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Organic Anion Transporting Polypeptides in the Hepatic Uptake of PBDE Congeners in Mice

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Abstract

BDE47, BDE99 and BDE153 are the predominant polybrominated diphenyl ether (PBDE) congeners detected in humans and can induce drug metabolizing enzymes in the liver. We have previously demonstrated that several human liver organic anion transporting polypeptides (humans: OATPs; rodents: Oatps) can transport PBDE congeners. Mice are commonly used to study the toxicity of chemicals like the PBDE congeners. However, the mechanism of the hepatic PBDE uptake in mice is not known. Therefore, the purpose of the current study was to test the hypothesis that BDE47, BDE99, and BDE153 are substrates of mouse hepatic Oatps (Oatp1a1, Oatp1a4, Oatp1b2, and Oatp2b1). We used Human Embryonic Kidney 293 (HEK293) cells transiently expressing individual Oatps and quantified the uptake of BDE47, BDE99, and BDE153. Oatp1a4, Oatp1b2, and Oatp2b1 transported all three PBDE congeners, whereas Oatp1a1 did transport 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. The importance of hepatic Oatps for the liver accumulation of BDE47 was confirmed using Oatp1a4^{-/-}, and Oatp1b2-null mice.

Keywords

Polybrominated diphenyl ethers; organic anion transporting polypeptides; liver; uptake; kinetics; mice

Introduction

Polybrominated diphenyl ethers (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

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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). 2,2',4,4'-Tetrabromodiphenyl ether (BDE47) is the predominant congener detected in human and wildlife samples, followed by 2,2',4,4',5-pentabromodiphenyl ether (BDE99), and 2,2',4,4',5,5'-hexabromodiphenyl ether (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 hydroxylated metabolites (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 monooxygenase *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 nuclear receptor, pregnane $\times$ receptor (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 nuclear receptors and induce metabolizing enzymes is that PBDE congeners can enter hepatocytes. Given that organic anion transporting polypeptides (OATPs: human; Oatps: rodents; Figure 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. Despite the fact that mouse and human OATP/Oatps share some overlapping substrates, many have unique substrate specificity. Of the Oatps expressed 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).

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 the 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, sulphobromophthalein, 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). In addition, it is informative to know that deletion of either Oatp1a4 or Oatp1b2 does not alter overall expression pattern or levels of other transporters and metabolism enzymes significantly (Lu *et al.*, 2008; Zaher *et al.*, 2008; Gong *et al.*, 2011).

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 hepatocytes in order to establish a scientific basis for the use of mouse models to study PBDE transport in humans.

Materials and Methods

Chemicals

Radiolabeled [^{14}C]BDE47 (36.5 mCi/mmol) was a gift from Dr. Kevin Crofton (U.S. EPA, National Health and Environmental Effects Laboratory). Radiolabeled [^{14}C]BDE99 (36.5 mCi/mmol) and [^{14}C]BDE153 (27.8 mCi/mmol) was a gift from Dr. Mike Sanders (National Toxicology Program, National Institute of Environmental Health Sciences). Unlabeled BDE47, BDE99 and BDE153 were obtained from Cerilliant (Round Rock, TX). Turbofect transfection reagent was purchased from Fermentas (Glen Burnie, MD). Reverse transcriptase PCR (RT-PCR) kit was purchased from Promega (Madison, WI).

Cloning of the mouse Oatp expression 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.

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

HEK293 cells were seeded in poly-D-lysine-coated 24-well plates and 1 μg of cDNA transfected with TurboFect according to the manufacturer's protocol. Transport assays were performed 48 hrs post-transfection. Cells were washed two times with 37°C, prewarmed uptake buffer [142mM NaCl, 5mM KCl, 1mM KH_2PO_4 , 1.2mM MgSO_4 , 1.5mM CaCl_2 , 5mM glucose and 12.5mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) (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 the radiolabeled substrate. Uptake was stopped at various times by removal of the uptake solution and washing of the cells 4 times with 1 mL of ice-cold uptake buffer. The cells were then solubilized with 300 μL of 1% Triton X-100, and 200 μL of the lysate was used for liquid scintillation counting (Research Products International Corp., Mt. Prospect, IL). To analyze the kinetics of Oatp-mediated PBDE transport, vector 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 portion of uptake. In all studies, cells transfected with empty vector served as the background control. For both time dependent and kinetic uptake studies, the total protein concentration in each well was measured using a BCA protein assay kit (Pierce Chemical, Rockford, IL), and the rate of uptake in each well

was normalized to its total protein concentration. In all studies, transporter-specific uptake was calculated by subtracting the background uptake of empty vector (nmol per mg of total protein per min) transfected cells from uptake of Oatp-transfected cells (nmol per mg of total protein per min).

Animals and treatments

Wild type (WT) mice with a C57BL/6 genetic background were obtained from Jackson Laboratories (Bar Harbor, ME). Oatp1a4 null mice (Deltagen, San Carlos, CA) were bred to homozygosity on the C57BL/6 background as described by Gong et al (Gong *et al.*, 2011). Homozygous Oatp1b2 null mice were also on the C57BL/6 background as described by Lu et al (2008). All mice were housed in an environmentally controlled room with a 12-h light/dark cycle and allowed free access to food and water. All protocols and procedures were approved by Institutional Animal Care and Use Committees (University of Kansas Medical Center or Bristol-Myers Squibb Co). All mice were housed in an environmentally controlled room with a 12-h light/dark cycle and allowed free access to feed and water. All protocols and procedures were approved by the University of Kansas Medical Center Animal Care and Use Committee. 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.). BDE47 was added directly to the dosing solution vial and dissolved in acetone. 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. Volatile solvents were evaporated under vacuum (Speed Vac, Savant Instruments, Inc. Farmingdale, NY). After indicated time points (0, 1, 3, and 8 hrs), mice were euthanized by CO_2 asphyxiation followed by cervical dislocation. Livers were removed and snap-frozen immediately. The gender, dose, and time post treatment for liver collection was selected based on a previous kinetic study on BDE47 (Staskal *et al.*, 2005).

Sample analysis

Sample preparation 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 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) and incubated an additional hour. Radioactivity was quantified using a MicroBeta TriLux liquid scintillation counter (Perkin Elmer, Waltham, MA).

Data analysis

Uptake experiments were performed in triplicate and repeated 2 to 4 times. Data with error bars represent the means $\pm$ the standard error. For the initial screen of PBDE congeners as substrates of Oatps and time-dependent uptake the student's t-test was used. In order to determine statistical differences between wild-type and Oatp1a4- and Oatp1b2-null mice, intergroup comparisons were performed by one-way ANOVA, followed by the Bonferroni t-test. For all tests, the p value for statistical significance was set at $p < 0.05$. All statistical analysis was performed using SigmaStat 3.5 (Systat Software, Inc., San Jose, CA) whereas kinetic parameters were calculated using the non-linear regression analysis module from SigmaPlot according to the Michaelis-Menten equation (Version 10.0; Systat Software, Inc., Point Richmond, CA).

Results

Uptake of PBDE congeners by hepatic Oatps expressed in HEK293 cells

The functional expression of Oatp1a1, Oatp1a4, Oatp1b2 and Oatp2b1 in HEK293 cells by transient transfection was confirmed by measuring uptake of well-known model substrates of these transporters (data not shown). 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 0.08 μ M (BDE47 and BDE99) or 0.1 μ M (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 2).

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

To further characterize the mechanism of PBDE transport by mouse Oatps expressed in HEK293 cells uptake of BDE47, BDE99 and BDE153 by Oatp1a4, Oatp1b2, and Oatp2b1 was measured in a time and concentration-dependent manner.

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

Uptake of BDE47, BDE99 and BDE153 by Oatp1b2 was linear for at least 1 min (Figure 4A–4C). Concentration-dependent transport of PBDEs by Oatp1b2 is shown in Figure 4D–4F. Kinetic analysis results for Oatp1b2 were similar to that of Oatp1a4 with BDE47 ($0.46 \pm 0.03 \mu$ M) transported with the highest affinity, followed by BDE99 ($0.72 \pm 0.08 \mu$ M) and BDE153 ($1.39 \pm 0.09 \mu$ M). 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 $\times$ min) followed by BDE99 (2.8 ± 0.4 nmol/mg protein $\times$ min) and BDE153 (18.8 ± 1.3 nmol/mg protein $\times$ min).

Oatp2b1-mediated uptake of BDE47, BDE99, and BDE153 was linear up to 1 min (Figure 5A–5C), similar to that of Oatp1a4 and Oatp1b2. As shown in Figure 5D–5F, Oatp2b1 transported all three congeners with similar affinities (BDE47: $K_m = 0.95 \pm 0.04 \mu$ M; BDE99: $K_m = 1.10 \pm 0.12 \mu$ M; BDE153: $K_m = 1.02 \pm 0.14 \mu$ M) and BDE47 (7.0 ± 1.02 nmol/mg protein $\times$ min) as well as BDE99 (4.4 ± 0.58 nmol/mg protein $\times$ min) with approximately the same rate whereas BDE153 (19.0 ± 0.58 nmol/mg protein $\times$ min) was transported with a higher V_{max} than BDE47 and BDE99.

All kinetic parameters are summarized in Table 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 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).

Contribution of Oatp1a4- and Oatp1b2-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 accumulation of BDE47 over an 8 hr period, with peak concentrations detected at the 3-hr time point (Figure 6A and 6B). In Oatp1a4-null mice, as shown in Figure 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 6B).

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, Oatp1b2, and Oatp2b1, are expressed (Hagenbuch *et al.*, 2000; Ogura *et al.*, 2000; van Montfoort *et al.*, 2002; Cheng *et al.*, 2005). However, the functional relationship between OATP/Oatps in humans and mice is not yet clear. Moreover, because the substrate selectivity of OATPs/Oatps is broad, and because several compounds are common substrates for plural OATP subtypes, the identity of the major contributors to the hepatic uptake of PBDEs in mice was unclear. 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.

The 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. Kinetic studies of PBDEs in mice showed that PBDE congeners initially accumulate in the 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 were lacking, however, we have shown that PBDEs are transported by OATPs expressed in human hepatocytes (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 (Pacyniak *et al.*, 2010). The rank order for transport affinities was similar for the OATP1B sub-family with BDE47 transported with the highest affinity ($K_m \approx 0.3$ – $0.4 \mu\text{M}$) followed by BDE99 ($K_m \approx 0.7$ – $0.9 \mu\text{M}$) then BDE153 ($K_m \approx 0.15$ – $2 \mu\text{M}$). The OATP2B subfamily transported all three PBDE congeners with approximately the same affinity ($K_m \approx 1 \mu\text{M}$). Oatp1a4 which does not have a human ortholog expressed in hepatocytes transported BDE47 with the greatest affinity followed by BDE99 and 153.

Of the two OATP1A subfamily members, only Oatp1a4 but not Oatp1a1 transports PBDEs. Consistent with the present findings, a dose dependent increase of Oatp1a4 mRNA expression was observed in mice exposed to PBDEs (Szabo *et al.*, 2009). Given that these

two proteins have 80% amino acids 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.

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.*, 2005; 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.*, 2010). Thus, we were interested in the contribution by Oatp1a4 and Oatp1b2 for hepatic BDE47 accumulation. Our *in vitro* data support the suggestion that Oatp1b2 is a high capacity transporter (Table 1), but because transporter expression levels depend on the expression system and because expression of Oatp1a4 and Oatp1b2 in HEK293 cells might be different from mouse hepatocytes, we cannot make any definitive conclusions from our *in vitro* studies. Therefore, in order to elucidate the roles of Oatp1a4 and Oatp1b2 in hepatic disposition of PBDEs we took advantage of the Oatp-null animal model. 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 6A). However, over the same time period, BDE47 concentrations in the liver were reduced approximately 50% in Oatp1b2-null mice (Figure 6B) suggesting that Oatp1b2 plays a more important role than Oatp1a4 in hepatic transport when BDE47 concentrations are achieving (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. These results could theoretically also be due to altered intestinal absorption, extrahepatic uptake or metabolism. However, because both Oatp1a4 and Oatp1b2 are essentially not expressed in mouse intestine (Cheng *et al.*, 2005) the involvement of the intestine is very unlikely. Furthermore, no significant compensatory changes in the hepatic expression of other Oatps and no significant changes in the expression of drug-metabolizing enzymes in the Oatp1a4- and Oatp1b2-null mice were noted (Lu *et al.*, 2008; Gong *et al.*, 2011). Comparison of the plasma area under the curve (AUC) of BDE47 in wild-type as well as the Oatp-null animals would further elucidate the effect of Oatp1a4 and Oatp1b2 transporters on BDE47 hepatic uptake. However, the current study design did not allow for such investigation. In the absence of any pharmacokinetic data the differences in hepatic levels of radioactivity observed in Oatp1a4- and Oatp1b2-null mice are very likely due to the missing Oatps. Although, we cannot exclude those differences in intestinal absorption, extrahepatic uptake, compartmentation, and metabolism prior reaching liver may also account for the differences in hepatic levels of PBDEs.

Gastrointestinal absorption of PBDE congeners 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). 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., 2003; Bronger *et al.*, 2005; Grube *et al.*, 2006). In mice, Oatp2b1 mRNA has been detected in multiple tissues including the liver, kidney, lung, brain, the 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, infant 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-mediated neurotoxicity.

In conclusion, the data presented in the current study demonstrate that uptake of BDE47, BDE99 and BDE153 by HEK293 cells expressing mouse Oatps. In addition, they suggest that Oatp1b2 is the major transporter for BDE47 uptake and that in hepatocytes Oatp1a4 plays a minor role. Furthermore, the data also suggest that transport of PBDEs into the brain can be mediated by Oatp1a4 and Oatp2b1 and establishes mice as a model system to study PBDE disposition in humans.

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Highlights

- PBDE congeners are substrates of OATPs expressed in human hepatocytes
- Mice are commonly used to study the toxicity of chemicals like the PBDE congeners
- Oatp1a4, Oatp1b2, and Oatp2b1 transported all three PBDE congeners *in vitro*
- *in vivo* Oatp1a4 plays a minor and Oatp1b2 a major role in BDE47 liver accumulation

Figure 1.
Oatps expressed in mouse hepatocytes.

Figure 2. Comparison of PBDE congener uptake by Oatps expressed in mouse liver
 HEK293 cells were transiently transfected with Oatp1b2, Oatp2b1, Oatp1a1, Oatp1a4 or empty vector. 48 hours later (A) Oatp1b2, (B) Oatp1b2, (C) Oatp1a1, (D) Oatp1a4 expressing or pcDNA5/FRT(Oatp1b2, Oatp2b1) or pExpress-1 (Oatp1a1, Oatp1a4) control cells were incubated with 0.08 μ M BDE47, 0.08 μ M BDE99 or 0.1 μ M BDE153 for 5 minutes. Values represent means $\pm$ S.E. of triplicate samples for at least two independent experiments. *, $p < 0.05$ compared to vector control.

Figure 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 37°C at the indicated time points. Filled in circles (●) represent vector control [pExpress-1] uptake while 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 was measured under initial linear rate conditions at 37°C with Oatp1a4-expressing and empty vector transfected HEK293 cells. After subtracting the values obtained with the empty vector transfected HEK293 and corrected for total protein concentration, 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. The unpaired Student t test was performed to determine statistical significance. Differences were considered significant at $P < 0.05$.

Figure 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 in circles (●) represent vector control [pcDNA5/FRT] uptake while 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 was measured under initial linear rate conditions at 37°C with Oatp1b2-expressing and empty vector transfected HEK293 cells. After subtracting the values obtained with the empty vector transfected HEK293 and corrected for total protein concentration, net Oatp1b2-mediated uptake was fitted to the Michaelis-Menten equation and plotted as a dashed line (---). Means $\pm$ S.E. of triplicate determinations are given. The unpaired Student t test was performed to determine statistical significance. Differences were considered significant at $P < 0.05$.

Figure 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 in circles (●) represent vector control [pcDNA5/FRT] uptake while 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 was measured under initial linear rate conditions at 37°C with Oatp2b1-expressing and empty vector transfected HEK293 cells. After subtracting the values obtained with the empty vector transfected HEK293 and corrected for total protein concentration, net Oatp2b1-mediated uptake was fitted to the Michaelis-Menten equation and plotted as a dashed line (---). Means $\pm$ S.E. of triplicate determinations are given. The unpaired Student t test was performed to determine statistical significance. Differences were considered significant at $P < 0.05$.

Figure 6. Contribution of Oatp1a4- and Oatp1b2-mediated uptake of BDE47 *in vivo*
Female, 10–12 week old C57BL/6 wild type, (A) Oatp1a4- or (B) Oatp1b2-knockout mice (n= 3–4) were administered a single oral dose 1 mg/kg dose of [¹⁴C] BDE47. Livers were harvested at time 0,1,3 and 8 hours post-administration. Livers were solubilized and radiolabeled BDE47 was quantified by liquid scintillation analysis. In order to determine statistical differences between wild type and either Oatp1a4- or Oatp1b2-knockout mice, intergroup comparisons were performed by one-way ANOVA followed by the Bonferroni t-test. The *p* value for statistical significance was set to *p* < 0.05.

Table 1
Kinetic parameters of PBDE congener uptake by Oatps expressed in mouse liver

Transporter	Substrate	K_m (μ M)	V_{max} (nmol/mg protein $\times$ 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 were determined 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 $\pm$ S.E. are given for 2-4 independent experiments.