
BDE99 Uptake (nmol/mg protein x min)
BDE99 Concentration (μM)
C
BDE99 Uptake (nmol/mg protein)
Time (s)
F
BDE99 Uptake (nmol/mg protein x min)
BDE99 Concentration (μM)

Figure 3-3 Time- and concentration-dependent uptake of BDE99 by OATP1B1, OATP1B3, and OATP2B1. (A) OATP1B1-, (B) OATP1B3-, or (C) OATP2B1-mediated uptake of [^{14}C]BDE99 at 37°C at the indicated time points. Filled circles (●) represent control (WT CHO; pcDNA5/FRT CHO) uptake, while open circles (○) represent OATP1B1, OATP1B3, or OATP2B1 uptake. Determination of kinetic parameters was performed in CHO cells expressing OATP1B1, OATP1B3, or OATP2B1. Uptake of increasing concentrations of [^{14}C]BDE99 was measured at 37°C for 30 s. After subtracting the values of the control (d) from OATP-expressing cells, net (D) OATP1B1-, (E) OATP1B3-, or (F) OATP2B1-mediated uptake data were fitted by nonlinear regression analysis to the Michaelis-Menten equation and plotted as a dashed line (- - -). Means ± SE of triplicate determinations are given. The unpaired Student's t-test was performed to determine statistical significance. Differences were considered significant at $p < 0.05$.

Saturation kinetics for OATP2B1-mediated transport for BDE47, BDE99, and BDE153 is shown in Figures 3-2F, 3-3F, and 3-4F, respectively. OATP2B1-mediated uptake exhibited similar transport affinities for the uptake of BDE47 (K_m: 0.81 ± 0.03μM), BDE99 (K_m: 0.87 ± 0.22μM), and BDE153 (K_m: 0.65 ± 0.07μM). BDE153 was transported with a higher affinity by OATP2B1 than by the members of the OATP1B subfamily. The capacity of OATP2B1 transport was approximately twofold higher for BDE47 and BDE153 than for BDE99, which resulted in an about twofold higher efficiency (V_{max}/K_m values) as compared to BDE99.

Figure 3-4

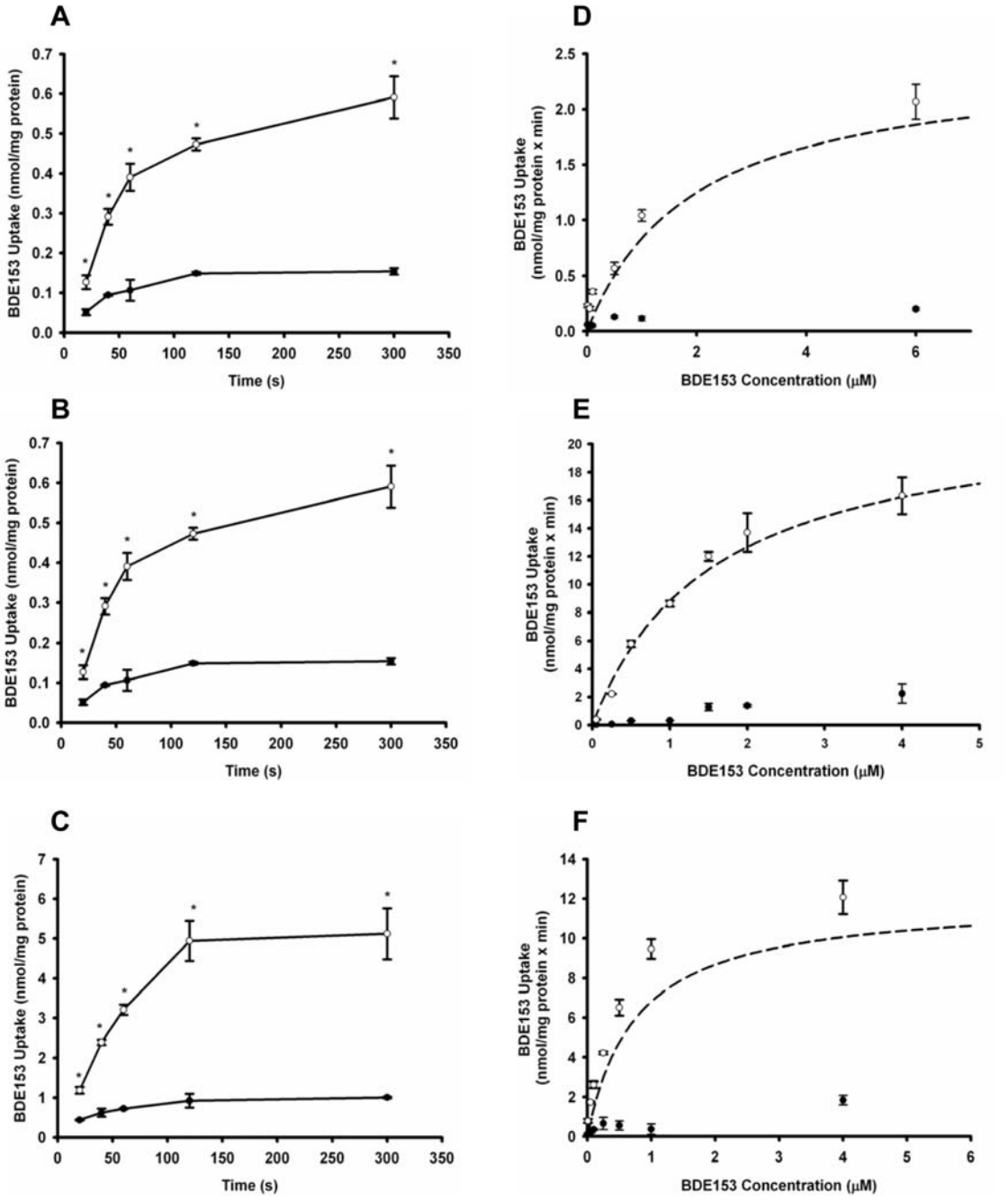

A
BDE153 Uptake (nmol/mg protein)
Time (s)
B
BDE153 Uptake (nmol/mg protein)
Time (s)
C
BDE153 Uptake (nmol/mg protein)
Time (s)
D
BDE153 Uptake (nmol/mg protein x min)
BDE153 Concentration (μM)
E
BDE153 Uptake (nmol/mg protein x min)
BDE153 Concentration (μM)
F
BDE153 Uptake (nmol/mg protein x min)
BDE153 Concentration (μM)

Figure 3-4 Time- and concentration-dependent uptake of BDE153 by OATP1B1, OATP1B3, and OATP2B1. (A) OATP1B1-, (B) OATP1B3-, or (C) OATP2B1-mediated uptake of [^{14}C]BDE153 at 37°C at the indicated time points. Filled circles (●) represent control (WT CHO; pcDNA5/FRT CHO) uptake, while open circles (○) represent OATP1B1, OATP1B3, or OATP2B1 uptake. Determination of kinetic parameters was performed in CHO cells expressing OATP1B1, OATP1B3, or OATP2B1. Uptake of increasing concentrations of [^{14}C]BDE153 was measured at 37°C for 30 s. After subtracting the values of the control (d) from OATP-expressing cells, net (D) OATP1B1-, (E) OATP1B3-, or (F) OATP2B1-mediated uptake data were fitted by nonlinear regression analysis to the Michaelis-Menten equation and plotted as a dashed line (- - -). Means ± SE of triplicate determinations are given. The unpaired Student's t-test was performed to determine statistical significance. Differences were considered significant at $p < 0.05$.

III - Discussion

The present study provides direct evidence that human hepatic OATPs (OATP1B1, OATP1B3, and OATP2B1) represent BDE47, BDE99, and BDE153 uptake systems in human liver. Additionally, we have demonstrated that OATP1B1 and OATP1B3 transport BDE47 with the highest affinity, while OATP2B1 transported all three congeners with similar affinities.

The predominant PBDE congeners detected in human liver are BDE47, BDE99, and BDE153 (Meironyte Guvenius et al., 2001; Schecter et al., 2007). Because of the anatomy of the hepatic circulation, any drug or chemical that is absorbed from the gastrointestinal tract into the portal vein must pass through the liver before reaching the systemic circulation. During this first pass effect, the potential for presystemic elimination is dependent upon the efficiency of the hepatic extraction process. PBDE concentration in the portal blood supply may be influenced by the efficiency of the hepatic extraction process. In human hepatocytes, OATP1B1, OATP1B3, and OATP2B1 function to mediate the portal clearance of large (MW > 350) amphipathic molecules (Hagenbuch and Gui, 2008). The BDE congeners with the highest serum concentration are BDE47 (~4.2nM), BDE99 (~1.0nM), and BDE153 (~0.9nM) (Sjodin et al., 2008b). At these concentrations, which are below the K_m values of the individual PBDE congeners determined in our study (Table 3-1), hepatic uptake, according to Michaelis-Menton kinetics, becomes first order and therefore depends on affinity

Table 3-1. Kinetic parameters of OATP1B1-, OATP1B3-, and OATP2B1-mediated uptake of PBDEs in CHO cells

Transporter	Substrate	K_m (μM)	V_{max} (nmol/mg protein × min)	V_{max}/K_m
OATP1B1	BDE47	0.31 ± 0.01	13.5 ± 1.33	44.0 ± 2.8
	BDE99	0.91 ± 0.02	13.9 ± 1.70	15.4 ± 2.2
	BDE153	1.91 ± 0.17	2.4 ± 0.19	1.2 ± 0.02
OATP1B3	BDE47	0.41 ± 0.06	15.7 ± 1.71	38.0 ± 1.1
	BDE99	0.70 ± 0.03	12.8 ± 0.76	18.3 ± 0.1
	BDE153	1.66 ± 0.48	22.6 ± 0.58	1.5 ± 5.3
OATP2B1	BDE47	0.81 ± 0.06	17.6 ± 1.1	22.6 ± 1.1
	BDE99	0.87 ± 0.22	8.9 ± 0.9	10.5 ± 1.6
	BDE153	0.65 ± 0.07	14.6 ± 3.1	28.1 ± 8.1

Note. Transport rates at increasing concentrations were determined at 37°C in OATP-expressing and WT CHO or vector-transfected cells. Transport values obtained from OATP-expressing cells were corrected with values obtained from WT cells, and the resulting net carrier-mediated uptake values were fitted by nonlinear regression analysis to the Michaelis-Menten equation. Means ± SE are given for three to four experiments.

for the transporters as well as blood flow. Thus, PBDEs are readily cleared from the portal blood supply and transported into the liver where they are subject to metabolic biotransformation. Importantly, it has been suggested that hydroxylated PBDES (OH-PBDEs) may have increased toxicological relevance (Meerts et al., 2000; Meerts et al., 2001; Zhou et al., 2001; Hamers et al., 2008). Disposition studies in rodents administered PBDEs by oral gavage suggest that BDE47 and BDE99 are preferentially transported into the liver when compared to BDE153 (Meerts et al., 2000; Meerts et al., 2001; Zhou et al., 2001; Chen et al., 2006; Sanders et al., 2006a; Sanders et al., 2006b; Darnerud et al., 2007; Hamers et al., 2008). The results from our study are in agreement with the published in vivo rodent studies. Transport efficiency for OATP1B1- and OATP1B3-mediated uptake, characterized by V_{max}/K_m, was greatest for BDE47 followed by BDE99 and then BDE153 (Table 3-1). These data suggest that OATP1B1 and OATP1B3 preferentially transport BDE47 and BDE99 compared to BDE153, which provides an explanation for the PBDE congener profile identified in human liver.

In addition, we investigated the role of OATP2B1 for the uptake of BDE47, BDE99, and BDE153. The results suggest that OATP2B1 does not preferentially transport any PBDE congeners since affinity and capacity were similar for BDE47, BDE99, and BDE153 (Table 3-1). It has been suggested that OATP2B1 may play a limited role in hepatic uptake since the pH of portal blood is unlikely acidic (Hagenbuch, 2010). Low extracellular pH has been shown to stimulate transport

activity of OATP2B1 localized at the apical membrane of human intestinal epithelial cells (Kobayashi et al., 2003a; Nozawa et al., 2004; Sai et al., 2006). The importance of histidine residues has been demonstrated for several pH-sensitive transporters (Miles, 1977; Grillo and Aronson, 1986; Ganapathy et al., 1987; Kato et al., 1989). Recent work has identified specific His residues that may explain the apparent pH dependency shown by OATP2B1. Specifically, in silico structural modeling of OATP2B1 revealed a His residue at position 579 in the 10th TM domain that is exposed to the extracellular medium and thus susceptible protonation changes applied by the extracellular pH (Meier-Abt et al., 2005). Additionally, stimulation of transport activity at a low extracellular pH (pH 6.5), shown for several OATPs including OATP2B1, was demonstrated to be dependent upon a His residue in the third transmembrane domain (Leuthold et al., 2009). Furthermore, OATP1C1, which lacks this His residues, did not exhibit the pH-dependent transport seen with other OATPs. PBDE congeners have been shown to be absorbed from the gastrointestinal tract in rodents. Specifically, gastrointestinal absorption has been estimated to be 80–90% for BDE47, 60–90% for BDE99, while 70% for BDE153 (Hakk et al., 2002; Staskal et al., 2005; Chen et al., 2006; Darnerud and Risberg, 2006; Sanders et al., 2006a; Sanders et al., 2006b). Given the expression of OATP2B1 at the apical membrane of human intestinal epithelial cells together with an increase in transport activity at a lower pH, it may be that OATP2B1 might play a greater role in uptake of PBDEs from the gastrointestinal tract.

Furthermore, the results of this study clarify why the congener patterns identified in humans do not reflect the composition of the commercial Penta product. For example, BDE99, which is the predominant congener in the mixture, is found to a lesser degree than BDE47 in human samples including blood and liver (Meironyte Guvenius et al., 2001; Mazdai et al., 2003; Hites et al., 2004; Schecter et al., 2005b; Schecter et al., 2007; Costa et al., 2008; Covaci et al., 2008; Sjodin et al., 2008b). The difference between the congeners profile found in the commercial mixture to that of human tissue, in particular the liver, can partly be explained by the higher affinity and greater overall transport efficiency for BDE47 by OATP1B1 and OATP1B3 compared to that for BDE99 (Table 3-1). Oxidation of many aromatic xenobiotic contaminants in the liver occurs through the catalytic action of the P450 enzymes of the hepatic mixed function oxidase system. OH-PBDE metabolites have been detected in human blood samples (Sandanger et al., 2007; Athanasiadou et al., 2008; Qiu et al., 2009). Furthermore, it was demonstrated that BDE99 is metabolized to the greatest extent followed by BDE47. Recently, two independent studies have identified oxidative metabolism of BDE47 and BDE99 through the use of human liver microsomes and cryogenically preserved human hepatocytes (Lupton et al., 2009; Stapleton et al., 2009). Again, BDE99 was shown to have a greater potential for metabolism followed by BDE47. Interestingly, BDE153 was shown to be resistant to oxidative metabolism. The authors explain this by the lack of unsubstituted adjacent carbons, which has been shown to be pivotal for the formation of the arene oxide intermediate during P450-mediated metabolism.

The greater abundance of BDE47 in human liver samples compared to that of BDE99 can be attributed to a greater uptake efficiency as well as lower rate of metabolism. Furthermore, although BDE99 is the primary component of the commercial penta mixture, its greater rate of metabolism by hepatic P450s seems to play a more important role than hepatic uptake regarding its decreased bioaccumulation. Hepatic uptake of BDE153 by OATP1B1 and OATP1B3 occurred in a low-affinity, low-capacity manner when compared to BDE47 and BDE99. However, OATP2B1-mediated transport occurs at a much higher affinity for BDE153, which is similar to K_m values for OATP1B1- and OATP1B3-mediated transport of BDE99. Additionally, BDE153 has been shown to be relatively resistant to metabolism (Sanders et al., 2006b; Lupton et al., 2009). Furthermore, in addition to DE-71, BDE153 is found in the octaBDE mixture (Darnerud et al., 2001). Taken together, high-affinity transport by OATP2B1 along with minimal metabolism can explain the greater bioaccumulation of BDE153 when compared to BDE47 and BDE99.

In conclusion, we have identified PBDE congeners BDE47, BDE99, and BDE153 as substrates of OATP1B1, OATP1B3, and OATP2B1. This has provided evidence for a transporter mediated mechanism for the hepatic accumulation of the predominant PBDE congeners. In addition, differential uptake efficiency and metabolism of BDE47, BDE99, and BDE153 together provides an explanation for the inconstancy found between ratios of PBDE concentrations in human samples to that of the commercial mixture.

Chapter 4

The Roles of Mouse Organic Anion Transporting Polypeptides in the Hepatic Uptake of PBDE Congeners

I - Introduction

PBDEs are flame retardants used in polymers incorporated into textiles, electronics, plastics and furniture. PBDEs are not covalently bound to products, but instead are chemical additives that are subject to leaching out into the environment. Consequently, PBDEs have been detected in sediment and wildlife, but potentially more pertinent to human health risks, are indoor air and house dust (Darnerud et al., 2001; Darnerud, 2003; Sjodin et al., 2003; Stapleton et al., 2006; Sjodin et al., 2008a). BDE47 is the predominant congener detected in human and wildlife samples, followed by BDE99 and BDE153 (Lorber, 2008).

Currently, health risks to humans following PBDE exposure are unknown. However, numerous animal studies have identified developmental, reproductive, neurological, and endocrine toxicity (Darnerud et al., 2001; Zhou et al., 2002; Birnbaum and Staskal, 2004; Costa and Giordano, 2007; Costa et al., 2008). It has been suggested that OH-PBDEs may play a prominent role in PBDE-mediated toxicity (Meerts et al., 2000; Zhou et al., 2001; Darnerud, 2003; Canton et al., 2008; Mercado-Feliciano and Bigsby, 2008; Szabo et al., 2009). Numerous rodent studies have reported that PBDEs induce hepatic monoxygenase *in vivo* (Sanders et al., 2005; Chen et al., 2006; Sanders et al., 2006a; Sanders et al.,

2006b; Qiu et al., 2007). Previous results from our laboratory demonstrated that PBDEs are capable of activating a xenobiotic NR, PXR (Pacyniak et al., 2007). Furthermore, rats and mice treated with the commercial mixture DE-71, consisting primarily of BDE47, BDE99 and BDE153, induced Cyp3a and Cyp2b enzymes resulting in the formation of OH-PBDEs (Zhou et al., 2001; Sanders et al., 2005; Qiu et al., 2007).

A prerequisite to activate xenobiotic NRs and induce metabolizing enzymes is that PBDE congeners can enter hepatocytes. Given that OATPs/Oatps (Figure 4-1) are polyspecific transporters that mediate uptake of numerous large, amphipathic substrates we previously tested whether human OATPs could transport PBDE congeners *in vitro*. Indeed, our recent study has shown that PBDE congeners are substrates of human OATP1B1, OATP1B3, and OATP2B1 (Pacyniak et al., 2010). Although mice are commonly used for toxicological characterization of PBDEs, nothing is known regarding transport by mouse hepatic Oatps**.** From the Oatps in mice, Oatp1a1 (*Slco1a1*), Oatp1a4 (*Slco1a4*), Oatp1b2 (*Slco1b2*), and Oatp2b1 (*Slco2b1*) are expressed in liver (Hagenbuch et al., 2000; van Montfoort et al., 2002; Cheng et al., 2005).

Figure 4-1

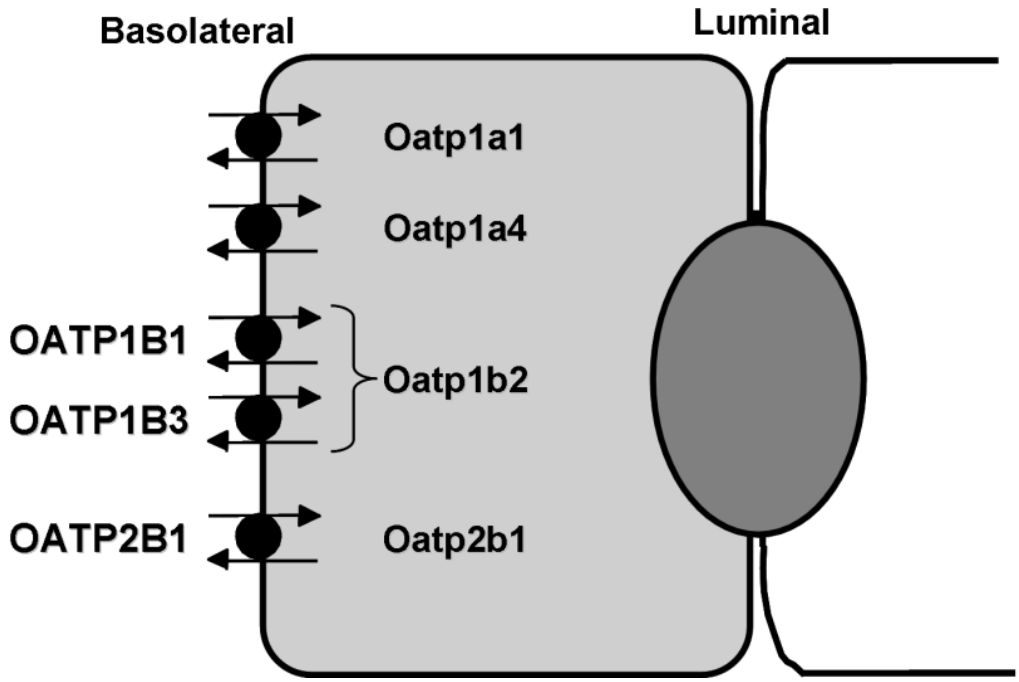

Basolateral
Luminal
Oatp1a1
Oatp1a4
OATP1B1
OATP1B3
Oatp1b2
OATP2B1
Oatp2b1

Figure 4-1 OATPs (human)/Oatps (mouse) expressed in hepatocytes.

The OATP1A sub-family has a single human member OATP1A2 (*SLCO1A2)* and two mouse members (Oatp1a1 and Oatp1a4). In contrast to its murine orthologues, OATP1A2 expression in liver is restricted to the epithelial cells of the bile duct (Lee et al., 2005). Oatp1b2 is the ortholog of both human OATP1B1 and OATP1B3 whereas Oatp2b1 is the mouse orthologue of human OATP2B1 (Cattori et al., 2000; Choudhuri et al., 2000; Ogura et al., 2000; Meyer Zu Schwabedissen et al., 2009).

In general, Oatp1a1, Oatp1a4, and Oatp1b2 demonstrate overlapping substrate specificity and transport substrates like taurocholic acid, BSP, and estrone-3-sulfate (Hagenbuch et al., 2000; van Montfoort et al., 2002; Meyer Zu Schwabedissen et al., 2009). However, unique substrates such as the cardiac glycoside digoxin, have been shown to be transported specifically by Oatp1a4 (Hagenbuch et al., 2000; van Montfoort et al., 2002). To date, no functional studies of Oatp2b1 exist.

In vitro functional characterization of Oatp substrates have significantly contributed toward our understanding of the mechanisms responsible for the absorption, distribution, and elimination of drugs/toxins. Unfortunately, quantitative extrapolation of *in vitro* data can be difficult in some cases due to differences in substrate specificity and differences in the relative expression levels of drug transporters. However, the development of Oatp-null mice are proving to be valuable tools to determine the *in vivo* contribution of hepatic Oatp

transporters to drug substrates identified using *in vitro* systems (Lu et al., 2008; Zaher et al., 2008).

Mice have been widely employed as an animal model to study the toxicity of PBDEs. The purpose of the current study was to identify the transport system responsible for uptake of PBDE congeners into mouse livers.

II - Results

4.1 Uptake of PBDE congeners by hepatic Oatps expressed in HEK293 cells

Uptake of BDE47, BDE99, and BDE153 into Oatp1a1-, Oatp1a4-, Oatp1b2- and Oatp2b1-expressing HEK293 cells was measured at a single time point of 5 mins at a single concentration of 0.08μM (BDE47 and BDE99) or 0.1μM (BDE153).

Except for Oatp1a1-expressing cells that did not transport BDE47, BDE99 or BDE153, uptake of BDE47, BDE99 and BDE153 into cells expressing Oatp1a4, Oatp1b2, or Oatp2b1 was significantly higher than uptake into the vector-transfected control cells (Figure 4-2).

Figure 4-2

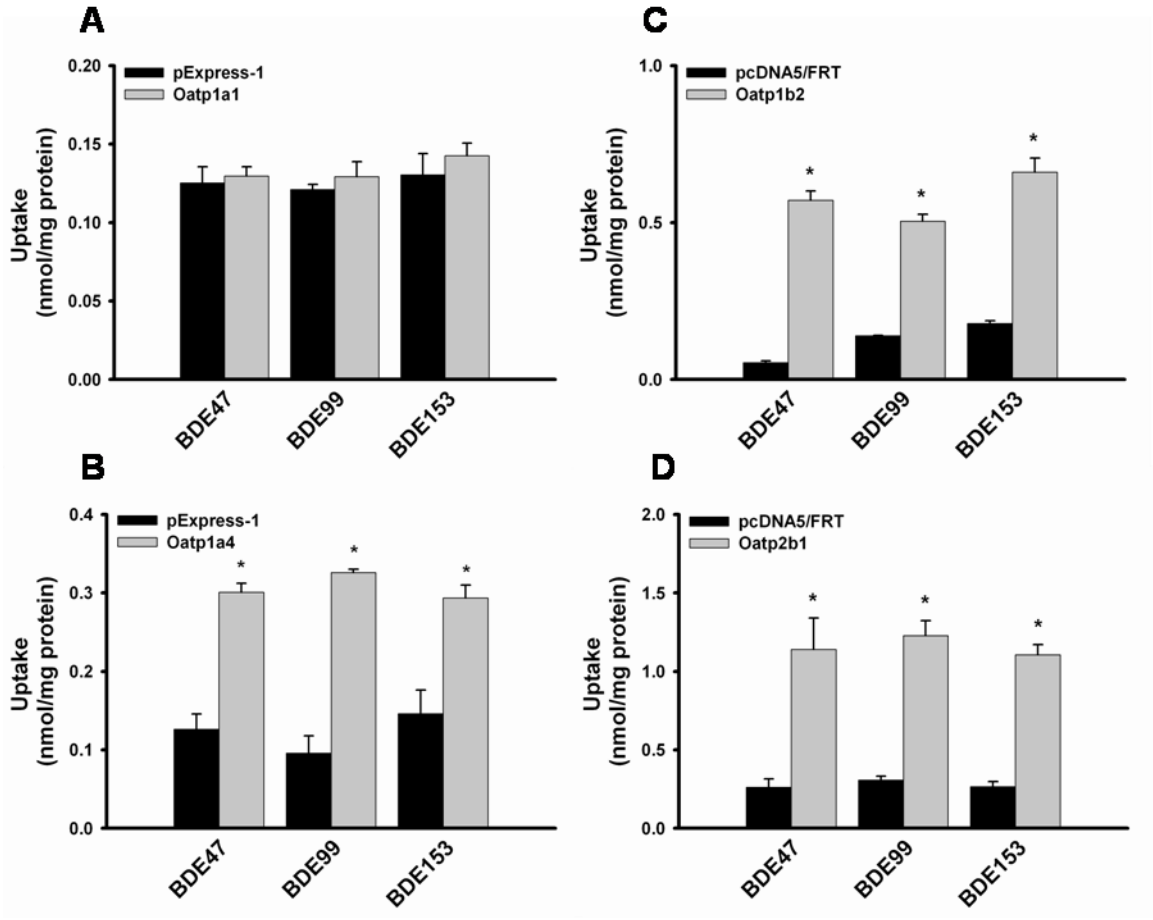

A
pExpress-1
Oatp1a1
Uptake (nmol/mg protein)
0.20
0.15
0.10
0.05
0.00
BDE47
BDE99
BDE153
C
pcDNA5/FRT
Oatp1b2
1.0
0.5
0.0
B
pExpress-1
Oatp1a4
0.4
0.3
0.2
0.1
0.0
D
pcDNA5/FRT
Oatp2b1
2.0
1.5
1.0
0.5
0.0
*

Figure 4-2 Comparison of PBDE congener uptake by mouse hepatic Oatps expressed in HEK293 cells. HEK293 cells were transiently transfected with Oatp1a1, Oatp1a4, Oatp1b2, Oatp2b1 or empty vector. Forty-eight hrs later (A) Oatp1a1-, (B) Oatp1a4-, (C) Oatp1b2-, or (D) Oatp2b1-mediated uptake was determined. Values represent means ± S.E. of triplicate samples for at least two independent experiments. *, $p<0.05$ compared to vector control.

4.2 Functional characterization of PBDE congener uptake of by Oatp1a4-, Oatp1b2-, and Oatp2b1-expressing HEK293 cells

To further characterize the uptake of PBDE by mouse hepatic transporters, BDE47, BDE99 and BDE153 transport by Oatp1a4, Oatp1b2, and Oatp2b1 was measured in a time- and concentration-dependent manner and is summarized in Table 4-1.

As shown in Figure 4-3A and 4-3C, Oatp1a4 mediated transport of BDE47, BDE99 and BDE153 was linear up to 1 min. Therefore, kinetic analysis was performed at 30 s and demonstrated that BDE47 was transported with the highest affinity (K_m = 0.41 ± 0.1μM), followed by BDE99 (K_m = 0.61 ± 0.15μM) and BDE153 (K_m = 2.0 ± 0.6μM) (Figure 4-3D, F). The V_{max} values for BDE47 (3.0 ± 0.22 nmol/mg protein x min) and BDE153 (3.0 ± 0.4 nmol/mg protein x min) were similar. BDE99, with a V_{max} of 1.1 ± 0 .1 nmol/mg protein x min, was transported with a slightly lower capacity than BDE47 and BDE153 (Fig. 4-3D, F).

Uptake of BDE47, BDE99 and BDE153 by Oatp1b2 was linear for at least 1 min (Fig. 4-4A, C). Concentration-dependent transport of PBDEs by Oatp1b2 is shown in Figure 4-4D, F. Kinetic analysis results for Oatp1b2 were similar to that of Oatp1a4 with BDE47 (0.46 ± 0.03μM) transported with the highest affinity, followed by BDE99 (0.72 ± 0.08μM) and BDE153 (1.39 ± 0.09μM).

Figure 4-3

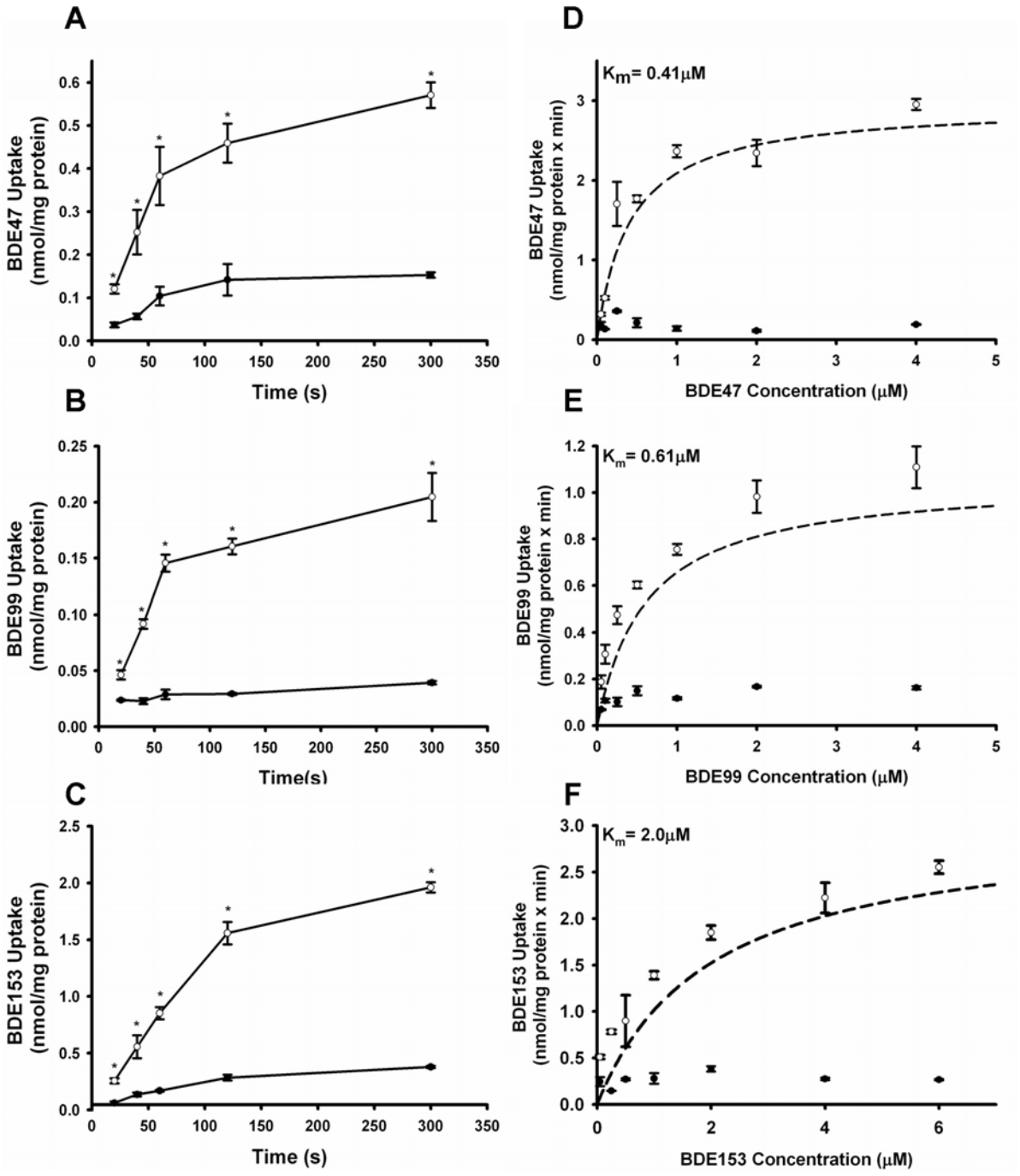

A
BDE47 Uptake (nmol/mg protein)
Time (s)
B
BDE99 Uptake (nmol/mg protein)
Time(s)
C
BDE153 Uptake (nmol/mg protein)
Time (s)
D
Km= 0.41μM
BDE47 Uptake (nmol/mg protein x min)
BDE47 Concentration (μM)
E
Km= 0.61μM
BDE99 Uptake (nmol/mg protein x min)
BDE99 Concentration (μM)
F
Km= 2.0μM
BDE153 Uptake (nmol/mg protein x min)
BDE153 Concentration (μM)

Figure 4-3 Time- and concentration-dependent uptake of PBDE congeners by Oatp1a4-expressing HEK293 cells. Time-dependent Oatp1a4-mediated uptake of (A) BDE47, (B) BDE99 and (C) BDE153 was measured at the indicated time points. Filled circles (●) represent vector control [pExpress-1] uptake whereas open circles (○) represent Oatp1a4-mediated uptake. Kinetic parameters of Oatp1a4-mediated uptake of (D) BDE47, (E) BDE99 and (F) BDE153 were determined with increasing concentrations of PBDEs under initial linear rate conditions at 37ºC with Oatp1a4-expressing and empty vector transfected HEK293 cells. Net Oatp1a4-mediated uptake was fitted to the Michaelis-Menten equation and plotted as a dashed line (- - -). Means ± S.E. of triplicate determinations are given. Differences were considered significant at $p < 0.05$.

Maximal transport rates (V_{max}) for Oatp1b2-mediated uptake had the same rank order as the K_m values with BDE47 (34.2 ± 1.3 nmol/mg protein x min) followed by BDE99 (2.8 ± 0.4 nmol/mg protein x min) and BDE153 (18.8 ± 1.3 nmol/mg protein x min). Oatp2b1-mediated uptake of BDE47, BDE99, and BDE153 was linear up to 1 min (Figure 4-5A, C), similar to that of Oatp1a4 and Oatp1b2. As shown in Figure 4-5D and 4-5F, Oatp2b1 transported all three congeners with similar affinities (BDE47: K_m = 0.95 ± 0.04μM; BDE99: K_m = 1.10 ± 0.12μM; BDE153: K_m = 1.02 ± 0.14μM) and BDE47 (7.0 ± 1.02 nmol/mg protein x min) as well as BDE99 (4.4 ± 0.58 nmol/mg protein x min) with approximately the same rate whereas BDE153 (19.0 ± 0.58 nmol/mg protein x min) was transported with a higher V_{max} than BDE47 and BDE99.

Table 4-1. Kinetic parameters of PBDE congener uptake by mouse hepatic Oatps

Transporter	Substrate	K_m (μM)	V_{max} (nmol/mg protein x min)	V_{max}/K_m
Oatp1b2	BDE 47	0.46 ± 0.03	34.2 ± 1.3	74.5 ± 3.4
	BDE 99	0.72 ± 0.14	2.8 ± 0.4	3.8 ± 0.14
	BDE 153	1.39 ± 0.09	18.8 ± 1.3	14.4 ± 1.6
Oatp2b1	BDE 47	0.95 ± 0.04	7.0 ± 1.02	7.8 ± 0.5
	BDE 99	1.10 ± 0.12	4.4 ± 0.58	4.0 ± 0.02
	BDE 153	1.02 ± 0.14	19.0 ± 0.58	20.2 ± 4.3
Oatp1a4	BDE 47	0.41 ± 0.1	3.0 ± 0.22	7.3 ± 0.7
	BDE 99	0.61 ± 0.15	1.1 ± 0.1	1.8 ± 0.6
	BDE 153	2.0 ± 0.6	3.0 ± 0.4	1.5 ± 0.5

Note: Transport rates at increasing concentrations were determined within the initial period of linearity at 37°C in Oatp expressing or vector-transfected cells. Transport values obtained from Oatp-expressing cells were corrected with values obtained from vector control cells and the resulting net carrier mediated uptake values were fitted by non-linear regression analysis to the Michaelis–Menten equation. Mean ± S.E. are given for 2-4 independent experiments.

Figure 4-4

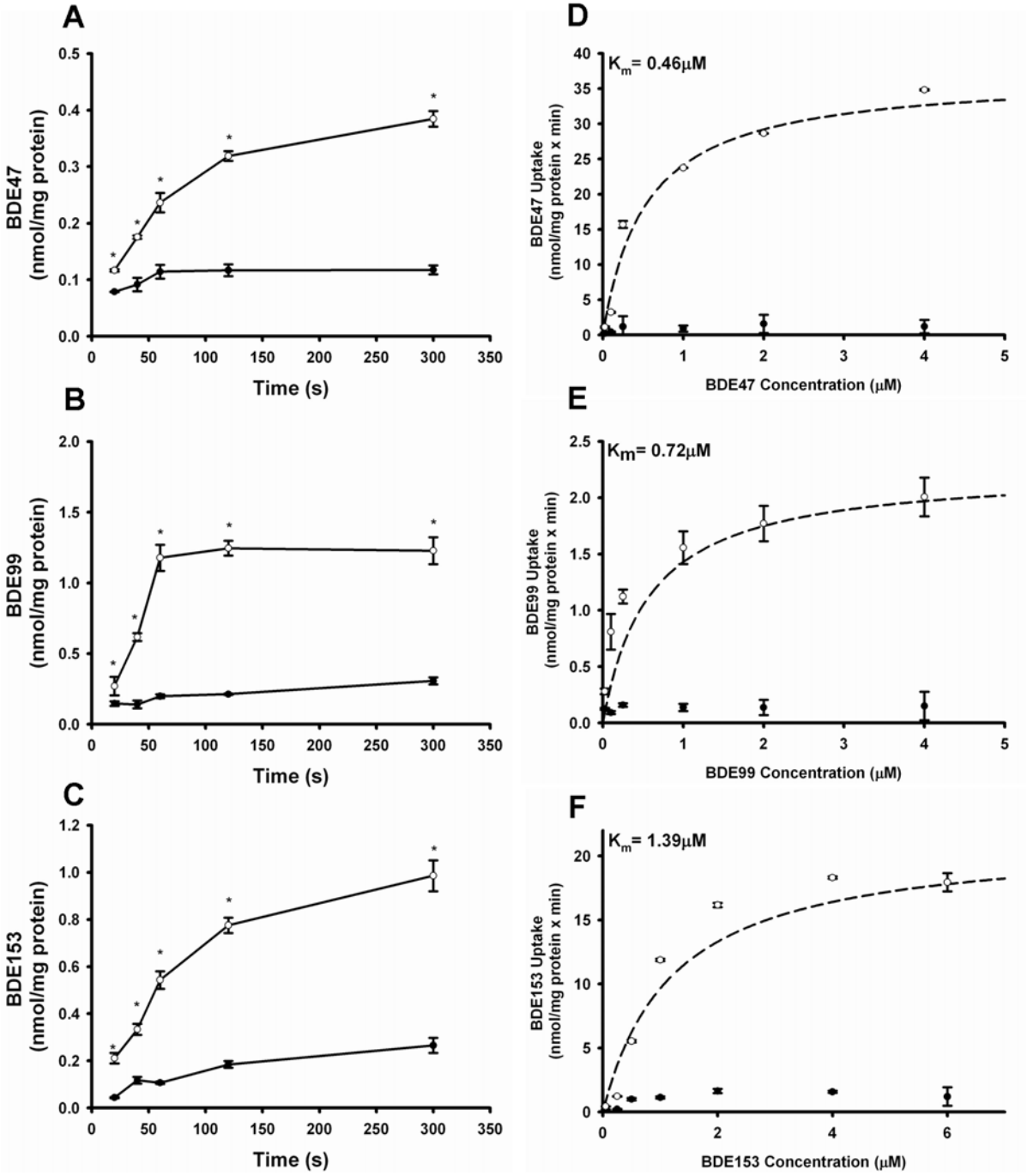

A
BDE47 (nmol/mg protein)
Time (s)
B
BDE99 (nmol/mg protein)
Time (s)
C
BDE153 (nmol/mg protein)
Time (s)
D
K_m= 0.46μM
BDE47 Uptake (nmol/mg protein x min)
BDE47 Concentration (μM)
E
K_m= 0.72μM
BDE99 Uptake (nmol/mg protein x min)
BDE99 Concentration (μM)
F
K_m= 1.39μM
BDE153 Uptake (nmol/mg protein x min)
BDE153 Concentration (μM)

Figure 4-4 Time- and concentration-dependent uptake of PBDE congeners by Oatp1b2 expressing HEK293 cells. Time-dependent Oatp1b2-mediated uptake of (A) BDE47, (B) BDE99 and (C) BDE153 was measured at 37°C at the indicated time points. Filled circles (●) represent vector control [pcDNA5/FRT] uptake whereas open circles (○) represent Oatp1b2-uptake. Kinetic parameters of Oatp1b2-mediated uptake of (D) BDE47, (E) BDE99 and (F) BDE153 were determined with increasing concentrations of PBDEs measured under initial linear rate conditions at 37ºC with Oatp1b2-expressing and empty vector transfected HEK293 cells. After subtracting the values obtained with the vector control cells and corrected for total protein concentration, net Oatp1b2-mediated uptake was fitted to the Michaelis-Menten equation and plotted as a dashed line (- - -). Means ± S.E. of triplicate determinations are given. The unpaired Student t -test was performed to determine statistical significance. Differences were considered significant at $p < 0.05$.

All kinetic parameters are summarized in Table 4-1. Intrinsic clearance, calculated as V_{max}/ K_m, for Oatp1a4-mediated transport was approximately 2- and 4-fold higher for BDE47 (7.3 ± 0.7) than for BDE153 (3.5 ± 0.5) and BDE99 (1.8 ± 0.6), respectively. Oatp1b2-mediated uptake clearance was about 20-fold higher for BDE47 (74.5 ± 3.4), than for BDE99 (3.8 ± 0.14) and 5-fold higher than for BDE153 (14.4 ± 1.6) while for Oatp2b1-mediated uptake it was highest for BDE153 (20.2 ± 4.3) followed by BDE47 (7.8 ± 0.5) and BDE99 (4.0 ± 0.02).

Figure 4-5

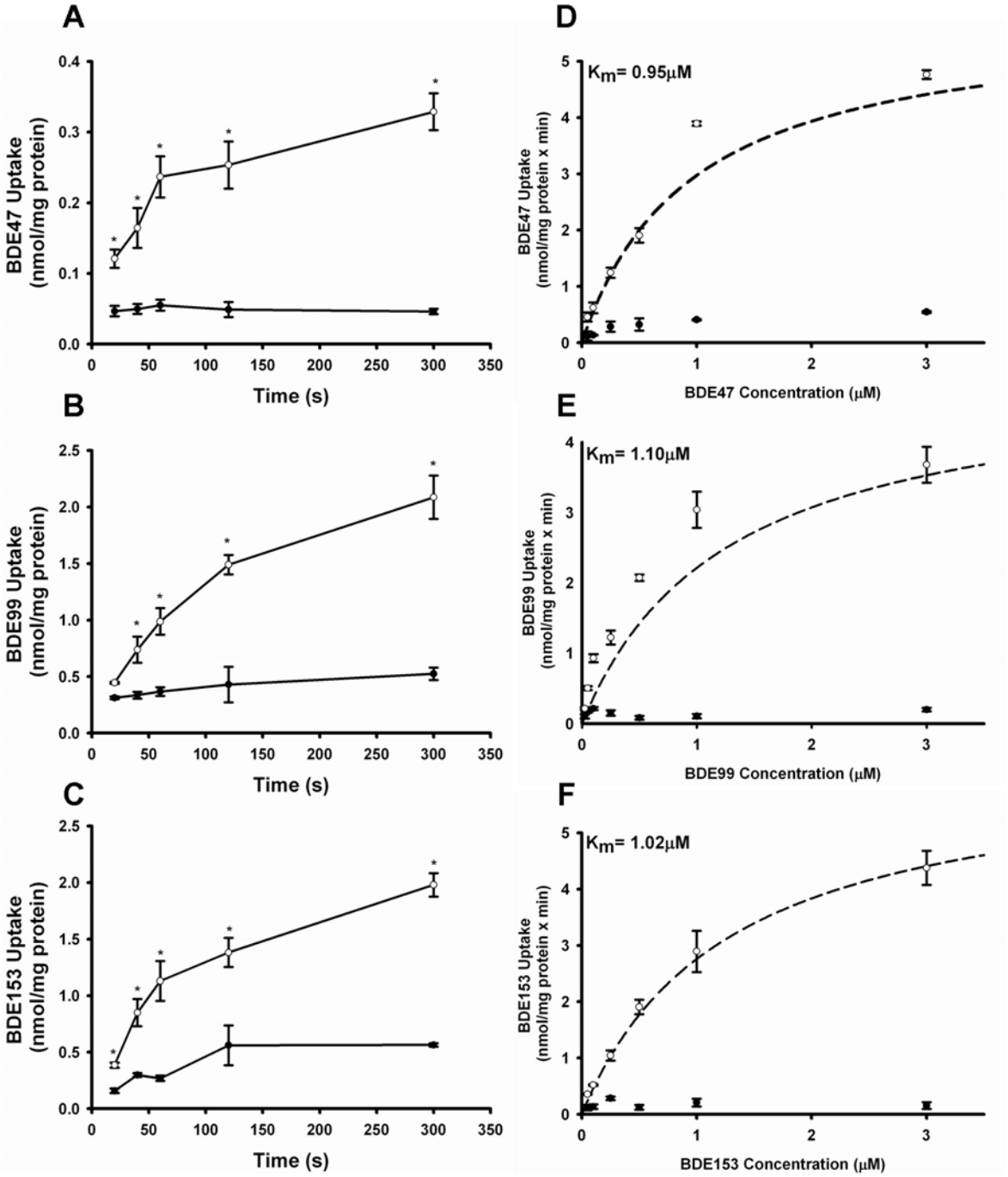

A
BDE47 Uptake (nmol/mg protein)
Time (s)
B
BDE99 Uptake (nmol/mg protein)
Time (s)
C
BDE153 Uptake (nmol/mg protein)
Time (s)
D
Km= 0.95μM
BDE47 Uptake (nmol/mg protein x min)
BDE47 Concentration (μM)
E
Km= 1.10μM
BDE99 Uptake (nmol/mg protein x min)
BDE99 Concentration (μM)
F
Km= 1.02μM
BDE153 Uptake (nmol/mg protein x min)
BDE153 Concentration (μM)

Figure 4-5 Time- and concentration-dependent uptake of PBDE congeners by Oatp2b1 expressing HEK293 cells. Time-dependent Oatp2b1-mediated uptake of (A) BDE47, (B) BDE99 and (C) BDE153 was measured at 37°C at the indicated time points. Filled circles (●) represent vector control [pcDNA5/FRT] uptake whereas open circles (○) represent Oatp2b1-uptake. Kinetic parameters of Oatp2b1-mediated uptake of (D) BDE47, (E) BDE99 and (F) BDE153 were determined with increasing concentrations of PBDEs measured under initial linear rate conditions at 37ºC with Oatp2b1-expressing and empty vector transfected HEK293 cells. After subtracting the values obtained with the vector control cells and corrected for total protein concentration, net Oatp2b1-mediated uptake was fitted to the Michaelis-Menten equation and plotted as a dashed line (- - -). Means ± S.E. of triplicate determinations are given. The unpaired Student t -test was performed to determine statistical significance. Differences were considered significant at $p < 0.05$.

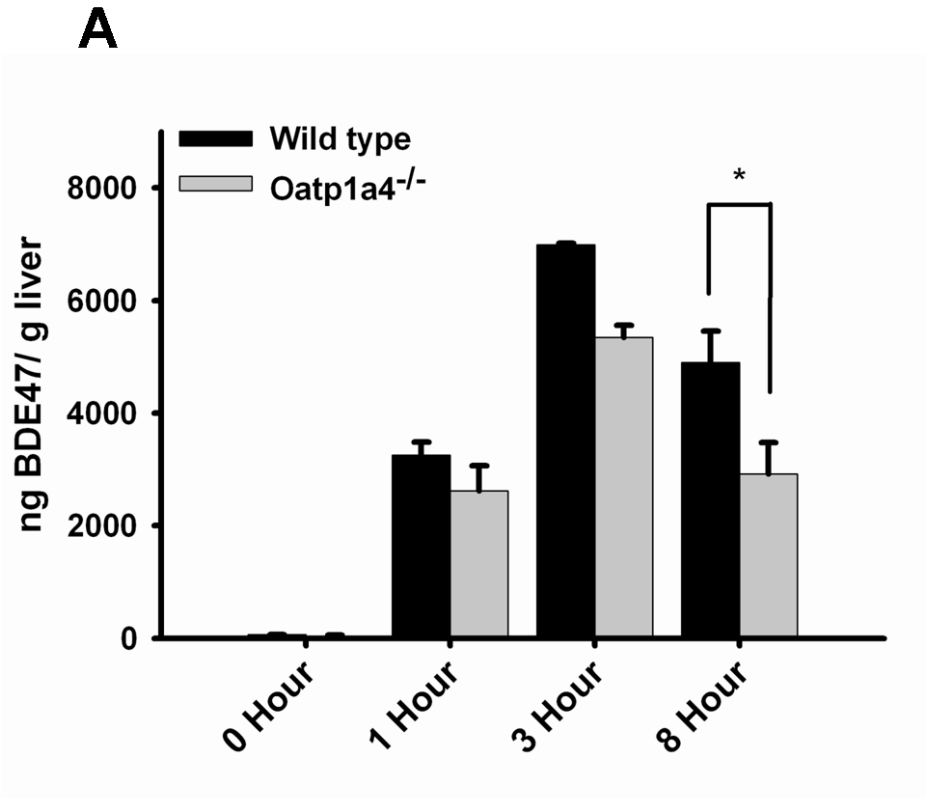
4.3 Contribution of Oatp1b2- and Oatp1a4-mediated hepatic uptake of BDE47 in vivo

To assess the *in vivo* relevance of Oatp1a4 and Oatp1b2 for PBDE disposition, hepatic uptake of BDE47 was compared between female WT and Oatp1a4- or Oatp1b2-null mice. Similar to a previously reported study (Staskal et al., 2005), WT mice demonstrated marked hepatic accumulation of BDE47 over an 8-hr period, with peak concentrations detected at the 3-hr time point (Figure 4-6A, B). In Oatp1a4-null mice, as shown in Figure 4-6A, hepatic BDE47 concentrations were reduced by 20, 24, and 41% for the 1-, 3-, and 8-hr time points, respectively. In Oatp1b2-null mice, hepatic BDE47 concentrations were decreased 47, 50, and 31% at the 1-, 3-, and 8-hr time points, respectively (Figure 4-6B).

III - Discussion

We have previously shown that PBDE congeners are substrates of OATPs expressed in human hepatocytes (Pacyniak et al., 2010). At the basolateral membrane of murine hepatocytes several Oatps, including Oatp1a1, Oatp1a4,

Figure 4-6


A
Wild type
Oatp1a4-/-
ng BDE47/ g liver
8000
6000
4000
2000
0
*
0 Hour
1 Hour
3 Hour
8 Hour

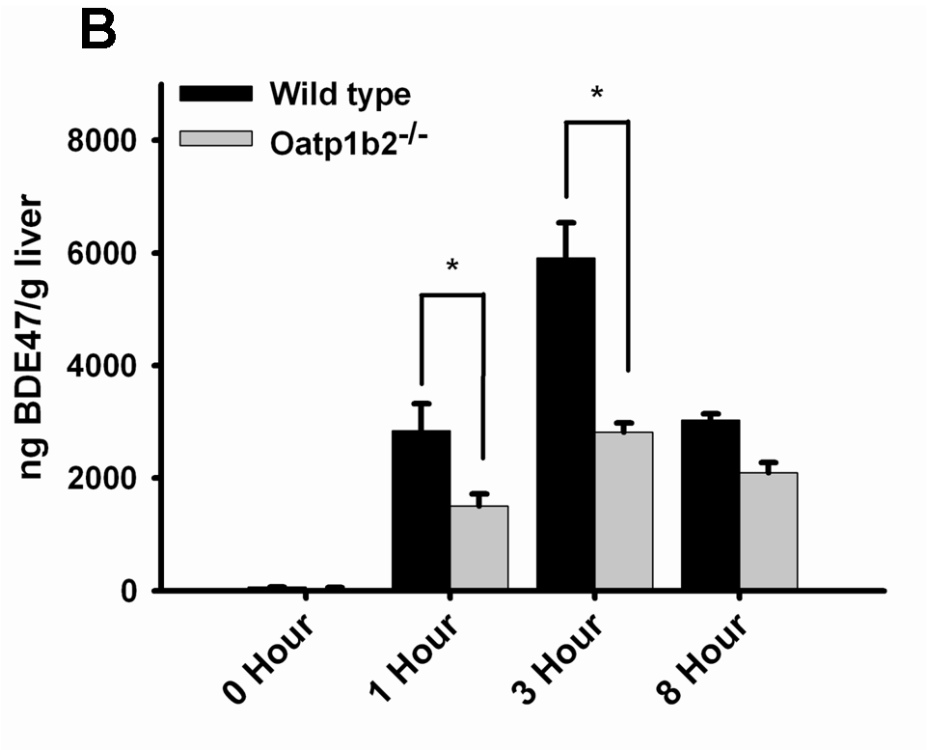

B
Wild type
Oatp1b2-/-
ng BDE47/g liver
8000
6000
4000
2000
0
*
*
0 Hour
1 Hour
3 Hour
8 Hour

Figure 4-6 Contribution of Oatp1a4 and Oatp1b2 to hepatic accumulation of BDE47 *in vivo*. Female, 10-12 week old WT, (A) Oatp1a4- or (B) Oatp1b2-null mice (n= 3-4) were administered a single oral dose of [^{14}C]BDE47 at 1mg/kg. After 0, 1, 3 and 8 hrs, livers were solubilized and radiolabeled BDE47 was quantified by liquid scintillation analysis. Statistical differences between wild type and Oatp1a4- or Oatp1b2-null mice were performed by one-way ANOVA followed by the Bonferroni t-test. The *p* value for statistical significance was set to $p < 0.05$.

Oatp1b2, and Oatp2b1, are expressed (Hagenbuch et al., 2000; Ogura et al., 2000; van Montfoort et al., 2002; Cheng et al., 2005). However, due to potential substrate differences between human OATPs and mouse Oatps it remained unclear whether mouse Oatps would also transport PBDE congeners. In the present study we demonstrated that several of the hepatic mouse Oatps can transport PBDE congeners. Specifically BDE47, BDE99 and BDE153 are substrates for Oatp1a4, Oatp1b2 and Oatp2b1, but not for Oatp1a1. Oatp1a4 and Oatp1b2 transported BDE47 with the highest affinities followed by BDE99 and BDE153. Oatp2b1 transported all three PBDE congeners with similar affinities. Using Oatp1a4- and Oatp1b2-null mice we demonstrated that *in vivo* Oatp1a4 plays a minor and Oatp1b2 plays a major role for hepatic accumulation of BDE47.

Liver is the major detoxification organ responsible for the elimination of endogenous and exogenous chemicals from the body. Hepatic uptake is a prerequisite for biotransformation and subsequent elimination. Therefore, activities of the transporters involved in the hepatic uptake process are critical factors in the systemic exposure to PBDEs. The predominant PBDE congeners detected in human liver are BDE47, BDE99, and BDE153 (Meironyte Guvenius et al., 2001; Schecter et al., 2007). The formation of OH-PBDE metabolites is of concern because greater adverse effects have been reported for the OH-PBDEs relative to the parent compound in laboratory studies (Meerts et al., 2000; Meerts et al., 2001; Hamers et al., 2006; Canton et al., 2008). Kinetic studies of PBDEs

in mice indicate that PBDE congeners initially accumulate in liver, and then redistribute to lipophilic tissues, such as the adipose tissue (Staskal et al., 2005; Staskal et al., 2006a; Staskal et al., 2006b). So far, functional studies investigating direct uptake of PBDEs by mouse hepatic uptake transporters are lacking, however, we have shown that PBDEs are transported by human hepatic OATPs (Pacyniak et al., 2010). The results obtained in the present study demonstrate that the K_m values of Oatp-mediated PBDE transport in mice are very similar to the previously published K_m values for their respective human orthologs (Table 4-2). The rank order for transport affinities was similar for the OATP1B sub-family with BDE47 transported with the highest affinity (K_m ≈0.3-0.4μM), followed by BDE99 (K_m ≈0.7-0.9μM), and then BDE153 (K_m ≈0.1.5-2μM). The OATP2B sub-family transported all three PBDE congeners with approximately the same affinity (K_m ≈1μM). Oatp1a4, which does not have a human ortholog, transported BDE47 with the highest affinity followed by BDE99 and 153. Of the two Oatp1a subfamily members, only Oatp1a4 but not Oatp1a1 transports PBDEs. Given that these two proteins have 80% amino acid sequence identity and numerous common substrates (Hagenbuch *et al.* 2000; van Montfoort *et al.* 2002), this result is somewhat surprising. However, it has been known for quite a while that the cardiac glycoside digoxin is specifically transported by Oatp1a4, (van Montfoort et al., 2002) and the three PBDEs can now also be considered to be Oatp1a4-specific substrates when comparing Oatp1a1 and Oatp1a4.

Table 4-2. Comparison of kinetic parameters of PBDE congener uptake by human and mouse hepatic OATPs/Oatps

	K_m (μM)		
OATP sub-family	BDE47	BDE99	BDE153
OATP1B1	0.31 ± 0.03	0.91 ± 0.02	1.91 ± 0.17
OATP1B3	0.41 ± 0.06	0.70 ± 0.03	1.66 ± 0.48
Oatp1b2	0.46 ± 0.03	0.72 ± 0.14	1.39 ± 0.09
OATP2B1	0.81 ± 0.06	0.87 ± 0.22	0.65 ± 0.07
Oatp2b1	0.95 ± 0.04	1.10 ± 0.12	1.02 ± 0.14

Note. K_m values obtained from human OATP-mediated transport are from our previously published study (Pacyniak et al., 2010). For all kinetic data, transport rates at increasing concentrations were determined at 37°C in OATP- or Oatp--expressing and WT CHO or vector-transfected cells. Transport values obtained from OATP- or Oatp-expressing cells were corrected with values obtained from WT or vector-transfected control cells, and the resulting net carrier-mediated uptake values were fitted by nonlinear regression analysis to the Michaelis-Menten equation. Means ± SE are given for three to four experiments.

BDE47 is a major component of the PentaBDE formulation which was widely used in the United States (Darnerud et al., 2001; Birnbaum and Cohen Hubal, 2006). As a result, BDE47 is the primary congener detected in humans (Sjodin et al., 2003; Schecter et al., 2005b; Sjodin et al., 2008b). From a toxicological standpoint, BDE47, and potentially more important, OH-BDE47, has been shown to have endocrine activity and produce developmental, reproductive, and neurotoxic effects (Costa et al., 2008; Szabo et al., 2009; Kodavanti et al., 2010a; Kodavanti et al., 2010b). Our *in vitro* studies suggest high affinity BDE47 uptake by both Oatp1a4 and Oatp1b2 (Table 4-1). Thus, we were interested in the *in vivo* contribution by Oatp1a4 and Oatp1b2 for hepatic BDE47 accumulation. Portal clearance of PBDEs is governed by blood flow and efficiency of the hepatic extraction process. It has been demonstrated that highly perfused tissues, such as the liver, achieved peak BDE47 concentrations 3 hrs after exposure (Staskal et al., 2005). Our *in vivo* data further support the idea that Oatp1b2 mediates BDE47 hepatic uptake in a high-capacity manner during the early time points. Liver accumulation of BDE47 over the 1- and 3-hr time period was only moderately reduced by 20 and 24%, respectively in Oatp1a4-null mice (Figure 4-6A). However, over the same time period, BDE47 concentrations in the liver were reduced approximately 50% in Oatp1b2-null mice (Figure 4-6B) suggesting that Oatp1b2 plays a more important role than Oatp1a4 in hepatic transport when BDE47 concentrations are approaching (1 hr) or are at peak (3 hr) levels. The 41% reduction in hepatic BDE47 accumulation in the Oatp1a4-null mice at the 8-hr time point suggests that as portal concentrations of BDE47 decrease transport

by Oatp1a4 may predominate. Our *in vitro* data support the suggestion that Oatp1b2 is a high capacity transporter, but because transporter expression levels depend on the expression system and because expression of Oatp1a4 and Oatp1b2 might not be controlled in HEK293 cells as they are in mouse hepatocytes, we cannot make any definitive conclusions from our *in vitro* studies.

Gastrointestinal absorption of PBDE congeners has been estimated to be 80–90% for BDE47, 60–90% for BDE99, and 70% for BDE153 (Hakk et al., 2002; Staskal et al., 2005; Chen et al., 2006; Darnerud and Risberg, 2006; Sanders et al., 2006a; Sanders et al., 2006b). In humans OATP2B1 has been detected at the protein level in liver, heart, placenta, brain, and the small intestine and has been suggested to be involved in drug uptake from the small intestine into the body (Kullak-Ublick et al., 2001; St-Pierre et al., 2002; Kobayashi et al., 2003a; Bronger et al., 2005; Grube et al., 2006). In mice, Oatp2b1 mRNA has been detected in multiple tissues including liver, kidney, lung, brain, and small intestine (Cheng et al., 2005). We have shown in this study that Oatp2b1 can transport all three PBDE congeners suggesting that besides its limited role in hepatocytes next to Oatp1a4 and Oatp1b2, it might be the major uptake system responsible for PBDE uptake in mouse enterocytes. However, in the absence of Oatp2b1 knockout mice this suggestion remains to be confirmed.

Neurotoxicity has been demonstrated for PBDEs and is considered an important age-dependent toxicological endpoint. Furthermore, infants and toddlers have

the highest body burden of PBDEs, due to exposure via maternal milk and house dust inhalation (Costa and Giordano, 2007; Costa et al., 2008). PBDEs have been shown to mediate numerous developmental neurotoxicity endpoints including the perturbation of intracellular signaling events, disturb development of neural progenitor cells and the production of oxidative stress (Alm et al., 2006; Huang et al., 2009; Alm et al., 2010; Belles et al., 2010; Tagliaferri et al., 2010). Notably, BDE99 has been suggested especially neurotoxic (Eriksson et al., 2001; Branchi et al., 2003; Costa and Giordano, 2007; Kuriyama et al., 2007). Oatp1a4 and Oatp2b1 are among the several OATPs/Oatps have been localized to the blood-brain barrier (Bronger, 2005; Hagenbuch, 2002; Hagenbuch, 2004) and thus could be responsible for the transport of PBDEs into the brain. This assumption is supported by a recent report that demonstrated Oatp1a4-dependent blood to brain uptake of various compounds including taurocholate and pravastatin using Oatp1a4-null mice (Ose et al., 2010). Thus, OATP/Oatp-mediated uptake of BDE99 could represent the underlying mechanism for PBDE uptake into the brain.

In conclusion, the data presented in the current study demonstrate that uptake of BDE47, BDE99 and BDE153 into mouse hepatocytes is mediated by Oatps. In addition, Oatp1b2 appears to be the major transporter for BDE47 uptake in liver and Oatp1a4 plays a minor role. Furthermore, the data also suggest that transport of PBDEs into the brain can be mediated by Oatp1a4 and Oatp2b1.

Overall the current study establishes mice as a model system to study PBDE disposition in humans.

Chapter 5

The Flame Retardants, Polybrominated Diphenyl Ethers, are PXR Activators

I - Introduction

The PBDE congeners are used as flame retardants by being incorporated into potentially flammable materials, such as plastics, rubbers, and textiles. The commercial products are produced by bromination of diphenyl oxide and contain mixtures of PBDE congeners (IPCS, 1994). PBDEs are released into the environment during their manufacture and disposal, and by recycling of plastic materials, such as TV sets and computer shells (Riess et al., 2000). Human exposure to PBDEs is chronic and is likely via the route of food consumption and inhalation. In the last several decades, increasing use and environmental contamination of PBDEs has led to a marked increase in PBDE levels in humans. PBDE congeners have been detected in plasma, liver, adipose tissue, and breast milk, suggesting that PBDEs are bioaccumulative and persistent (Hardell et al., 1998; Meneses et al., 1999; Sjodin et al., 1999; Ryan, 2000; Darnerud et al., 2001; Sjodin et al., 2001; Covaci et al., 2003). BDE47 and BDE99 are the predominant congeners detected in humans, whereas BDE209 predominates in computer wipes and house-dust (Schecter et al., 2003; Schecter et al., 2004; Schecter et al., 2005a; Schecter et al., 2005b; Schecter et al., 2006). In general, human samples from the US have higher PBDEs than those from Europe or Asia (Darnerud et al., 2001; Schecter et al., 2005b). Of particular concern is the

unexpected high levels of PBDEs in US women and milk (Petreas et al., 2003). This is because transporting PBDEs from mother to breast-fed babies can significantly increase the exposure levels of PBDEs to infants.

Numerous studies have shown that PBDEs have adverse effects on laboratory animals. Accumulated evidence shows that PBDEs reduce serum total and free T_4 levels (Fowles et al., 1994; Hallgren et al., 2001; Zhou et al., 2001; Zhou et al., 2002), are neurotoxins (Madia et al., 2004; Kodavanti and Ward, 2005), cause hepatic oxidative stress (Fernie et al., 2005), and are labeled as rodent carcinogens (NTP, 1986). These chemicals markedly induce P450 enzyme activities (Fowles et al., 1994; Zhou et al., 2001; Zhou et al., 2002), with a recent report showing that PBDEs induce Cyp3a and Cyp2b in rats (Sanders et al., 2005), however, the mechanism(s) by which PBDEs induce P450 enzymes remains unknown.

Several mechanisms may be responsible for induction of P450 enzymes, including transcriptional regulation, mRNA stabilization, and post-translational modification of the proteins (Jaeschke et al., 2002). However, the most common mechanism for inducing Cyp3a is via activation of PXR, a ligand-activated transcription factor in the NR superfamily (Kliewer et al., 2002). PXR can be activated by a wide range of chemicals, including several endogenous compounds, such as pregnanes (Kliewer et al., 1998) and lithocholic acid (Staudinger et al., 2001; Xie et al., 2001), as well as xenobiotics, including

rifampicin (antibiotic), tamoxifen (anticancer), troglitazone (anti-type II diabetic), ritonavir (antiviral), and herbal supplements, such as St John's wort (Bertilsson et al., 1998; Blumberg et al., 1998; Kliewer et al., 1998; Lehmann et al., 1998; Zhang et al., 1999; Jones et al., 2000; Maloney et al., 2000). The human counterpart for rodent PXR is SXR (Blumberg et al., 1998). There is a clear species difference between rodent PXR and human SXR (Kliewer et al., 2002). For example, PXR is activated by PCN but not by rifampicin, whereas SXR is activated by rifampicin but not by PCN. In addition, activated PXR and SXR bind to different response elements in the promoters/enhancers of their target genes: the most common response element for PXR is a direct repeat of AGG/TTCA separated by 3 nucleotides (DR3). Additionally, a recent study identified a novel PXR binding site with a (5n+4) spacer (Cui et al., 2010). The most common response element for SXR is an everted repeat of AGG/TTCA separated by 6 nucleotides (ER6).

PXR/SXR is crucial to the regulation of metabolism and transport of xenobiotic and endogenous chemicals. Activation of PXR/SXR induces a network of genes important in xenobiotic disposition, such as the uptake transporter Oatp1a4, phase-I and -II metabolizing enzymes, exemplified by Cyp3a11 and Ugt1a9, and efflux transporters, such as Mdr1a and Mrp2 (Abe et al., 1998; Kliewer et al., 1998; Lehmann et al., 1998; Guo et al., 2002). Activation of PXR/SXR results in enhanced elimination of some endogenous chemicals and xenobiotics that are substrates for transporters and/or drug-metabolizing enzymes. However,

activation of PXR can also be detrimental, because enhanced elimination of endogenous hormones, such as T_3/T_4, leads to endocrine disruption (Zhou et al., 2001; Zhou et al., 2002; Kretschmer and Baldwin, 2005; Zhai et al., 2006). In addition, disruption of the PXR gene in mice reveals severe defects in drug and chemical metabolism and disposition, including the inability to induce Cyp3a11, the phase-I enzyme that metabolizes over 50% of pharmaceuticals. Furthermore, PXR-null mice exhibit dysfunction in apoptosis and response to hepatic oxidative stress, which may affect cell proliferation and tumor formation (Gong et al., 2005; Zucchini et al., 2005).

Thus, the goal of the current study is to test the hypothesis that PBDEs induce P450 Cyp3a by activating PXR.

II - Results

5.1 PBDE congeners are Cyp2b10 and Cyp3a11 inducers in mice

Induction of rat Cyp3a and 2b by PBDEs has been demonstrated by Sanders et al. (2005). To determine whether PBDEs induce gene expression of these two enzymes in mice, we treated C57BL/6 male mice with various chemicals that are known to induce these two enzymes, together with several forms of PBDEs, namely, PBDEs 47, 99, 209, and DE-71, a mixture of BDE47 and BDE99 (Table 5-1). Levels of mRNA that are used to assess gene expression were determined and quantified by two independent methods, Northern-blot analysis and bDNA

assay, shown in Figures 5-1 and 5-2, respectively. The fold induction determined from these two methods was summarized in Table 5-2.

Our data show that all the positive controls induced their corresponding target genes. For example, PCB126 induced a 106 fold increase of cyp1a1/2 by Northern-blot analysis and 478 fold increase by bDNA assay. PCB153 induced 5 fold of Cyp2b10 by Northern-blot and 5.5 fold by bDNA. TCPOBOP induced Cyp2b10 more than 2000 fold by Northern-blot, and 319 fold by bDNA. PCN and TCPOBOP induced 2 fold of Cyp3a11, determined by both Northern-blot and bDNA assay. Treatment with DE-71 (containing PBDEs 47 and 99), PBDEs 47, 99, and 209 induced the mRNA levels of Cyp3a11 and Cyp2b10. Specifically, 300 μmol/kg/day of DE-71 and 10 and 100μmol/kg/day of PBDEs 47, 99, and 209 induced Cyp3a11 to a similar degree, 4 to 5 fold as revealed by Northern-blot (Figure 5-1) analysis and 2 fold by bDNA assay (Figure 5-2). Cyp2b10 is highly induced compared to Cyp3a11, with 110 and 58 fold induction by DE-71 (300 μmol/kg/day), determined by Northern-blot analysis and bDNA assay, respectively.

Figure 5-1

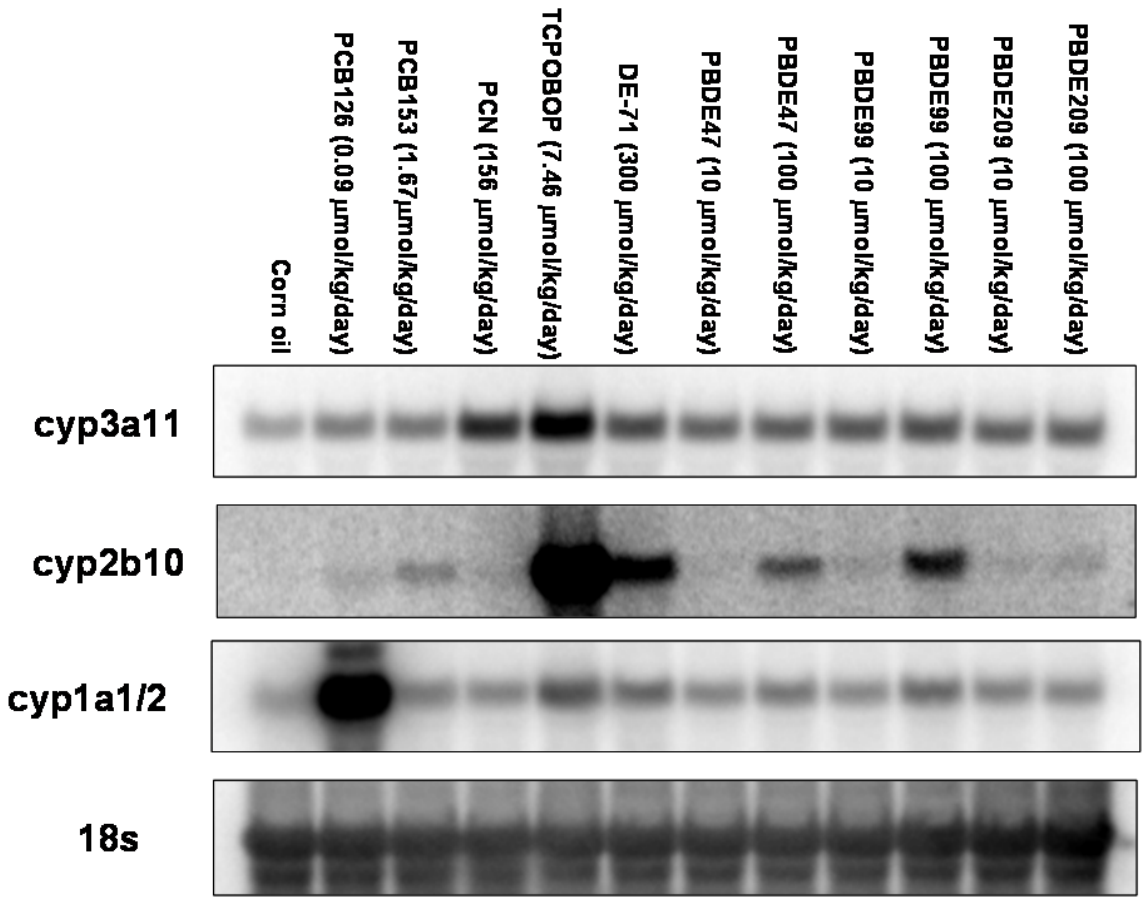

Corn oil
PCB126 (0.09 µmol/kg/day)
PCB153 (1.67µmol/kg/day)
PCN (156 µmol/kg/day)
TCPOBOP (7.46 µmol/kg/day)
DE-71 (300 µmol/kg/day)
PBDE47 (10 µmol/kg/day)
PBDE47 (100 µmol/kg/day)
PBDE99 (10 µmol/kg/day)
PBDE99 (100 µmol/kg/day)
PBDE209 (10 µmol/kg/day)
PBDE209 (100 µmol/kg/day)
cyp3a11
cyp2b10
cyp1a1/2
18s

Figure 5-1 Northern-blot analysis of hepatic Cyp3a11, Cyp2b10, and Cyp1a1/2 mRNA levels after treatment of mice with various chemicals shown in Table 5-1. The 12 lanes are the pooled total RNA from the 12 groups of mice. Specifically, lane 1 is for corn oil, lane 2 for PCB126, lane 3 for PCB153, lane 4 for PCN, lane 5 for TCPOBOP, lane 6 for DE-71, lanes 7 and 8 for PBDE47 at 10 and 100 μmol/kg/day, respectively, lanes 9 and 10 for PBDE99 at 10 and 100 μmol/kg/day, respectively, and lanes 11 and 12 for PBDE209 at 10 and 100 μmol/kg/day, respectively. The internal loading control is the 18s ribosomal RNA because several housekeeping genes, such as GAPDH, may be induced by microsomal enzyme inducers. The fold induction is summarized in Table 5-2.

Table 5-1 Animal groups for P450 enzyme induction

Groups	Chemical	Activated nuclear receptor	Dose
1	Corn oil	Vehicle	0.1 ml/10 g of body weight
2	PCB126	AhR	0.09 μmol or 0.03 mg/kg/day
3	PCB153	CAR	1.67 μmol or 0.6 mg/kg/day
4	PCN	PXR	156 μmol or 50 mg/kg/day
5	TCPOBOP	CAR	7.46 μmol or 3 mg/kg/day
6	DE-71	Unknown	300 μmol or 150 mg/kg/day
7	PBDE47	Unknown	10 μmol or 4.9 mg/kg/day
8	PBDE47	Unknown	100 μmol or 49 mg/kg/day
9	PBDE99	Unknown	10 μmol or 5.7 mg/kg/day
10	PBDE99	Unknown	100 μmol or 57mg/kg/day
11	PBDE209	Unknown	10 μmol or 9.6 mg/kg/day
12	PBDE209	Unknown	100 μmol or 96 mg/kg/day

Note. Twelve groups of male C57BL/6 mice (10 weeks old, n = 3–4 per group) were treated with PBDE congeners or the appropriate vehicle or positive controls. Corn oil was used as the vehicle, and PCB126 was used as the positive control for activation of aryl hydrocarbon receptor (De Gottardi et al.) and induction of Cyp1a1/2, PCB153, and TCPOBOP for activation of CAR and induction of Cyp2b10, and PCN activation of PXR and induction of Cyp3a11. The animals were dosed daily by ip injection for 4 days, followed by removal of the liver 24 hrs after the last injection.

BDE47, 99, and 209, at 10μmol/kg/day, induced Cyp2b10 11, 4, and 3 fold, respectively, determined by Northern-blot analysis; and 2, 5 and 4, respectively, by bDNA assay. At 100μmol/kg/day, these PBDE congeners induced Cyp2b10 to a higher level with 42, 74, and 5 fold induction, respectively, by Northern-blot analysis, and 22, 37, and 7 fold induction, respectively, by bDNA assay. The levels of Cyp1a1/2 mRNA were induced by all the chemicals treated when determined by Northern-blot analysis. Because Northern-blot analysis is not as specific as bDNA assay, mostly due to cross react of the Northern-blot probes to transcripts with similar sequences, we also measured Cyp1a1/2 mRNA levels by bDNA assay, with the results clearly showing that except for PCB126 and PCB153, none of the chemicals induced Cyp1a1/2, including PBDE congeners.

5.2 PBDEs are PXR/SXR activators *in vitro*

Most Cyp3a11 and 2b10 inducers are activators of PXR, so we tested whether PBDEs are activators of mouse PXR and human SXR. Shown in Figure 5-3, at lower concentrations (100nM and 1μM), only PBDE99 activated PXR. At higher concentrations (10μM), PBDEs 47 and 99 activated mouse PXR to a similar

Figure 5-2

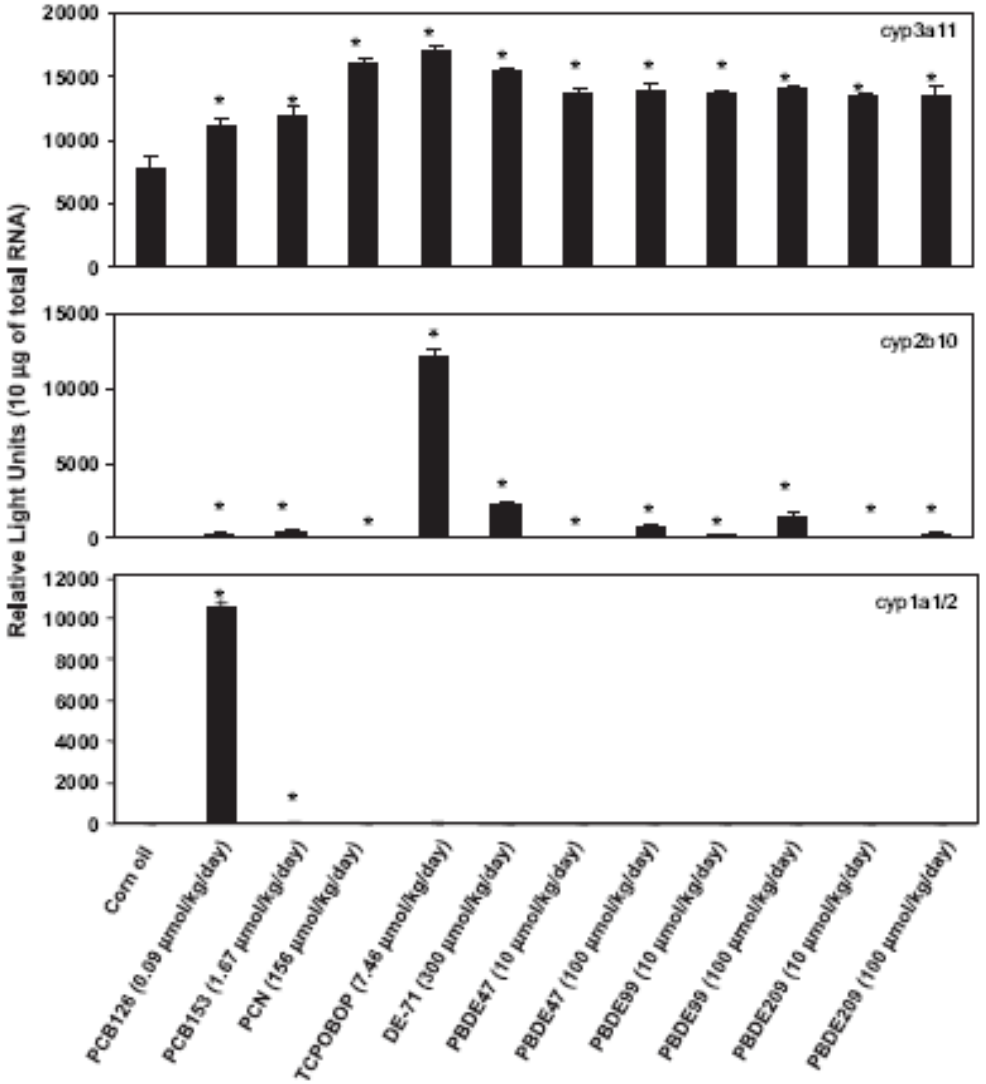

cyp3a11
cyp2b10
cyp1a1/2
Relative Light Units (10 µg of total RNA)
20000
15000
10000
5000
0
12000
8000
6000
4000
2000
Corn oil
PCB126 (0.09 µmol/kg/day)
PCB153 (1.67 µmol/kg/day)
PCN (156 µmol/kg/day)
TCPOBOP (7.46 µmol/kg/day)
DE-71 (300 µmol/kg/day)
PBDE47 (10 µmol/kg/day)
PBDE47 (100 µmol/kg/day)
PBDE99 (10 µmol/kg/day)
PBDE99 (100 µmol/kg/day)
PBDE209 (10 µmol/kg/day)
PBDE209 (100 µmol/kg/day)

Figure 5-2 Levels of hepatic Cyp3a11, Cyp2b10, and Cyp1a1/2 mRNA determined by bDNA assay. Total RNA was isolated from livers of mice treated with the chemicals shown in Table 5-1. The mRNA levels of Cyp3a11, Cyp2b10, and Cyp1a1/2 were determined by bDNA assay. The asterisk indicates that *p* value is < 0.05 comparing chemical- and corn oil–treated groups.

Table 5-2. Fold induction of hepatic Cyp3a11, Cyp2b10, and Cyp1a1/2 following PBDE Treatment (obtained from Northern-blot analysis and bDNA assay)

		cyp3a11		cyp2b10		cyp1a1/2	
Groups	Chemicals (μmol/kg/day)	Northern blot	bDNA	Northern blot	bDNA	Northern blot	bDNA
1	Corn oil	1	1	1	1	1	1
2	PCB126 (0.09)	3	1.4	5	5.5	106	477.7
3	PCB153 (1.67)	4	1.5	21	12.3	6	3.1
4	PCN (156)	10	2.0	4	3.8	9	1.0
5	TCPOBOP (7.46)	17	2.2	2139	319.4	37	1.8
6	DE-71 (300)	5	2.0	110	57.5	17	1.0
7	PBDE47 (10)	5	1.8	11	2.1	10	0.7
8	PBDE47 (100)	5	1.8	42	21.9	16	0.8
9	PBDE99 (10)	5	1.8	4	5.1	9	1.0
10	PBDE99 (100)	5	1.8	74	36.9	16	0.9
11	PBDE209 (10)	4	1.7	3	3.6	9	0.5
12	PBDE209 (100)	4	1.7	5	7.0	6	0.8

Note. The fold induction was obtained by comparing mRNA levels between corn oil- and chemical-treated mice. Northern blot was quantified by ImageQuant computer software.

degree, and at the highest concentration tested (100μM), PBDE209 also activated PXR. Compared to the known PXR activator, PCN, which activates PXR at relatively higher concentrations (10 and 100μM), BDE47 and BDE99 activated PXR at lower concentrations (1 μM for PBDE47 and 100 nM and 1μM for PBDE99). However, at higher concentration (10 and 100μM), PCN was stronger than PBDE congeners in activating PXR. Activation of human SXR is shown in Figure 5-4A. All three PBDEs activated human SXR dose-dependently. In contrast to activating mouse PXR, the potency of BDE47 and 99 is similar in activating human SXR, followed by PBDE209. In addition, one striking observation for these PBDE congeners was they activated SXR to a similar degree compared to a known SXR potent ligand, rifampicin. We also tested whether PBDEs activated AhR *in vitro* in a HepG2 cell line stably transfected with human AhR cDNA and AhR-response element in a luciferase vector (DRE cell line). The results showed that at concentrations that activated SXR, these three PBDEs activated AhR to a much less degree than the classical AhR activator, TCDD (Figure 5-4B). Therefore, the *in vitro* experiments show that PBDEs are mouse PXR and human SXR activators, but are very weak or not in activating human AhR.

Figure 5-3

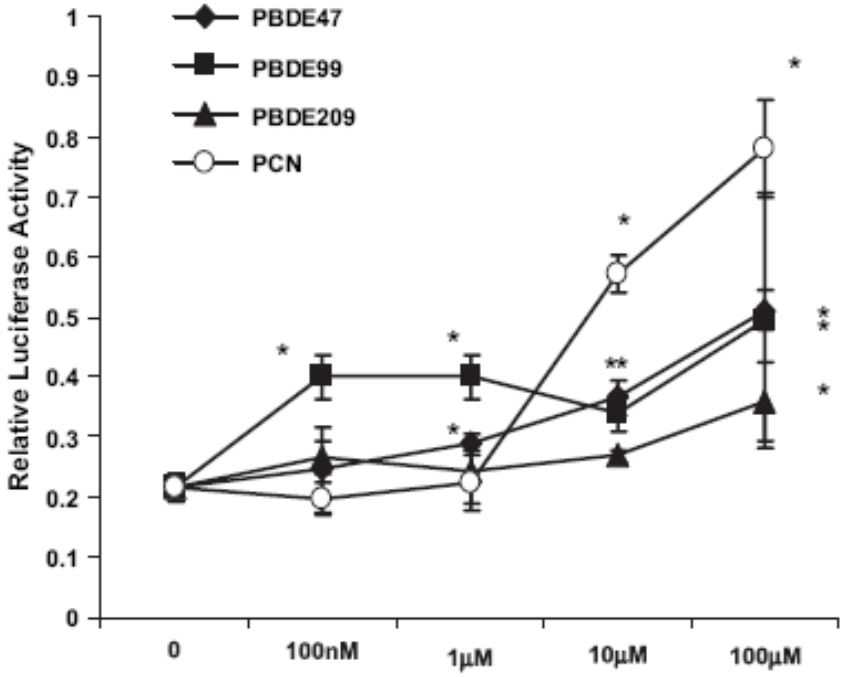

PBDE47
PBDE99
PBDE209
PCN
Relative Luciferase Activity
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
100nM
1μM
10μM
100μM

Figure 5-3 *In vitro* activation of mouse PXR by BDE47, BDE99, and BDE209. HepG2 cells were transiently transfected with mouse PXR expression vector (pSG5-mPXR), three copies of PXR-response element DR3 in a firefly luciferase vector pGL4-TK, and pCMV-renilla luciferase vector that serves as the control for transfection efficiency. Twenty-four hrs following addition of PCN or PBDEs at indicated concentrations, firefly and renilla luciferase activities were quantified, and the transfection efficiency was normalized by comparing firefly and renilla luciferase activities. The asterisk indicates that p value is < 0.05 comparing DMSO–treated cells (concentration 0).

5.3 PBDEs are PXR activators *in vivo*

After determining that PBDEs are PXR/SXR activators *in vitro*, we tested whether PBDEs activate PXR *in vivo* by using PXR-null mice on a pure C57BL/6 genetic background. As shown in Figure 5-5A and C, treatment of WT mice with BDE47, 99, and 209 induced Cyp3a11 mRNA and protein, compared to corn-oil treated group. However, the induction of Cyp3a11 mRNA and protein in the PXR-null mice was markedly suppressed when comparing the levels of Cyp3a11 between corn-oil and PBDEs-treated groups, but not completely diminished. The induction of Cyp2b10 mRNA by PBDEs in PXR-null mice was suppressed and the induction of Cyp2b10 protein was abolished (Figure 5-5B, C).

III - Discussion

The current study provides convincing evidence that PBDEs induce Cyp3a11 and Cyp2b10, but not Cyp1a1/2 in mouse livers. We also provide the first evidence that PBDEs activated both PXR and SXR *in vitro*. In addition, induction by PBDEs of the genes encoding these enzymes was suppressed in PXR-null mice compared to WT mice. This study is important, because for the first time the molecular mechanism by which PBDEs induce P450 enzymes is elucidated. In addition, because little information regarding human exposure and toxicity of PBDEs is available, our study helps to provide a scientific basis for

Figure 5-4

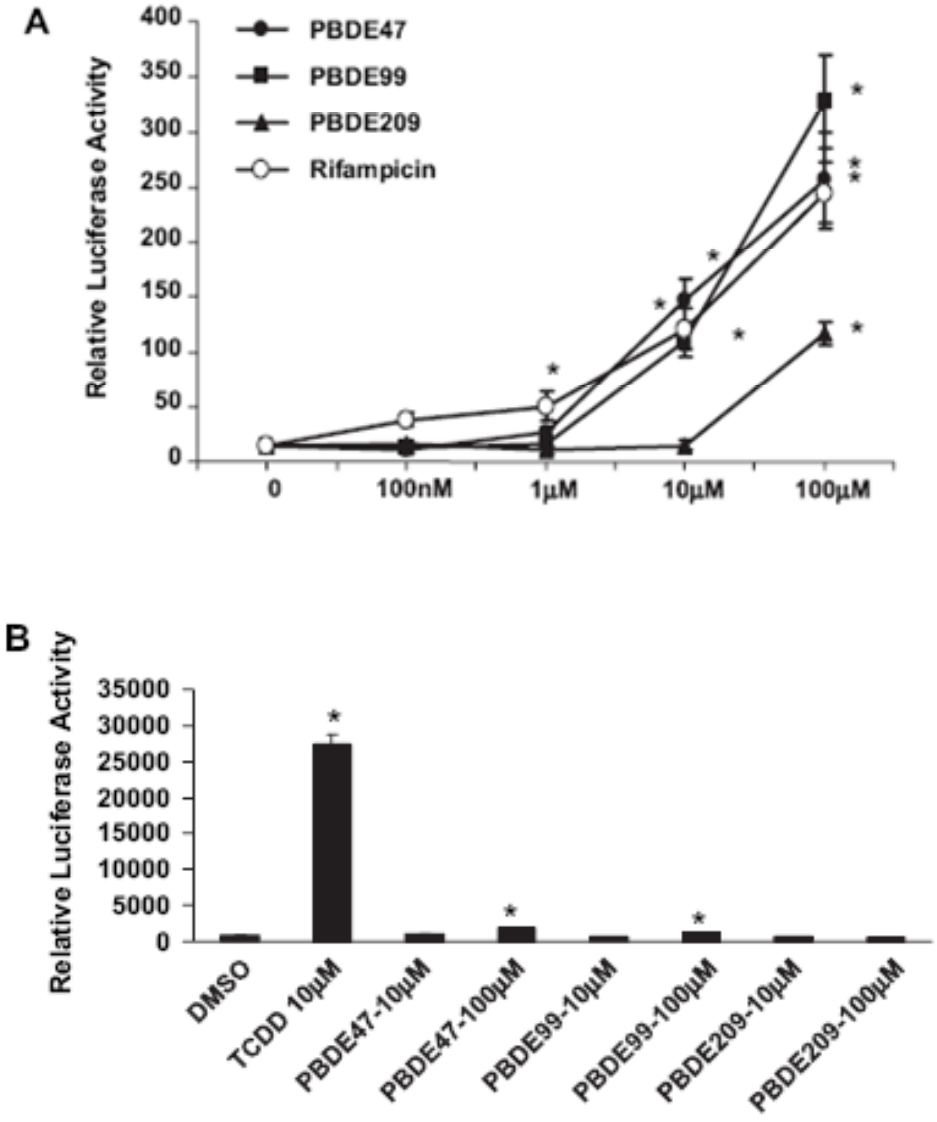

A
Relative Luciferase Activity
400
350
300
250
200
150
100
50
0
PBDE47
PBDE99
PBDE209
Rifampicin
0
100nM
1μM
10μM
100μM
B
Relative Luciferase Activity
35000
30000
25000
20000
15000
10000
5000
0
DMSO
TCDD 10μM
PBDE47-10μM
PBDE47-100μM
PBDE99-10μM
PBDE99-100μM
PBDE209-10μM
PBDE209-100μM

Figure 5-4 *In vitro* activation of human SXR and AhR by BDE47, BDE99, and BDE209. The DPX2 cells with stably transfected human SXR and SXR-response element in a firefly luciferase vector or the DRE cells with stably transfected human AhR and AhR-response element in a firefly luciferase vector were treated with various concentrations of PBDEs and rifampicin, or TCDD (10μM). Twenty-four hours following addition of PBDEs at the indicated concentrations, luciferase activity was quantified, and the activation of receptors was normalized by total protein concentration. Panel (A) is for SXR and panel (B) for AhR. Asterisks indicate that p value is < 0.05 comparing chemical- and dimethyl sulfoxide–treated cells (concentration 0). This experiment was repeated three times with similar results.

extrapolating data from rodents into humans by comparing the data obtained *in vitro* from interaction of PBDEs with both rodent and human xenobitic receptors. In this regard, knowledge obtained from this study will aid in understanding and future prediction of PBDE toxicities in humans.

The present study not only shows that PBDEs induce mouse Cyp3a11 and 2b10, it also indicates that the effectiveness of PBDEs in inducing the genes encoding these two enzymes is different. While the ability of BDE47, BDE99, and BDE209 in inducing mouse Cyp3a11 is similar regardless of the degree of bromination and dosage, they are different in inducing Cyp2b10, withBDE99 the strongest, followed by BDE47 and 209. We think that the different efficacies of PBDEs in inducing these two enzymes relies on the basal expression levels of these two P450 enzymes as well as their ability to respond to inducers. The basal gene expression level of Cyp3a11 is high, which makes it relatively easier to reach the saturation point where Cyp3a is no longer responsive to inducers, thus the fold induction of this enzyme is relatively low, even the total amount of protein is abundant. In contrast, the basal level of Cyp2b is very low, thus the fold induction can be high. This may also be the reason that treatment with 3 times higher concentration of DE-71, a mixture of BDE47 and BDE99, did not result in 3 fold higher induction of Cyp3a11 and 2b10 compared to BDE47 and BDE99.

Figure 5-5

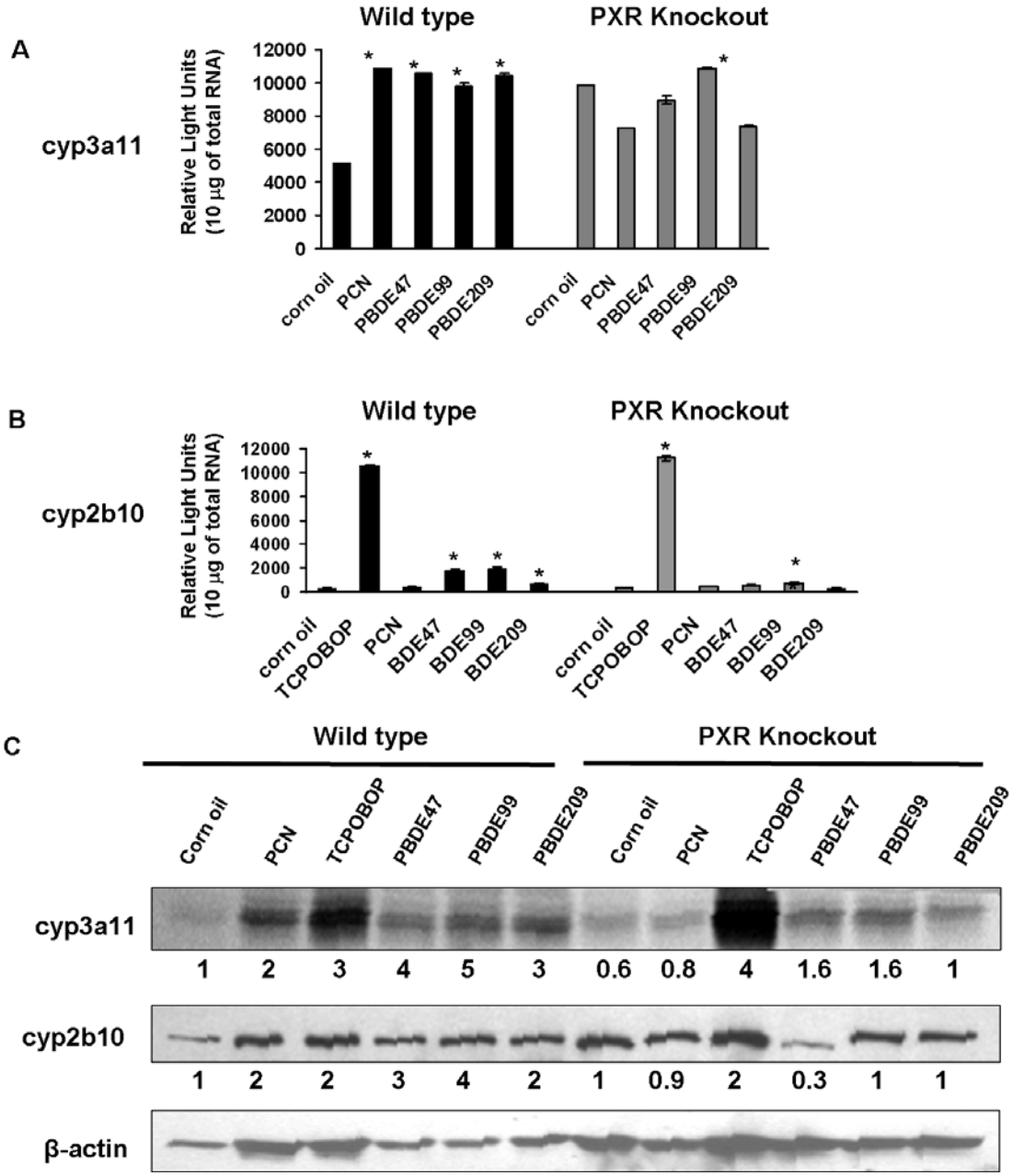

A
cyp3a11
Wild type
PXR Knockout
Relative Light Units (10 μg of total RNA)
12000
10000
8000
6000
4000
2000
0
corn oil
PCN
PBDE47
PBDE99
PBDE209
B
cyp2b10
Wild type
PXR Knockout
Relative Light Units (10 μg of total RNA)
corn oil
TCPOBOP
PCN
BDE47
BDE99
BDE209
C
Wild type
PXR Knockout
Corn oil
PCN
TCPOBOP
PBDE47
PBDE99
PBDE209
cyp3a11
1 2 3 4 5 3 0.6 0.8 4 1.6 1.6 1
cyp2b10
1 2 2 3 4 2 1 0.9 2 0.3 1 1
β-actin

Figure 5-5 Hepatic levels of Cyp3a11 and Cyp2b10 mRNA and protein in WT and PXR-null mice after treatment with PBDEs. Male WT or PXR-null mice, n = 4, at 10–12 weeks of age were ip injected with corn oil, PCN (50 mg/kg/day), TCPOBOP (3 mg/kg/day), BDE47, 99, and 209, at 100 μmol/kg/day, for 4 days. Twenty-four hrs after the last injection, the livers were removed and total RNA were isolated. The mRNA levels of Cyp3a11 (panel A) and Cyp2b10 (panel B) were determined by bDNA assay. The protein levels of these two enzymes, and β-actin that is the loading control, from pooled samples were determined by Western blot analysis (panel C). The Western blot signal was quantified by using ImageQuant software (GE Healthcare). The fold induction was indicated below each blot. For panels (A) and (B), the statistical comparison was made between corn oil– and chemical-treated groups with the same genotype. The asterisk indicates $p < 0.05$ between corn oil– and chemical-treated groups.

Although structurally similar to PCBs, PBDEs appear unlikely to be inducers for Cyp1a1/2 in mice. We used two methods to determine the mRNA levels of Cyp1a1/2 after treatment with various PBDEs and PCB126, a chemical known to activate AhR and to induce Cyp1a1/2. The results obtained from Northern-blot analysis showed an induction of Cyp1a1/2 by all the compounds tested. However, Northern-blot analysis is not as specific as bDNA assay, due to the fact that probes used in Northern blot are fragment of cDNA, which can cross react with other transcripts with sequence similar to Cyp1a1/2. Therefore, we also determined Cyp1a1/2 mRNA levels by bDNA assay that specifically detects a single gene transcript. The results obtained from bDNA assay clearly showed that PBDEs are not Cyp1a1/2 inducers. This is consistent with the studies reported previously that PBDEs are not AhR activators and do not induce Cyp1a1/2 (Peters et al., 2004; Sanders et al., 2005).

Our study provides the first evidence that PBDEs are activators for xenobiotic nuclear receptor, PXR. The induction of Cyp3a11 and 2b10 by PBDE congeners suggests that PBDEs may be activators for xenobiotic NRs, namely PXR and/or CAR. In the current study, we focus on investigating the interaction between PBDEs and PXR. Our *in vitro* studies show that BDE47, BDE99, and BDE209 are activators of both mouse PXR and human SXR. Based on our data, the potency for these three PBDEs is similar in activating mouse PXR, which is consistent with gene expression data that induction of Cyp3a11 by these three PBDEs is similar regardless of the magnitude of bromination. However, BDE47

and 99 activated SXR at a much lower concentration than BDE209, indicating that they are more potent activators for SXR than BDE209. The activation of PXR/SXR by PBDEs is likely the mechanism for induction of Cyp3a11 and 2b10 *in vivo*, which is further confirmed by the results gathered from the PXR-null mice, showing that disruption of the PXR gene abolished the induction markedly. PBDEs may induce Cyp3a11 and 2b10 via activating CAR. This is because PBDEs induce Cyp2b10, a classical target gene for not only PXR, but also for CAR. In addition, treatment with the PBDEs in the PXR-null mice induced Cyp3a11 and 2b10 to a less degree, but the induction was not completely abolished. This suggests that factors other than PXR are also involved in induction of these P450 enzymes. Furthermore, we also can not exclude other factors, independent of the xenobiotic NRs, might be involved for induction of Cyp3a11 and 2b10. These two possibilities will be further tested by using CAR- and PXR/CAR double knockout mice in the future studies.

In summary, we have demonstrated that PBDEs, the widely used brominated flame retardants, are inducers of P450 enzymes, Cyp3a11 and 2b10 in mice. The mechanism for this induction is, at least partly, due to activation of the xenobiotic NR, PXR.

Chapter 6

Polybrominated diphenyl ethers are CAR Activators

I – Introduction

Flame retardants are used in a variety of industrial and consumer products and have contributed to a reduction in the incidence of fires. Among fire retardants, several are brominated compounds, such as PBDEs. Examples of products containing PBDEs include many components of electronic devices, specifically in cabinets for circuit boards, personal computers and television sets, building materials, and textiles (Pijnenburg et al., 1995). PBDEs have been marketed in three primary formulations, the penta formulation, commercially known as DE-71 and Bromkal 70–5DE, the octa formulation (DE-79), and the deca formulation (DE-83R or Saytex 102E). The penta and octa formulations were voluntarily withdrawn from the marketplace in United States at the end of 2004, but the deca formulation, predominantly composed of BDE209, is still used in commercial products(Darnerud et al., 2001; Lorber, 2008). Nevertheless, even with both the penta and octa formulations having been withdrawn from the US market, PBDEs are still present in many consumer products and as a result increasing concentrations have been detected in humans (Petreas et al., 2003; Schecter et al., 2003; Focant et al., 2004).

The persistence of PBDEs is attributed to its lipophilic properties and bioaccumulative nature. Exposure to PBDEs is likely via the route of inhalation

and food consumption (Schecter et al., 2005a; Schecter et al., 2006; Allen et al., 2007; Lorber, 2008; Sjodin et al., 2008a). In almost all cases, BDE47 is the predominant congener detected in human and wildlife samples, followed by BDE99 (Meironyte Guvenius et al., 2001; Schecter et al., 2007).In Europe and Asia PBDE body burdens are approximately 2 orders of magnitudes less than levels detected in the United States (Petreas et al., 2003; Schecter et al., 2003; Hites et al., 2004; Johnson-Restrepo et al., 2005; Schecter et al., 2005b; Lorber, 2008). PBDEs have been detected in plasma and breast milk as well as liver, kidney, and adipose (Hites, 2004). The presence of PBDEs in human liver is particularly alarming because PBDEs can be converted to OH-PBDEs in the liver and more severe adverse effects have been documented for the OH-PBDEs relative to the PBDEs. For example, OH-PBDEs have been shown to significantly affect aromatase activity in human adrenocortical carcinoma cells, whereas PBDEs had no effect (Canton et al., 2008). In addition, OH-PBDEs have an order of magnitude higher potency than do PBDEs in their ability to compete with thyroid hormones for binding sites on serum transporters (Meerts et al., 2000; Meerts et al., 2001; Hamers et al., 2006).

Hepatic metabolism is an important factor in determining the bioaccumulation as well as pharmaco- and toxicokinetics of PBDEs. The liver is the most important organ for xenobiotic metabolism because of its location between the portal and systemic circulations and the expression of a wide range of xenobiotic-metabolizing enzymes. A number of studies have shown that exposure to PBDEs

induce hepatic microsomal enzyme activities involved in the metabolism of xenobiotics. For example, in rats PBDEs induce DE-71 induced Cyp2b and Cyp3a expression (Sanders et al., 2005). The expression of Cyp2b and 3a can be induced upon activation of xenobiotic nuclear receptors, PXR and CAR. We have shown PBDEs are activators of PXR and deletion of PXR in mice reduces PBDE-mediated induction of phase I metabolizing enzymes (Chapter 5; (Pacyniak et al., 2007) however, the role of CAR activation by PBDEs has not been fully characterized.

CAR and PXR are two closely related NRs that activate the transcription of genes involved in xenobiotic detoxification and elimination from the body (Liddle and Goodwin, 2002; Maglich et al., 2002; Handschin and Meyer, 2003). CAR is primarily expressed in the liver and small intestine. CAR was originally shown to bind to the PBRE in the CYP2B gene promoter, as a heterodimer with RXR. CAR null mice showed a lack of induction of Cyp2b10 in addition to other Phase I/II enzymes and drug transporters by in the liver (Wei et al., 2000). Certain CAR activators exhibit species specificities. For example, TCPOBOP and CITCO are the only compounds shown to specifically bind to mouse CAR and human CAR, respectively. CAR and PXR have been established as xenosensors and master regulators of xenobiotic responses.

Although it is commonly accepted that CAR preferentially induces Cyp2b10 and PXR preferentially induces Cyp3a11 there is evidence for cross-talk between these two nuclear receptor signaling pathways. For example, the CYP2B and CYP3A genes are both induced in rodents and humans by several of the same xenobiotics, including PB, clotrimazole, and rifampicin (Strom et al., 1996). This co-induction is due to the ability of both CAR and PXR to recognize each other's response elements through binding to common regulatory sequence elements in CYP3A and CYP2B promoters (Blumberg et al., 1998; Xie et al., 2000b; Goodwin et al., 2001; Smirlis et al., 2001). For example, CAR has been shown to bind to CYP3A response elements (IR-6, DR-3) regulating downstream target genes whereas PXR has regulated CYP2B, both in cultured cells and in transgenic mice, via recognition of the PBRE (Xie et al., 2000b). Thus, the establishment of a metabolic safety net that enables dual enzyme activation seems advantageous by expanding the protective capacity of the xenobiotic response system.

Induction of the genes encoding enzymes and transporters critical for endobiotic and xenobiotic disposition have been demonstrated in rodents exposed to relatively high doses of PBDEs (10-100mg/kg) (Sanders et al., 2005; Sanders et al., 2006a; Pacyniak et al., 2007; Richardson et al., 2008a; Szabo et al., 2009). PBDE intake has been estimated to be approximately 0.003mg/kg daily for adults(Johnson-Restrepo and Kannan, 2009). As a result, toxic effects and biomarkers drawn from exposures several fold higher than the levels of exposure of the general human population cannot be transferred directly to the studies of

the general population since most of chemicals exert different effects at different doses. Moreover, induction of metabolizing enzymes obtained from dose-response studies performed at high doses cannot be extrapolated to existing low-dose exposures of the general population. Thus, the effect of low dose exposure of PBDEs on hepatic gene induction remains to be clarified.

The goal of the current study is twofold, to clarify whether PBDEs are CAR activators and to establish a dose-response of the effects of PBDE exposure on mouse hepatic Cyp2b10 and 3a11 expression. Identification of PBDEs as CAR activators may help to predict the effects of PBDE exposure on liver physiology, drug metabolism and pathology. Data obtained from the dose-reponse study in mice may help to extrapolate effects of induction of hepatic metabolism of PBDE exposure in humans.

II - Results

6.1 PBDEs are activators of human and mouse CAR *in vitro*

As the positive control, PB exhibited a strong activation of both human and mouse CAR as determined by induction of luciferase activity (Figure 6-1A). Activation of human CAR by PBDEs is shown in Figure 6-1B. BDE47 and BDE209 demonstrated CAR activation at the 10μM concentration and greater. BDE99 activated human CAR at concentrations of 0.1μM and higher. As shown

in Figure 6-1C, BDE47, BDE99, and BDE209 were all capable of activating mouse CAR at concentrations of 1μM and higher.

6.2 Effects of PBDEs on Cyp2b10 and Cyp3a11 mRNA and protein expression levels in WT and CAR-null mice

Treatment of WT mice with BDE47, BDE99, and BDE209 significantly induced Cyp2b10 mRNA and protein, compared to the vehicle (corn oil)-treated group (Figure 6-2A, 2C). CAR deficiency in mice markedly reduced the induction of Cyp2b10 mRNA for all treatment groups (Figure 6-2A). In comparison to WT mice, Cyp2b10 protein induction was reduced in CAR-null mice following PB or BDE99 treatment, but not following BDE47 and BDE209 treatment (Figure 6-2C). Induction of Cyp3a11 mRNA and protein by BDE47, BDE99, and BDE209 was observed in WT mice and CAR-null mice but CAR deficiency decreased the degree of induction (Figure 6-2B, 2C).

Figure 6-1

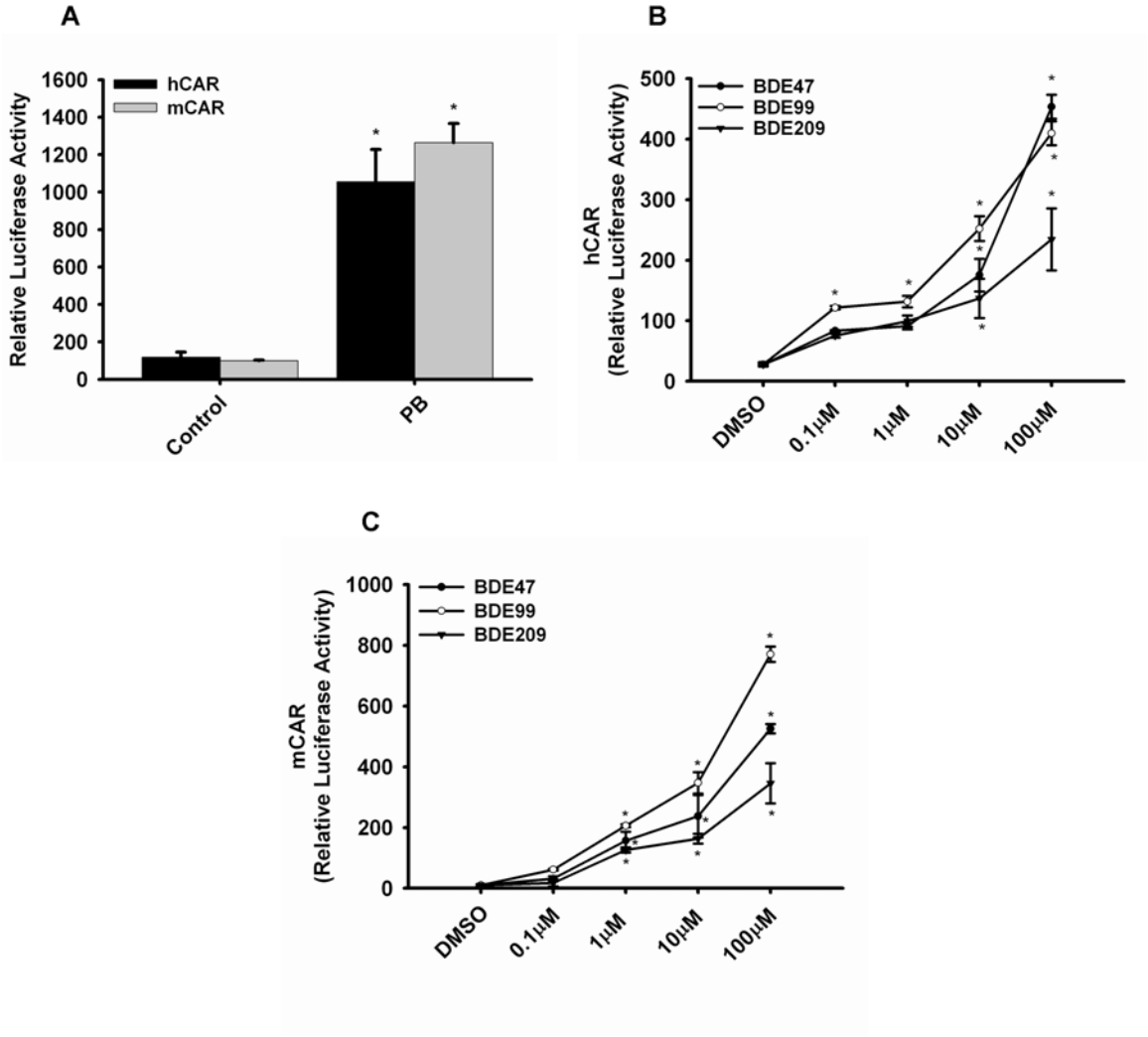

A
Relative Luciferase Activity
1600
1400
1200
1000
800
600
400
200
0
hCAR
mCAR
Control
PB
B
hCAR
(Relative Luciferase Activity)
500
400
300
200
100
0
BDE47
BDE99
BDE209
DMSO
0.1μM
1μM
10μM
100μM
C
mCAR
(Relative Luciferase Activity)
1000
800
600
400
200
0
BDE47
BDE99
BDE209
DMSO
0.1μM
1μM
10μM
100μM

Figure 6-1 *In vitro* activation of human and mouse CAR by PBDEs. CV-1 cells were transiently transfected with human or mouse CAR expression vector (pSG5-hCAR; pSG5-mCAR), three copies of PBRE-response element in a firefly luciferase vector pGL4-TK, and pCMV-renilla luciferase vector that serves as the control for transfection efficiency. Twenty-four hrs following addition of (A) 1mM PB or (B, C) PBDE congeners at the indicated concentrations in the presence of 1μM 5-α-Androstan-3-α-ol, firefly and renilla luciferase activities were quantified, and the transfection efficiency was normalized by comparing firefly and renilla luciferase activities.. The asterisk (*) indicates that *p* value is < 0.05.

Figure 6-2

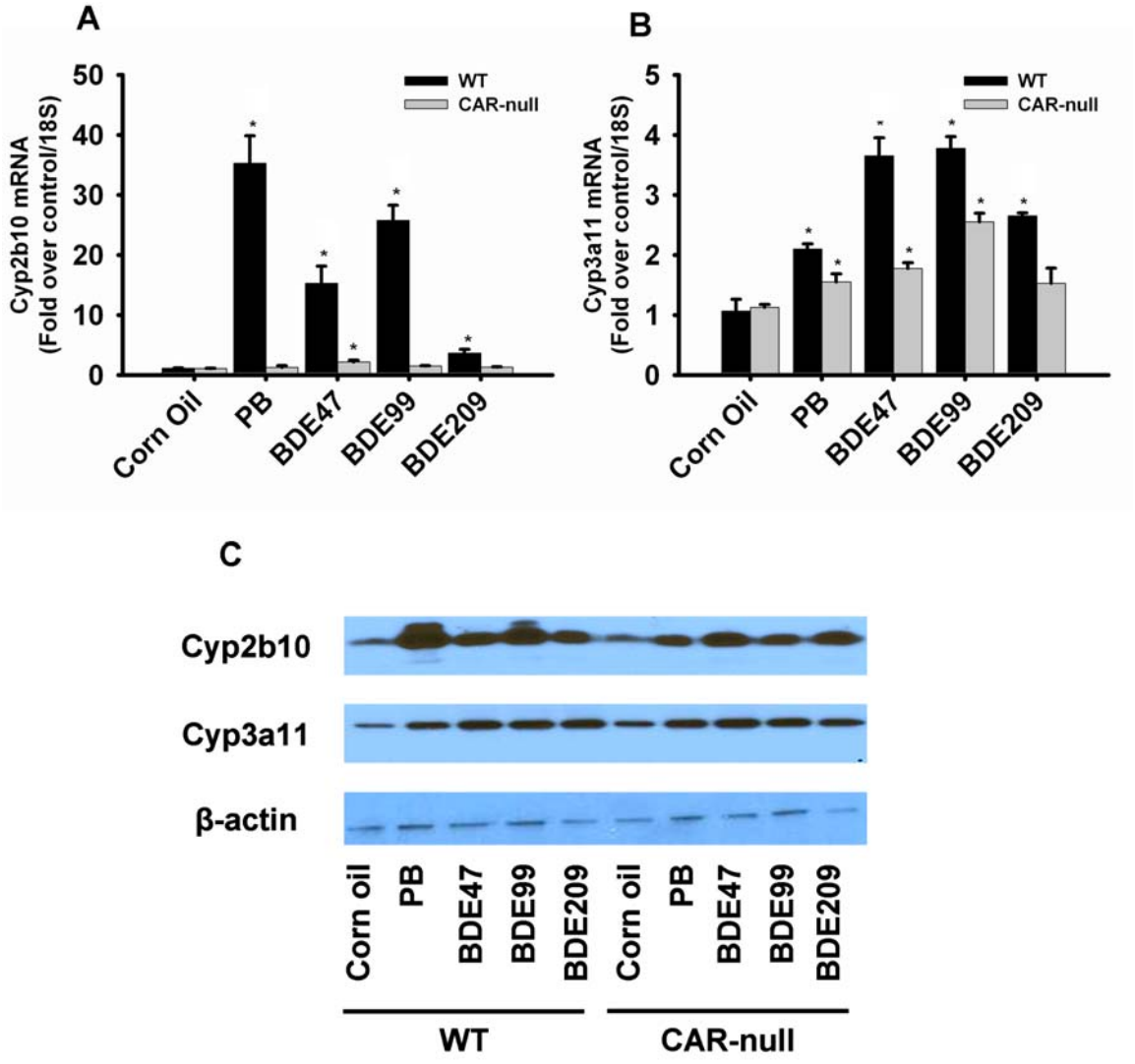

A
B
C
WT
CAR-null
Cyp2b10 mRNA (Fold over control/18S)
Cyp3a11 mRNA (Fold over control/18S)
Corn Oil
PB
BDE47
BDE99
BDE209
Cyp2b10
Cyp3a11
β-actin
Corn oil
PB
BDE47
BDE99
BDE209
WT
CAR-null

Figure 6-2 Effects of PBDEs on mRNA and protein expression of Cyp2b10 and Cyp3a11 in mouse livers. Male WT or CAR-null mice, (n = 3-4, at 10–12 weeks of age) were injected, ip, with corn oil, PB (50mg/kg), BDE47 (47mg/kg), BDE99 (57mg/kg) or BDE209 (96mg/kg) for 4 days. Twenty-four hrs after the last injection, the livers were removed and total RNA and protein homogenates were prepared. mRNA expression levels of (A) Cyp2b10 and (B) Cyp3a11 were determined by qPCR (SYBR Green) whereas (C) protein levels of Cyp2b10, Cyp3a11, and β-actin (loading control), from pooled samples, were determined by Western-blot analysis. Asterisks (*) indicate statistically significant differences between control and PBDE-treated ($p < 0.05$).

6.3 Dose-dependent induction of Cyp2b10 and Cyp3a11 mRNA and protein by BDE47 and BDE99 in mouse livers

As shown in Figure 3A, BDE47 dose-dependently induced Cyp2b10 and 3a11 mRNA expression. However, a lower dosage (0.49mg/kg) was required to induce Cyp2b10 compared to that required to induce Cyp3a11 (15mg/kg). In addition, the magnitude of Cyp2b10 mRNA induction was much higher than that of the induction of Cyp3a11 mRNA. To determine whether the induction of Cyp mRNA by BDE47 also results in changes in protein expression, Cyp2b and 3a protein levels were measured in these liver samples (Figure 3B). The results showed that similar to mRNA expression, BDE47 induced Cyp2b and 3a protein levels in a dose-dependent manner with a higher magnitude of Cyp2b10 protein induction than that of Cyp3a protein. Although the concentration for BDE47 required to induce Cyp2b10 and 3a11 mRNA is 0.49 and 15mg/kg, respectively, BDE47 increased protein levels for both Cyp2b and Cyp3a at doses as low as 0.049mg/kg.

Similar to BDE47, BDE99 dose-dependently induced Cyp2b10 and 3a11 mRNA and protein levels, except that BDE99 induced Cyp3a11 mRNA at a lower dosage (1.7mg/kg) (Figure 4A and 4B). Moreover, BDE99 increased Cyp2b protein levels at doses as low as 0.017mg/kg whereas Cyp3a protein was increased starting from doses of 0.17mg/kg.

Figure 6-3

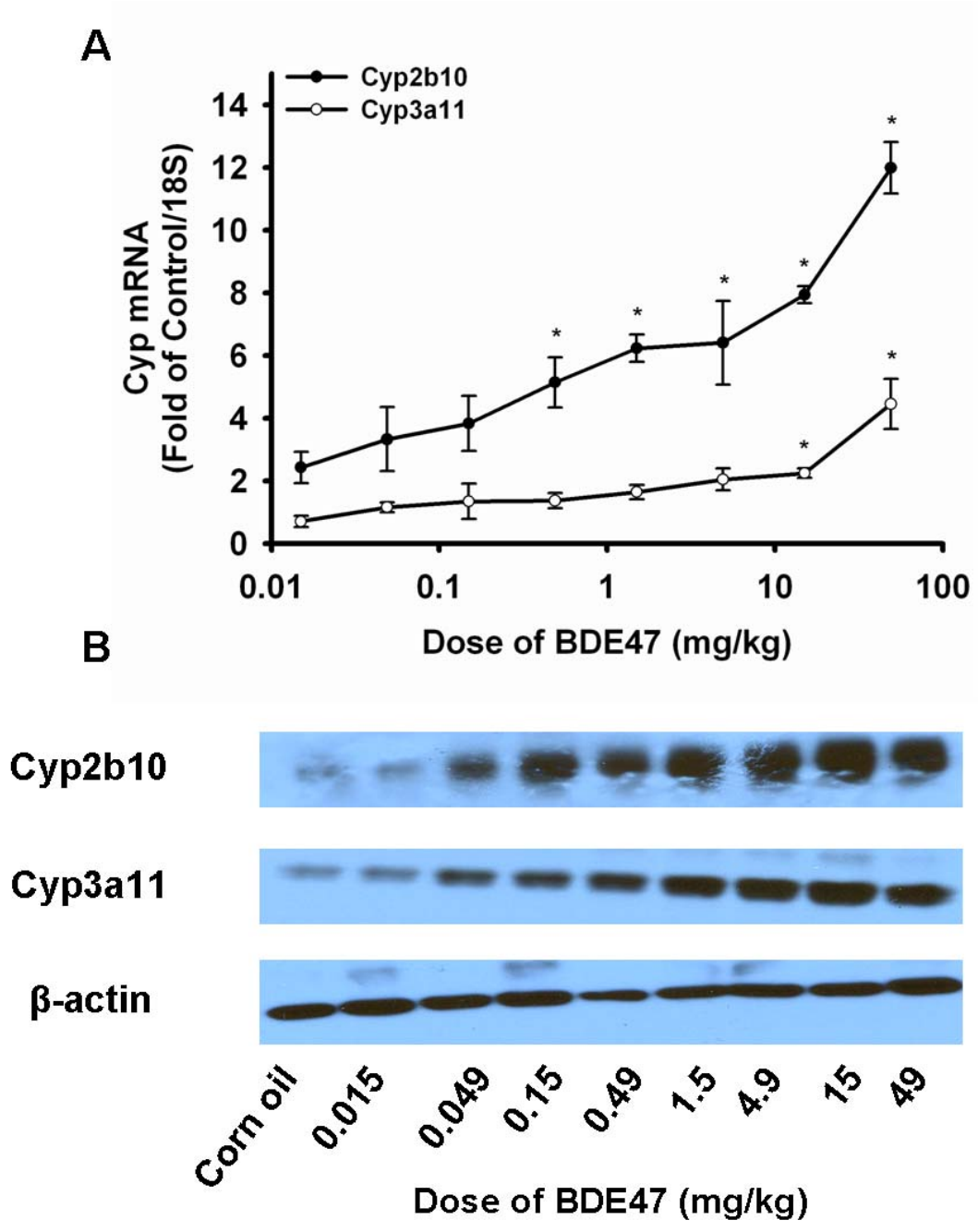

A
Cyp2b10
Cyp3a11
Cyp mRNA
(Fold of Control/18S)
0
2
4
6
8
10
12
14
0.01
0.1
1
10
100
Dose of BDE47 (mg/kg)
B
Cyp2b10
Cyp3a11
β-actin
Corn oil
0.015
0.049
0.15
0.49
1.5
4.9
15
49
Dose of BDE47 (mg/kg)

Figure 6-3. Dose-response of BDE47 on mouse Cyp2b10 and Cyp3a11 mRNA expression. Adult C57BL/6 male mice (n = 6) were i.p. administered the vehicle control (corn oil) or BDE47 at various doses (0.015, 0.047, 0.15, 0.47, 1.5, 4.7, 15, or 47 mg/kg of body weight) for four days. Twenty-four hrs after the last injection, the livers were removed and RNA and protein homogenates were prepared. (A) mRNA expression levels from treated male mouse livers (n = 6) were determined by qPCR (SYBR Green) whereas (B) protein levels of Cyp2b10,Cyp3a11, and β-actin (loading control), from pooled samples, were determined by Western-blot analysis. Asterisks (*) indicate statistically significant differences between control and BDE47-treated ($p < 0.05$).

Figure 6-4

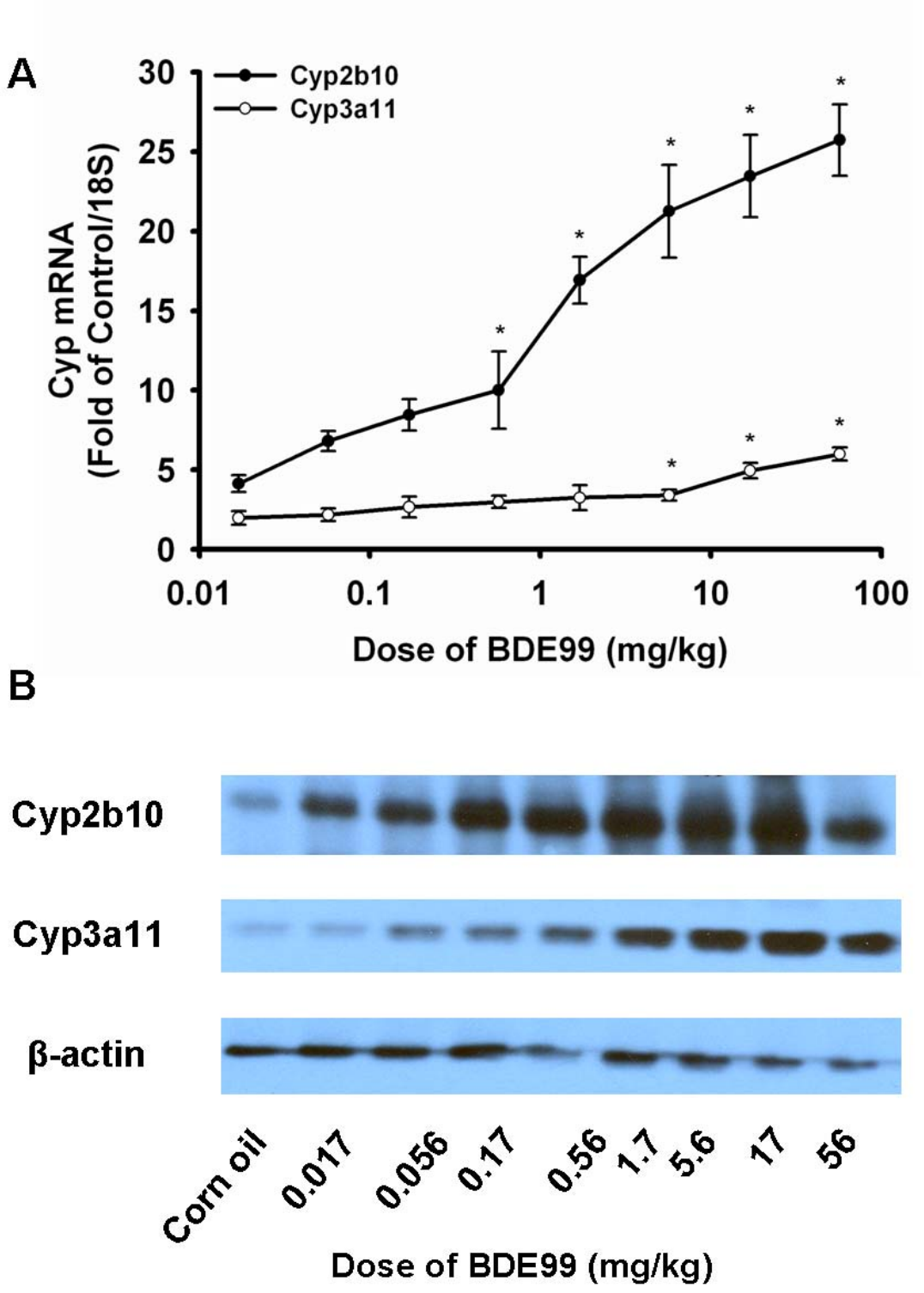

A
Cyp2b10
Cyp3a11
Cyp mRNA
(Fold of Control/18S)
30
25
20
15
10
5
0
0.01
0.1
1
10
100
Dose of BDE99 (mg/kg)
B
Cyp2b10
Cyp3a11
β-actin
Corn oil
0.017
0.056
0.17
0.56
1.7
5.6
17
56
Dose of BDE99 (mg/kg)

Figure 6-4 Dose-response of BDE99 on mouse Cyp2b10 and Cypaa11 mRNA expression. Adult C57BL/6 male mice (n = 6) were i.p. administered the vehicle control (corn oil) or BDE99 at various doses (0.017, 0.057, 0.17, 0.57, 1.7, 5.7, 17, or 57 mg/kg of body weight) for four days. Twenty-four hrs after the last injection, the livers were removed and RNA and protein homogenates were prepared. (A) mRNA expression levels from treated male mouse livers (n = 6) were determined by qPCR (SYBR Green) whereas (B) protein levels of Cyp2b10,Cyp3a11, and β-actin (loading control), from pooled samples, were determined by Western-blot analysis. Asterisks (*) indicate statistically significant differences between control and BDE99-treated ($p < 0.05$).

III - Discussion

In the present study, we show that PBDEs directly activate the xenobiotic nuclear receptor, CAR and CAR deficiency in mice reduced the PBDE-mediated induction of the CAR target gene Cyp2b10. Furthermore, we have performed a dose-response curve for induction of Cyp2b10 and 3a11 mRNA and protein by acute BDE47 and BDE99 treatments. The result demonstrates a preferential induction of Cyp2b10 at low doses of PBDEs and induction of both Cyp2b10 and 3a11 mRNA and protein at high doses of PBDEs.

BDE47 has previously been shown to induce CAR target genes such as Cyp2b10; however, it is unknown whether induction of Cyp2b10 by BDE47 is through direct CAR activation (Richardson et al., 2008a). Together with PXR, CAR coordinately regulates xenobiotic metabolism in the liver. Our *in vitro* studies provide direct evidence that PBDEs activate human and mouse CAR (Figure 1B-1C). Based on our data, BDE47 and BDE99 exhibit the highest potency for human CAR activation followed by BDE209 (Figure 1B). BDE47 and BDE99 have been shown to be metabolized by human hepatocytes in a P450-mediated manner (Lupton et al., 2009; Stapleton et al., 2009). BDE99 was shown to have a greater potential for metabolism followed by BDE47. Interestingly our previous (Pacyniak et al. 2007) and current findings also demonstrate that BDE99 is a more potent activator of human CAR and PXR than BDE47. Furthermore, extensive metabolism of BDE99 may help to explain why congener

patterns identified in humans do not reflect the composition of the commercial penta product. BDE99, which is the predominant congener in the commercial mixture, is found to a lesser degree than BDE47 in human liver (Meironyte Guvenius et al., 2001; Schecter et al., 2007; Covaci et al., 2008). We have previously demonstrated that the hepatic basolateral uptake transporters, OATP1B1 and OATP1B3, transport BDE47 with the highest affinity and efficiency compared to BDE99 (Pacyniak et al., 2010). Together, differential hepatic uptake coupled with a greater rate of metabolism of BDE99 by hepatic P450s may be the primary factor responsible for its decreased bioaccumulation in human liver.

No specific metabolites of BDE209 were identified after incubation with human cyropreserved hepatocytes suggesting that metabolism of BDE209 did not occur (Stapleton et al., 2009). It is possible that metabolism of BDE209 led to reactive metabolites that covalently bound to lipids or proteins and were not recovered during the extraction process. This has been reported for rats exposed to radiolabeled BDE209 (Morck et al., 2003). However, it is clear that *in vitro*, BDE209 is capable of activating human CAR and PXR, thus suggesting the potential for enzyme induction. Based upon rodent studies BDE209 is shown to be extensively metabolized through oxidative dehalogenation reactions to form phenolic metabolites and debrominated to form a variety of lower brominated congeners. (Morck et al., 2003). This may be a cause for concern since lower brominated congeners have been proposed to have greater toxicity, at least in rodents (Costa et al., 2008; Szabo et al., 2009).

The present study not only shows that PBDEs induce mouse cyp2b10 and 3a11 but also provides an explanation for the differing induction of the genes encoding these enzymes. Reporter gene assays identified BDE99 as the most potent activator of mouse CAR, followed by BDE47 and BDE209. These results were further confirmed *in vivo* using CAR-null mice. The decrease of Cyp2b10 mRNA and protein induction in CAR-null mice after exposure to BDE47, BDE99 and BDE209 suggest these congeners are CAR activators *in vivo* (Figure 2A, 2C). However, for BDE47 and BDE209, Cyp2b10 induction was not completely abolished in CAR-null animals, suggesting a preference for PXR activation since PXR is known to induce Cyp2b10 in mice. This is further confirmed by our previous study showing ablation of Cyp2b10 expression in PXR-null mice exposed to BDE47 and BDE209 (CH. 5; Pacyniak et al., 2007). All tested PBDE congeners markedly induced Cyp3a11 compared to corn oil treated animals and furthermore this induction was not completely abolished in the CAR-null animals further supporting the role of PXR for Cyp3a11 induction (Figure 2B, 2C). This finding is not surprising since reciprocal induction of Cyp2b and Cyp3a has been shown for certain CAR and PXR ligands (clotrimazole, PB) due to their ability to bind to CAR and PXR response elements (Strom et al., 1996; Blumberg, 1998; Xie et al., 2000b). Treatment of PXR/CAR double-null could be used in the future to further confirm the role of PXR and CAR for induction of phase I enzymes upon exposure to PBDEs.

BDE47 and BDE99, the primary components of the DE-71 formulation widely used in the United States, are capable of inducing Cyp2b and Cyp3a enzymes in rodents (Darnerud et al., 2001; Birnbaum and Cohen Hubal, 2006). Basal P450 levels directly affect their ability to respond to inducers. Importantly, the basal activities of these two enzymes differ with Cyp2b10 having low activity whereas Cyp3a11 has a high activity. Cyp2b10 is mainly induced by CAR activation whereas Cyp3a11 is mainly induced by PXR activation. However, it is not clear which Cyp isoform, if any, is preferentially induced upon exposure to BDE47 or BDE99 at lower doses. Clearly we don't know which CYP enzyme is preferably induced upon low-dose PBDE exposure, doses relevant to human exposure.. Previous studies show an increased induction in CYP2B as compared with CYP3A suggesting that PBDE congeners may have a preference for CAR over PXR (Sanders et al., 2005; Richardson et al., 2008b; Szabo et al., 2009). Our dose response studies reveal that BDE47 and BDE99 induce Cyp2b10 mRNA and protein with a greater potency (Figure 3 and 4). The magnitude of Cyp2b10 mRNA induction was much higher than that of Cyp3a11 mRNA, likely due to low basal expression levels of Cyp2b10. Furthermore, saturation of Cyp3a11 enzymatic activity may provide an explanation for the relatively flat dose-response curve and low fold of induction resulting in a decreased responsiveness to BDE47 and BDE99 at the higher doses. Results from the current study agree with previous findings showing greater induction for Cyp2b10 over Cyp3a11. Together with expression data from CAR- and PXR-null mice, the current study

suggests that induction of Cyp2b10 likely occurs in a PXR- and CAR-dependent manner for BDE47 and BDE99, respectively.

The activation of CAR and PXR by PBDE congeners may play an important role in overall liver physiology. In addition to the well known effects on regulation of genes encoding metabolizing enzymes and transporters, emerging evidence link CAR and PXR activation with maintaining glucose and lipid homeostasis in the liver (Manenti et al., 1987; Herzig et al., 2001; Kodama et al., 2004; Zhou et al., 2006; Kodama et al., 2007). For example, PB treatment decreased mitochondrial carnitine palmitoyltransferase 1 and the enoyl-CoA-isomerase mRNA content in WT mice but not in CAR-null mice thus inhibiting lipid catabolism (Kassam et al., 2000). Moreover, treatment with the mouse PXR activator PCN decreased blood glucose levels in fasting WT but not PXR-null mice. Recently, a correlation was shown between PBDEs and individuals with diabetes and metabolic syndrome (Lim et al., 2008). Moreover, it has recently been demonstrated that exposure to PBDEs results in activation of metabolic pathways including lipid and fatty acids metabolism which may be dependent upon the activation of CAR and/or PXR (Suvorov and Takser, 2010). Thus, to better investigate the role of CAR and PXR activation and their affect on hepatic energy homeostasis chronic low dose exposure of young animals, situation similar to humans, may be a better model to determine the effects of PBDEs on glucose and lipid homeostasis.

In conclusion, the data presented in the current study demonstrate that PBDEs are activators of both human and mouse CAR *in vitro*. Additionally, BDE99 is a potent activator of CAR *in vivo* whereas BDE47 and BDE209 activate CAR and PXR at high doses (100μmoles/kg). Finally, we have shown that BDE47 and BDE99 induce Cyp2b10 at lower doses than that of Cyp3a11 suggesting a dose-dependent activation of CAR and PXR. Risk assessment of PBDE congeners is ongoing and results from this study aids in a better evaluation of the possible risks for human beings. Currently, daily exposure of PBDEs is estimated to be 0.003mg/kg for adults (Johnson-Restrepo and Kannan, 2009). Although the administered doses used here are higher than human exposure levels, this study shows that effects of PBDEs are seen in animal models at concentrations within ~10-fold of the high end of the human population.

Chapter 7

Summary and overall discussion of dissertation

The present dissertation has characterized the molecular mechanisms responsible for the hepatic disposition of PBDEs. This was achieved by two specific aims. First, the current work has identified human and mouse hepatic OATPs/Oatps as the transport systems for PBDEs uptake. Secondly, activation of CAR and PXR by PBDEs results in the induction of the phase I metabolizing enzymes, Cyp2b10 and 3a11.

PBDEs are ubiquitous, persistent, and have the potential to bioaccumulate in humans (Hites, 2004; Schecter et al., 2005b). In human blood, milk, and tissues (liver, kidney, adipose), total PBDE levels have increased exponentially over the past 30 years, doubling every 5 years (Hites, 2004). Of particular interest are PBDE levels in the liver. The presence of PBDEs in human liver is particularly alarming since more severe adverse effects have been documented for the OH-PBDEs relative to the PBDEs. For example, HO-PBDEs, but not the PBDE congeners themselves, behave as ligands for human transthyretin suggesting the importance for bioactivation (Meerts et al., 2000). Biotransformation of xenobiotics in the liver occurs through the catalytic action of the P450 enzymes. A prerequisite for induction of metabolizing enzymes is that PBDE congeners can enter the liver. Prior to this dissertation work, very little was known about the mechanism of PBDE uptake in the liver. Therefore, the goal of the first specific

aim was to elucidate the mechanism of PBDE uptake into human and mouse liver.

In the first part of **specific aim 1**, CHO cells expressing OATP1B1, OATP1B3, and OATP2B1 were used to test the hypothesis that OATPs expressed in human hepatocytes would be responsible for the uptake of PBDE congeners 47, 99, and 153. The results demonstrated that PBDE congeners inhibited OATP1B1- and OATP1B3-mediated uptake of estradiol-17β-glucuronide as well as OATP2B1-mediated uptake of estrone-3-sulfate in a concentration-dependent manner suggesting that PBDEs might be substrates of OATP1B1, OATP1B3 and OATP2B1. Direct uptake studies confirmed that all three PBDE congeners are substrates for the three tested hepatic OATPs. Detailed kinetic analysis revealed that OATP1B1 transported BDE47 with the highest affinity followed by BDE99 and BDE153. For OATP1B3, the order was the same, while OATP2B1 transported all three congeners with similar affinities.

The intestine functions as a barrier to xenobiotics and directly effects their systemic concentration. A major route of exposure to PBDEs occurs through the ingestion of contaminated food products (Schecter et al., 2006; Schecter et al., 2008). OATP2B1 is expressed at the apical membrane of human intestinal epithelial cells. Due to the acidic microenvironment of the small intestine, OATP2B1 is thought to play a more important role in the small intestine rather than in hepatocytes where the pH of the portal blood is unlikely acidic. In rodents,

PBDEs are known to be relatively well absorbed from the gastrointestinal tract. Together, this suggests an important role for OATP2B1 in the transport of PBDE congeners from the lumen of the gastrointestinal tract.

Hepatic uptake and metabolizing enzymes determine the xenobiotic concentration in the liver and may affect potential toxic side effects (Giacomini and Sugiyama, 2005). The primary components of the penta mixture, which was widely employed in the US, is BDE99, BDE47 and BDE153 (Lorber, 2008) whereas the predominant PBDE congeners detected in the human liver are BDE47, BDE99 and BDE153 (Meironyte Guvenius et al., 2001). The difference between the congeners profile found in the commercial mixture to that of human tissue, in particular the liver, can partly be explained by the higher affinity and greater overall transport efficiency for BDE47 by OATP1B1 and OATP1B3 compared to that for BDE99 and BDE153. The higher concentration of BDE47 in the liver is of importance. It has been shown that OH-BDE47 is a potent inhibitor of human TTR potentially resulting in increased TH elimination. Metabolic conversion of BDE47 to its hydroxylated metabolite is thought to play an important role in the disruption of TH homeostasis. Together, the results from the first part of **specific aim 1** illustrate the importance of hepatic uptake on hepatic PBDE concentrations.

Although mice are commonly used for toxicological characterization of PBDEs, nothing was known regarding transport by mouse hepatic Oatps. Therefore, in

the second part of **specific aim 1**, we tested the hypothesis that BDE47, BDE99, and BDE153 are substrates of mouse hepatic Oatps (Oatp1a1, Oatp1a4, Oatp1b2, and Oatp2b1). We used HEK293 cells transiently expressing individual Oatps and quantified the uptake of BDE47, BDE99, and BDE153. The results showed Oatp1a4, Oatp1b2, and Oatp2b1 transported all three PBDE congeners, whereas Oatp1a1 transported none. Kinetic studies demonstrated that Oatp1a4 and Oatp1b2 transported BDE47 with the greatest affinity, followed by BDE99 and BDE153. In contrast, Oatp2b1 transported all three PBDE congeners with similar affinities. A potential drawback of *in vitro* functional characterization of Oatp substrates is differences in substrate specificity and differences in the relative expression levels of drug transporters. However, the generation of Oatp-null mice is proving to be valuable tools to determine the *in vivo* contribution of hepatic Oatp transporters to drug substrates identified using *in vitro* systems. Using Oatp1a4- and Oatp1b2-null mice we further demonstrated that *in vivo* Oatp1a4 plays a minor and Oatp1b2 plays a major role in the hepatic accumulation of BDE47.

The first part of **specific aim 1** demonstrated that PBDE congeners are substrates of OATPs expressed in human hepatocytes. The results of the second part of **specific aim 1** showed Oatps expressed in mouse hepatocytes can transport PBDEs as well. Furthermore, kinetic analysis exhibited that transport affinity was conserved between orthologous members. For example, OATP1B1 and OATP1B3 both transported BDE47 with the highest affinity. This held true for

Oatp1b2, the mouse member of the OATP1B sub-family, which also transported BDE47 in a high affinity manner. Additionally, OATP2B1 and Oatp2b1 both transported all three PBDE congeners with similar affinities.

PBDEs are known to induce Cyp2b and Cyp3a in rat liver; however the mechanism of induction remains unclear. The goal of **specific aim 2** was to clarify the mechanism by which PBDE congeners induce phase I metabolizing enzymes. Treatment of C57BL/6 mice with BDE47, BDE99, and BDE209 induced gene expressions of Cyp2b10 and 3a11, but not Cyp1a1/2. Because Cyp2b10 and Cyp3a11 are target genes of CAR and PXR, respectively, we hypothesized that PBDE congeners are CAR and/or PXR activators. Using reporter-gene luciferase assays, the data showed that BDE47, 99, and 209 activated humanand mouse CAR/PXR PXR but not AhR. Furthermore, induction Cyp2b10 and 3a11 by BDE47, 99, and 209 was markedly suppressed in CAR- and PXR-null mice, respectively, indicating that PBDE congeners activate CAR and PXR *in vivo*.

In rodents, metabolic activation of PBDEs in the liver are thought to be the mechanism for endocrine disruption observed characterized by reduction in serum T_4 levels (Zhou et al., 2002). Phase I CYPS catalyze the hydroxylation of PBDEs. Hydroxylated metabolites are thought to be responsible for the reduction in TH through competitive inhibition of TTR. Additionally, TH elimination occurs through via conjugation with UDPGT. UDPGT has been identified as a CAR and

PXR target genes (Tolson and Wang) further underlying the impact upon liver physiology via activation of CAR and PXR by PBDEs.

Induction of the genes encoding enzymes xenobiotic disposition have been demonstrated in rodents exposed to relatively high doses of PBDEs (10-100mg/kg) (Sanders et al., 2005; Sanders et al., 2006; Pacyniak et al., 2007; Richardson et al., 2008a; Szabo et al., 2009). In the second part of **specific aim 2**, I showed induction of hepatic Cyp2b10 and 3a11 by BDE47 and BDE99 was distinctly dose-dependent, with Cyp2b10 induced at lower doses and Cyp3a11 at much higher doses. Because CAR preferentially induces Cyp2b10 and PXR Cyp3a11, the results indicate PBDEs may serve as stronger activators of CAR than PXR. This study has shown that effects of PBDEs are seen in animal models at concentrations within 10-fold of the low end of the human population in North America (McDonald, 2005).

Overall, in this dissertation, I examined the hepatic disposition of PBDEs from two perspectives. First, I demonstrated that BDE47, BDE99, and BDE153, the most common PBDEs congeners detected in humans, were transported by human and murine hepatic OATPs/Oatps. Secondly, I have shown that induction of the phase I target genes, Cyp2b10 and Cyp3a11, was due to the activation of CAR and PXR. Together, my dissertation work has provided the mechanism for hepatic uptake and enzyme induction upon exposure to PBDE congeners.

Chapter 8

Future directions

PBDEs and PCBs belong to the same class of chemicals, polyhalogenated hydrocarbons. Despite potential differences in mechanism of action, the history of research on PCBs may shed lights on future research directions of PBDEs. The history of research on PCBs demonstrates serious delays in the accumulation of necessary data for the purpose of toxic-substance policy making and regulatory action (Grandjean and Landrigan, 2006). It can not be over emphasized that intensive animal testing must precede marketing of a new substance or be concomitant to its arrival upon the market in order to avoid contamination of the environment and human population by chemicals with unknown hazardous properties.

An important question to ask is whether current studies employ doses relevant to human exposure levels? Toxic effects and biomarkers drawn from exposures several fold higher than the levels of exposure of the general human population cannot be transferred directly to the studies of the general population since most of toxic substances exert different effects at different doses. Moreover, toxic effects obtained from dose-response studies performed at high doses cannot be extrapolated to existing low-dose exposures of the general population. This is so because a number of environmental toxicants display non-linear dose-response relationships. Non-monotonic dose-response curves were already reported for

the structurally related PCBs (Fisher et al., 2006). Experimental testing exclusively at only high doses can therefore lead to a false sense of security with respect to the safety of a substance for the general population. Infant and toddlers have been shown to have the highest PBDE body burdens (Petreas et al., 2003). The greatest concern for potential adverse health effects of PBDEs relates to their developmental neurotoxicity (Costa and Giordano, 2007). Thus, top-priority should be given to experiments aimed at identifying the most sensitive stages of development in order to identify and protect the most vulnerable segment of the human population, using doses relevant to human exposure (0.003mg/kg) (Johnson-Restrepo and Kannan, 2009).

We identified that PBDEs are activators of CAR and PXR. Besides inducing drug metabolizing enzymes and transporters, activation of CAR and PXR alters hepatic energy metabolism. PBDEs have been associated with diabetes and metabolic syndrome (Lee et al., 2010). We have preliminary microarray data suggesting exposure to BDE99 results in the alteration of hepatic energy metabolism in male mice. Of interest were genes involved in gluconeogenesis as well as fatty acid and lipid metabolism. However, the current study design may not be the best model. One potential reason is PBDEs exposure in humans is chronic while our study design is for an acute exposure. In addition, we used 10 week old C57BL/6 mice which are considered adults. Importantly, toddlers and children, not adults, are the most sensitive population. To this end, a better study design would be to treat neonatal and/or perinatel mice on both a regular and

high-fat diet with chronic exposure to PBDEs to accurately mimic human diet and PBDE interaction.

Additionally, endpoints such as hepatic enzyme induction may not be the best biomarker because they are inapplicable to human studies due to potential specie differences. Systematic screening of the most sensitive endpoints is necessary for each potentially hazardous substance. The powerful new tools of "omics" research which appeared over the last decades will no doubt increasingly contribute to intensifying the investigation into the sensitive endpoints of toxicity.

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