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Sulfated Metabolites of Polychlorinated Biphenyls Are High-Affinity Ligands for the Thyroid Hormone Transport Protein Transthyretin

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Key Words: hydroxylated PCB, OHPCB, PCB, PCB sulfates, polychlorinated biphenyl, sulfation, thyroid disruption, transthyretin

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List of Abbreviations:

ANS, 8-anilinoanthracene-1-sulfonic acid

hSULT1A1, human cytosolic sulfotransferase 1A1

K_d , equilibrium dissociation constant

LC-PCB, lower-chlorinated PCB

NaOH, sodium hydroxide

OHPCB, hydroxylated PCB

PAP, adenosine-3', 5'-diphosphate

PAPS, adenosine-3'-phosphate-5'-phosphosulfate

PCB, polychlorinated biphenyl

PDB, Protein Data Bank

SULT, cytosolic sulfotransferase

T4, 3,3',5,5'-tetraiodo-L-thyronine/ L-thyroxine

TTR, transthyretin

Abstract

Background: The displacement of L-thyroxine (T4) from binding sites on transthyretin (TTR) is considered a significant contributing mechanism in PCB-induced thyroid disruption. Previous research has discovered hydroxylated PCB metabolites (OHPCBs) as high-affinity ligands for TTR, but the potential for binding of conjugated PCB metabolites such as PCB sulfates has not been explored.

Objectives: We evaluated the binding of five lower-chlorinated PCB sulfates to human TTR and compared their binding characteristics to those determined for their OHPCB precursors and for T4.

Methods: We utilized fluorescence probe displacement studies and molecular docking simulations to characterize the binding of PCB sulfates to TTR. The stability of PCB sulfates and the reversibility of these interactions were characterized by HPLC analysis of PCB sulfates following their binding to TTR. The ability of OHPCBs to serve as substrates for human sulfotransferase 1A1 (hSULT1A1) was assessed by OHPCB-dependent formation of adenosine-3', 5'-diphosphate, an end product of the sulfation reaction.

Results: All five PCB sulfates were able to bind to the high-affinity binding site of TTR with equilibrium dissociation constants (K_d values) in the low nanomolar range (4.8 – 16.8 nM), similar to that observed for T4 (4.7 nM). Docking simulations provided corroborating evidence for these binding interactions and indicated multiple high-affinity modes of binding. All OHPCB precursors for these sulfates were found to be substrates for hSULT1A1.

Conclusions: Our findings show that PCB sulfates are high-affinity ligands for human transthyretin and therefore indicate, for the first time, a potential relevance for these metabolites in PCB-induced thyroid disruption.

Introduction

Polychlorinated biphenyls (PCBs) comprise a group of 209 former industrial chemicals that are classified as toxic, bioaccumulative and environmentally persistent (Safe 1993). Many PCBs, particularly the lower-chlorinated congeners (LC-PCB), possess semi-volatile properties and are globally detected in air, with the highest concentrations being found in industrialized urban areas (Breivik et al. 2007; Hu et al. 2010; Wethington and Hornbuckle 2005). In contrast to the higher-chlorinated congeners, LC-PCBs exhibit higher susceptibility for metabolic conversion, but their fate and toxicities within the human body remain relatively poorly understood (Robertson and Ludewig 2011). Considering the abundance of LC-PCBs, and the resulting public health concerns, the identification of their toxicologically relevant metabolites and their molecular targets is essential for risk assessment purposes. To date, research has been primarily focused on the toxicological effects of parent PCBs and their hydroxylated metabolites (OHPCBs).

Certain OHPCBs have been identified as excellent substrates for cytosolic sulfotransferases (SULTs), a family of enzymes that catalyze the formation of sulfate conjugates (Ekuase et al. 2011; Liu et al. 2009; van den Hurk et al. 2002; Wang et al. 2006). It was recently reported that the formation of sulfate conjugates is a major metabolic pathway for PCB 3 in rats, thereby providing evidence for the formation of PCB sulfates *in-vivo* (Dhakal et al. 2012). Despite these indications of the potential for the formation of PCB sulfates *in-vivo*, their fates and toxicities have not yet been characterized.

The thyroid gland is a target of PCB toxicity. Chronic exposure to commercial PCB mixtures produces increases in the mass of the thyroid gland and in the number of thyroid neoplasms (Mayes et al. 1998). Both of these changes, it has been suggested, may be linked to PCB-driven

reduction in serum levels of thyroid hormones, a commonly measured result of PCB exposure (Knerr and Schrenk 2006; Pearce and Braverman 2009). Parent PCBs and OHPCBs are known thyroid disruptors that induce multiple effects on the thyroid homeostatic system in a congener-dependent manner (Boas et al. 2009; Brouwer et al. 1999; Patrick 2009). Epidemiological studies have revealed a correlation between prenatal PCB exposure and behavioral effects, decreased cognitive function and mental retardation in infants, and these effects may be caused by PCB-induced alterations of the thyroid status in the fetal brain (Boucher et al. 2009; Darras 2008; Schantz and Widholm 2001). A significant contributing mechanism in PCB-induced hypothyroxinemia is the displacement of L-thyroxine (T4) from its binding sites on the thyroid hormone transport protein transthyretin (TTR) (Gutleb et al. 2010; Kodavanti and Curras-Collazo 2010; Ucan-Marin et al. 2009; Ucan-Marin et al. 2010). TTR is the main transporter of T4 in the cerebrospinal fluid and, in addition to serum albumin and thyroxine binding globulin, one of three thyroid hormone carriers in human plasma (Larsson et al. 1985; Petitpas et al. 2003). Certain OHPCBs were previously identified as ligands for TTR that were capable of competing with T4 for its two binding sites (Lans et al. 1993; Purkey et al. 2004; Rickenbacher et al. 1986). These sites exhibit negative cooperativity and as a result, only one molecule of T4 is bound to TTR under physiological conditions (Ferguson et al. 1975). Due to TTR's suggested role as a mediator for the transport of thyroid hormones across the blood-brain barrier and the placenta, structurally specific binding of PCB metabolites to T4 binding sites on TTR is assumed to interfere with the delivery of thyroid hormones to target tissues in the brain and the fetus and may also facilitate the transport of bound ligands to these compartments (Brouwer et al. 1999; Brouwer et al. 1998; Mortimer et al. 2012; Schreiber et al. 1995).

Structural similarities between PCB sulfates and T4 led us to hypothesize that PCB sulfates represent a class of high-affinity ligands for human transthyretin. In order to test this hypothesis, we characterized the binding of five lower-chlorinated PCB sulfates to TTR, and compared their equilibrium dissociation constants (K_d values) to those determined for their hydroxylated precursors and for T4. In addition, we conducted docking simulations to determine the potential molecular interactions of these sulfates within the T4 binding site of TTR. Additional studies addressed the reversibility of the interaction of PCB sulfates with TTR and the likelihood that a human sulfotransferase, hSULT1A1, can catalyze the formation of these PCB sulfates.

The parent PCBs of all metabolites utilized in this study have been detected in Chicago air (Hu et al. 2010). Of particular interest is the sulfate ester derived from PCB 11. The parent PCB congener is a major component of PCBs present in Chicago and Cleveland air, yet it was never contained in any of the commercial PCB mixtures (Hu et al. 2010; Persoon et al. 2010). Current research suggests that PCB 11 is a by-product in paint production, thus representing a continuing source for PCB exposure (Hu and Hornbuckle 2010).

Methods

Chemicals: Hydroxylated PCBs (4'-chloro-biphenyl-2-ol, 4'-chloro-biphenyl-3-ol, 4'-chloro-biphenyl-4-ol, 3,3'-dichloro-biphenyl-4-ol, and 3',4'-dichloro-biphenyl-4-ol) and the ammonium salts of 2'-sulfooxy-4-chloro-biphenyl, 3'-sulfooxy-4-chloro-biphenyl, 4-chloro-4'-sulfooxy-biphenyl, and 3,4-dichloro-4'-sulfooxy-biphenyl were synthesized and characterized as previously described (Lehmler and Robertson 2001; Li et al. 2010). The ammonium salt of 3,3'-dichloro-4'-sulfooxy-biphenyl was synthesized and characterized as described in the Supplemental Material. 8-Anilinonaphthalene-1-sulfonic acid (ANS), L-thyroxine sodium salt

pentahydrate (T4), adenosine-3'-phosphate-5'-phosphosulfate lithium salt hydrate (PAPS), adenosine-3', 5'-diphosphate sodium salt (PAP), sodium phosphate monobasic and transthyretin purified from human plasma (> 95%) were purchased from Sigma Aldrich (St.Louis, MO). TTR was used without further purification and its purity was routinely confirmed by SDS-PAGE. The protein concentration was determined by the Bradford assay. PAPS was purified to a purity equal to or higher than 98% according to a procedure published by Sekura (1981). Cytosolic extract of *E. coli* expressing recombinant human sulfotransferase 1A1 (cytosolic protein concentration of 10 mg/ml) was purchased from Xenotech (Lenexa, KS) and used without further purification. Acetonitrile was purchased from Fisher Scientific (Hampton, NH). Sodium chloride, potassium phosphate monobasic and ammonium chloride were obtained from Research Products International (Mt. Prospect, IL).

ANS displacement assay: ANS displacement studies have frequently been used to determine dissociation constants for potential ligands of TTR (Cao et al. 2010; Cheng et al. 1977; Smith et al. 1994). We determined K_d values for PCB metabolites and T4 utilizing a modified version of a previously published procedure (Cheng et al. 1977). A solution containing 0.5 μ M TTR and 5 μ M ANS (total volume 1000 μ L) in phosphate buffer (50 mM sodium phosphate, 100 mM NaCl, pH 7.4) was titrated with small aliquots of PCB metabolites or T4 (structures shown in Figure 1) using a glass syringe (Hamilton, Reno, NV). The displacement of ANS was monitored by measuring the decrease in fluorescence intensity at 470 nm upon excitation of the molecule at 410 nm in a Spectramax M5 fluorimeter (see Supplemental Material, Figure S1). Three fluorescence measurements were averaged per determination, and at least three separate determinations were made at each ligand concentration. The protocol was optimized for ligand concentrations up to 2000 nM, and assays were conducted at 25°C (\pm 0.2°C) in quartz cuvettes

with 1 cm path length. The fluorescence was corrected for dilution (up to 4.6% of the total volume) and was found to be unaffected by the solvent of the ligands (0.5 mM NaOH) and the duration of the assay (see Supplemental Material, Figure S2). The total change in pH was less than 0.01 pH units. The concentration of ANS in phosphate buffer was determined spectrophotometrically at 350 nm using a molar extinction coefficient of $6.3 \times 10^3 \text{ cm}^{-1} \text{ M}^{-1}$ (Kolb and Weber 1975). Binding data were evaluated using both a one-site plus nonspecific binding equation ($y = (B_{\text{max}1} * x)/(K_{\text{d}1} + x) + N_s$) and a two-site binding equation ($y = (B_{\text{max}1} * x)/(K_{\text{d}1} + x) + (B_{\text{max}2} * x)/(K_{\text{d}2} + x)$). In these equations, $K_{\text{d}1}$ is the dissociation constant for the TTR-ligand complex and $K_{\text{d}2}$ is the dissociation constant for the TTR complex with two bound ligands, $B_{\text{max}1}$ and $B_{\text{max}2}$ are the relative changes in fluorescence required to saturate the respective binding sites and N_s is a constant representing low-affinity interactions. In this case, the N_s term includes both the low-affinity second T4 binding site in TTR and any other low-affinity interaction with the protein. X and y represent ligand concentrations and changes in fluorescence (Δ fluorescence), respectively. Best fits for $K_{\text{d}1}$ were obtained by fitting data in the 0-100 nM range to the one-site plus nonspecific binding equation. In order to determine $K_{\text{d}2}$ values, all available data points were fit to the two-site binding equation.

HPLC analysis of PCB sulfates: 0.5 μM TTR and 5 μM PCB sulfate were incubated in phosphate buffer (50 mM sodium phosphate, 100 mM NaCl, pH 7.4) at 25°C ($\pm 0.5^\circ\text{C}$) for 120 minutes. After 0, 60 and 120 minutes, 20 μl samples were analyzed for PCB sulfates by chromatography on a C18 HPLC column with a linear acetonitrile gradient (15% - 95%) in triethylammonium acetate (1% v/v, pH 7.5). Detailed HPLC conditions and representative chromatograms are provided in Supplemental Material, Figure S3.

Molecular Docking Simulations: All docking simulations were performed using SYBYL X software (Tripos, St. Louis, MO). From the protein data bank (PDB), we selected the crystal structure complex of TTR with bound 2,'6'-difluorobiphenyl-4-carboxylic acid (PDB # 2F7I) to constitute the receptor model for human transthyretin. The receptor structure was prepared according to protocols provided by Tripos. Briefly, protein chain termini were set into their charged states, and hydrogen atoms were added before staged minimizations were performed with the Powell method using the MMFF94s force field. Ligands present in the crystal structure were extracted from the receptor and used to define the active site (protomol). Default settings were utilized for protomol generation. Ligands (2'PCB 3 sulfate, 3'PCB 3 sulfate, 4'PCB 3 sulfate, 4'PCB 11 sulfate, 4'PCB 12 sulfate) were modeled in ChemBioDraw 12.0 (Perkin Elmer, Waltham, MA) and imported into a SYBYL X database. All ligands were energy-minimized using the Tripos force field and docked into prepared receptor structures using the Surflex-Dock module in Geom mode. Twenty different binding poses were generated per molecule and ranked according to their binding energies. Images were created using the PyMOL Molecular Graphics System (v. 1.5.0.4; Schrödinger, LLC).

Sulfotransferase assay: The assays for sulfation of OHPCBs catalyzed by hSULT1A1 were conducted according to a previously published procedure (Liu et al. 2009). Assay mixtures containing 0, 10, 20 or 30 μM of the potential substrate were pre-incubated at 37°C in the presence of 200 μM PAPS and 7.5 mM 2-mercaptoethanol in 250 mM potassium phosphate at pH 7.0. Following the addition of 1 μl cytosolic extract of *E. coli* expressing hSULT1A1 (10 μg cytosolic protein), the reaction mixture (total volume 30 μl) was incubated for 10 minutes before the reaction was terminated by the addition of 30 μl methanol. 20 μl samples were subsequently analyzed by HPLC to determine the amount of PAP formed in the reaction (Sheng et al. 2001).

Results

Binding of T4 and PCB metabolites to transthyretin: We determined K_d values for T4 of 4.7 ± 1.1 nM and 234 ± 11 nM at the high and low-affinity binding sites on TTR, respectively (see Figure 2A and Supplemental Material, Figure S4A). These values are in agreement with previously published data for T4 binding to TTR (Cheng et al. 1977; Smith et al. 1994; Ucan-Marín et al. 2009; Ucan-Marín et al. 2010). Following the validation of the assay conditions, we determined K_d values for five PCB sulfates and their hydroxylated precursors (see Figure 2 and Supplemental Material, Figure S5). All five PCB sulfates were found to be ligands for the high-affinity binding site with K_{d1} values ranging from 4.8 nM to 16.8 nM. 2'PCB 3 sulfate, 3'PCB 3 sulfate, and 4'PCB 11 sulfate exhibited significantly higher affinities (i.e., lower K_{d1} values) than their respective OHPCBs (Table 1). While the K_{d1} for 4'PCB 3 sulfate (14.5 nM) was lower than the one for 4'OHPCB 3 (19.3 nM), the difference was not significant. The lowest K_{d1} was obtained for 4'PCB 11 sulfate (4.8 nM) (see Figure 2C and Table 1). The highest-affinity binding for a metabolite in this study was 4'OHPCB 12, which exhibited a significantly lower dissociation constant ($K_{d1} = 2.7$ nM) than its sulfate (16.8 nM) and T4 (4.7 nM). K_d values determined for the low-affinity binding site revealed a clustered pattern for mono- and dichlorinated PCB metabolites. While dichlorinated PCB metabolites bound with K_{d2} values ranging from 624 to 825 nM (see Table 1 and Supplemental Material, Figure S4A), the chosen concentration range of up to 2000 nM was insufficient to facilitate quantitative displacement of ANS from the second binding site by PCB 3 metabolites (see Supplemental Material, Figure S4B). All lower chlorinated PCB metabolites exhibited significantly higher K_{d2} values than T4.

Stability of PCB sulfates in the assay: In order to determine the stability of PCB sulfates under the conditions of the assay for binding to TTR, we developed an HPLC procedure for the

separation and quantification of PCB sulfates and their respective OHPCBs. Using a linear 15-95% acetonitrile gradient, we were able to separate all five matching pairs of PCB sulfates from their OHPCB precursors (see Supplemental Material, Figure S3). Utilizing this procedure, we were able to quantitatively recover 5 μ M samples of 2'PCB 3 sulfate, 3'PCB 3 sulfate, 4'PCB 3 sulfate, 4'PCB 11 sulfate and 4'PCB 12 following incubation with 0.5 μ M TTR under ANS assay conditions (see Supplemental Material, Figure S6). Moreover, quantitative recovery of the PCB sulfates by HPLC indicates that the binding to TTR is reversible under the conditions of HPLC analysis.

Selection of a receptor structure for docking simulations: TTR is a 55 kDa homotetrameric protein consisting of a dimer of dimers in a twofold symmetrical arrangement. The interface between the two dimers forms a channel that contains two T4 binding sites (Blake and Oatley 1977; Wojtczak et al. 1996). Each T4 binding site is lined with mostly hydrophobic residues that form two symmetrical sets of three halogen binding pockets, P1 – P3 and P1' – P3' (Blake and Oatley 1977). P1 and P1' are located on the outside of the channel and comprise side chains of residues Met13, Lys15, Thr106 and Ala108. On the outermost part of this pocket, Lys15 and Glu54 form a charged region that provides the possibility for polar interactions between protein and ligands. The side chains of Lys15, Leu17, Ala109 and Leu110 form P2 and P2'. P3 and P3', the innermost binding pockets, are formed by side chains of Ala108, Leu110, Ser117 and Thr119. The crystal structure of T4 bound to TTR (PDB #: 2ROX) indicates a binding orientation in which the alanyl moiety of T4 facilitates hydrogen bonding interactions with Lys15 and Glu54, while the phenol is oriented towards Ser117 and Thr119 (Wojtczak et al. 1996). Iodine substituents in the 3 and 5 position of thyroxine are positioned in P2 and P3, respectively. We initially selected three TTR crystal structure complexes with different bound

ligands (PDB #: 2ROX, 2G9K, and 2F7I) from the PDB to constitute the receptor model for transthyretin. Following extraction of the ligands and subsequent docking of the extracted ligands into the generated protomol, the closest match was obtained for the complex of 2',6'-difluorobiphenyl-4-carboxylic acid and TTR (PDB # 2F7I). The docked ligand revealed similar positioning and molecular interactions within the binding site as compared to the crystal structure (Figure 3A). In particular, the carboxylate of 2',6'-difluorobiphenyl-4-carboxylic acid engages in hydrogen bonding interactions with Lys15. The 2' and 6' fluorine substituents are located in P3 and P3'. Due to the lack of fluorines on the unsubstituted ring, it shows some flexibility and is twisted approximately 90° in the docked structure as compared to the crystal structure.

Docking simulations for PCB sulfates: Using PDB # 2F7I as the receptor model, we were able to dock all five PCB sulfates into the T4 binding site (Figure 3B-F). In order to be consistent in the interpretation of our docking results, only the lowest energy binding conformation is indicated per PCB sulfate. The lowest energy binding conformations indicated an orientation that enables hydrogen bonding interactions between the sulfate groups and lysine 15. The only exception was 2'PCB 3 sulfate, where hydrogen bonding with lysine 15 residues was sterically unfavorable. Instead, the sulfate moiety of 2'PCB 3 sulfate formed a hydrogen bond with Leu110, resulting in a reverse orientation of this metabolite. The binding modes of 4'PCB 11 sulfate and 4'PCB 12 sulfate, the only two compounds with chlorines in the meta position were similar to those previously determined for T4. While the sulfate group facilitates hydrogen bonding with Lys15, the meta-positioned chlorines appeared to be inserted, analogously to the iodines in T4, into P2 and P3. It should be noted that some of the higher energy binding poses

obtained for PCB sulfates exhibited an antiparallel orientation with the sulfate group pointing towards the central Ser117.

OHPCBs as substrates for hSULT1A1: Since formation of PCB sulfates requires their metabolic formation from OHPCBs, we determined the rate of sulfation of OHPCBs by recombinant human SULT1A1. The five OHPCBs that were tested in this study were substrates for the enzyme and exhibited a concentration-dependent increase in the rate of sulfation (Figure 4). The highest specific activities were determined for the ortho-hydroxylated 2'OHPCB 3 and for the meta-hydroxylated 3'OHPCB 3. The para-hydroxylated 4'OHPCB 3, 4'OHPCB 11 and 4'OHPCB 12 exhibited slightly lower specific activities. Thus, the hSULT1A1, a major human cytosolic sulfotransferase, catalyzes the sulfation of these OHPCBs.

Discussion

A number of recent studies have demonstrated the potential of OHPCB metabolites to serve as substrates for sulfotransferases *in-vitro* and *in-vivo* (Dhakal et al. 2012; Ekuase et al. 2011; Liu et al. 2009). Importantly, utilizing a rat model it was shown that serum concentrations of PCB 3 sulfates greatly exceeded those of their respective OHPCBs, thereby suggesting that in contrast to the higher-chlorinated PCBs and OHPCBs that tend to be retained in blood and adipose tissue, LC-PCBs undergo relatively rapid oxidation and conjugation upon absorption (Dhakal et al. 2012). Considering the abundance of LC-PCBs in indoor and outdoor air samples (Hu et al. 2010; Ludewig et al. 2008), these discoveries raise concern about both the fate and potential toxicities of PCB sulfates.

In this study, we demonstrate the ability of five LC-PCB sulfates to bind with high-affinity to the most physiologically relevant binding site for T4 on human TTR. Utilizing a fluorescence displacement assay, K_d values determined for the high-affinity binding site ranged from 4.8 nM to 16.8 nM and were thus comparable to T4 (4.7 nM). Importantly, four of the five PCB sulfates, 2'PCB 3 sulfate, 3'PCB 3 sulfate, 4'PCB 3 sulfate and 4'PCB 11 sulfate interacted with higher affinities than their corresponding OHPCBs. Although OHPCBs have been previously shown to be high-affinity ligands for TTR that are capable of competing with T4 (Gutleb et al. 2010; Lans et al. 1993; Rickenbacher et al. 1986), our results now establish the potential for binding of PCB sulfates to TTR. Some of the higher-chlorinated OHPCBs are several times more potent ligands for TTR than T4 (Chauhan et al. 2000; Rickenbacher et al. 1986; Ucan-Marín et al. 2009; Ucan-Marín et al. 2010). It is possible that some higher-chlorinated PCB sulfates may exhibit even higher affinities for TTR than the lower-chlorinated ones utilized in this study, although testing of this hypothesis and evaluating the role of sulfation in the metabolism of such higher-chlorinated PCBs will await further studies. Besides PCB mono-sulfates, which constituted the largest group of metabolites in serum of PCB 3 exposed rats, the recent metabolism study on PCB 3 also reported detectable levels of mercapturic acid conjugates and sulfate esters derived from dihydroxylated metabolites, albeit at lower levels (Dhakal et al. 2012). The binding characteristics of such metabolites remain to be characterized in subsequent studies, since they might contribute to the displacement of T4 from binding sites on TTR. Although glucuronide conjugates were not major metabolic products for PCB 3 (Dhakal et al. 2012), we also cannot rule out the possibility of biological effects of these conjugates.

Supporting evidence for high-affinity binding of PCB sulfates to TTR was provided by molecular docking simulations. With the exception of 2'PCB 3 sulfate, the sulfate moiety

facilitated hydrogen bonding interactions with Lys15 residues, thereby dictating the binding orientation of the PCB sulfate. The *ortho*-sulfate group in 2'PCB 3 sulfate appears to sterically prevent this interaction. Instead, the orientation of the molecule is reversed, which enables hydrogen bonding of the sulfate with Leu110. Among the PCB 3 sulfates, 2'PCB 3 sulfate bound significantly better to the high-affinity binding site than 3'PCB 3 sulfate or 4'PCB 3 sulfate (Table 1), and this may be due to the alternate binding orientation within the T4 binding site. In addition, the observed increased affinity of 4'PCB 11 sulfate as compared to 4'PCB 12 sulfate can be explained by the presence of a second meta-chlorine that provides the opportunity for additional anchorage within binding pockets P3 or P3'. This finding is consistent with previous reports indicating that meta- and para-chlorination increases affinities of OHPCBs to TTR (Chauhan et al. 2000; McKinney et al. 1987; Rickenbacher et al. 1986).

Similar to T4, K_d values determined for the second binding site on TTR were found to be at least two orders of magnitude higher than those determined for the high-affinity binding site (Cheng et al. 1977; Smith et al. 1994). K_{d2} values determined for all PCB metabolites were significantly higher than for T4. These weaker interactions with the second binding site are consistent with the negative cooperativity between these sites that is observed with T4 (Ferguson et al. 1975). T4, while structurally similar to PCB metabolites, is a larger molecule with a higher degree of halogenation than the PCB metabolites utilized in this study. These differences may account for a sterically more favorable binding to the second site. However, under physiological conditions, concentrations of T4 are too low to allow binding to the low-affinity binding site (Liz et al. 2010). Consequently, the displacement of T4 by PCB metabolites primarily affects the high-affinity binding site *in-vivo*, whereas both sites may have relevance in the (inter-tissue) transport and retention of xenobiotics.

While in cerebrospinal fluid TTR is the only TH transporter, in human serum the displacement of T4 from binding sites on two additional T4 transport proteins, albumin and thyroxine binding globulin, may be a contributing factor in the saturation of T4 binding sites and should be addressed in further studies. However, previous research has emphasized the displacement of T4 from TTR as a key contributing mechanism in organohalogen induced hypothyroxinemia (Gutleb et al. 2010; Kodavanti and Curras-Collazo 2010). TTR has additional relevance due to its function as a mediator for the transport of thyroid hormones across the placenta and the blood-brain barrier and it has been suggested that the binding of PCB metabolites to TTR may facilitate their transport to the cerebrospinal fluid and to the fetus (Brouwer et al. 1999; Brouwer et al. 1998; Schreiber et al. 1995; Southwell et al. 1993). Recently, it was reported that TTR has the potential for translocation across the placenta into the fetal circulation and represents a potential shuttle system for thyroid hormones and for exogenous compounds (Mortimer et al. 2012). Considering their ability to bind with high affinity to TTR, the PCB sulfates utilized in this study possess the basic requirements for such inter-tissue transport. Moreover, the observations that their binding affinities do not exceed those determined for T4, and that their interactions are non-covalent, points towards their ability to dissociate from TTR upon delivery to potential target tissues. Although higher-chlorinated PCB sulfates remain to be examined for their binding to TTR, previous reports suggest that the higher-chlorinated OHPCBs may be less able to dissociate and may be retained bound to TTR in the circulation (Bergman et al. 1994; Brouwer et al. 1998). Likewise, future research will be required to further assess levels of human exposure to PCB sulfates as well as the *in-vivo* effects of PCB sulfates on T4 concentrations in susceptible tissues and/or transport of PCB sulfates into those tissues.

Conclusions

We have identified five lower-chlorinated PCB sulfates as high-affinity ligands for human TTR. Notably, their binding affinities were similar to those determined for their respective OHPCB precursors and for L-thyroxine. The binding interactions between all five PCB sulfates and TTR were found to be non-covalent. We utilized docking simulations to calculate their lowest energy binding conformations within the thyroxine binding sites of human TTR, thereby providing corroborating evidence for their high-affinity binding, and insight into potential binding orientations. Moreover, the corresponding OHPCBs were found to be substrates for human SULT1A1, a major human cytosolic sulfotransferase. Thus, these results indicate, for the first time, a potential toxicological relevance of PCB sulfates in disruption of thyroid hormone homeostasis in tissues dependent upon TTR-mediated transport.

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Table 1: K_d values determined for PCB metabolites and T4

Compound	n	K_{d1} (nM)	p^a	p^b	K_{d2} (nM)	p^c
L-thyroxine (T4)	4	4.7 ± 1.1	-	-	234 ± 11	-
2'OHPCB 3	4	19.8 ± 6.0	-	0.004	^d	-
2'PCB 3 sulfate	3	5.4 ± 1.2	0.015	0.458	^d	-
3'OHPCB 3	4	14.8 ± 2.0	-	<0.001	^d	-
3'PCB 3 sulfate	3	8.2 ± 1.0	0.007	0.008	^d	-
4'OHPCB 3	3	19.3 ± 3.8	-	<0.001	^d	-
4'PCB 3 sulfate	4	14.5 ± 4.0	0.206	0.005	^d	-
4'OHPCB 11	8	13.9 ± 1.8	-	<0.001	624 ± 207^e	0.004
4'PCB 11 sulfate	3	4.8 ± 0.7	<0.001	0.897	624 ± 63	<0.001
4'OHPCB 12	3	2.7 ± 0.5	-	0.035	825 ± 47	<0.001
4'PCB 12 sulfate	4	16.8 ± 5.8	0.014	0.008	742 ± 58	<0.001

^aStatistical significance levels for PCB sulfates as compared to their OHPCBs (K_{d1})

^bStatistical significance levels for PCB sulfates as compared to T4 (K_{d1})

^cStatistical significance levels for PCB sulfates as compared to T4 (K_{d2})

^dDue to the very low-affinity binding, K_d values could not be determined for the second site

^eBest fit was obtained using a one-site plus nonspecific binding equation

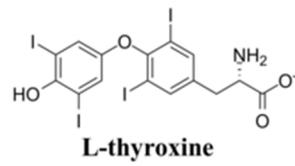
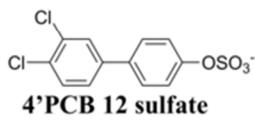
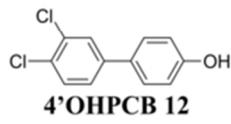
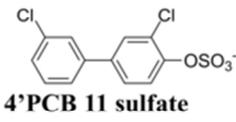
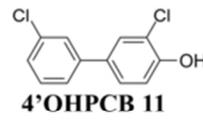
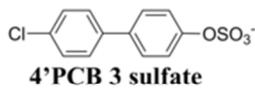
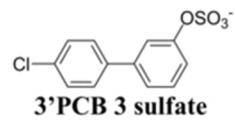
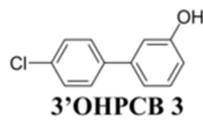
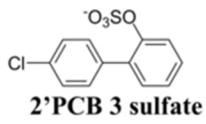
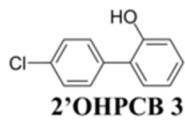
Figure Legends

Figure 1: Chemical structures of PCB sulfates, OHPCBs and L-thyroxine. PCB sulfates utilized in this study were synthesized as ammonium salts.

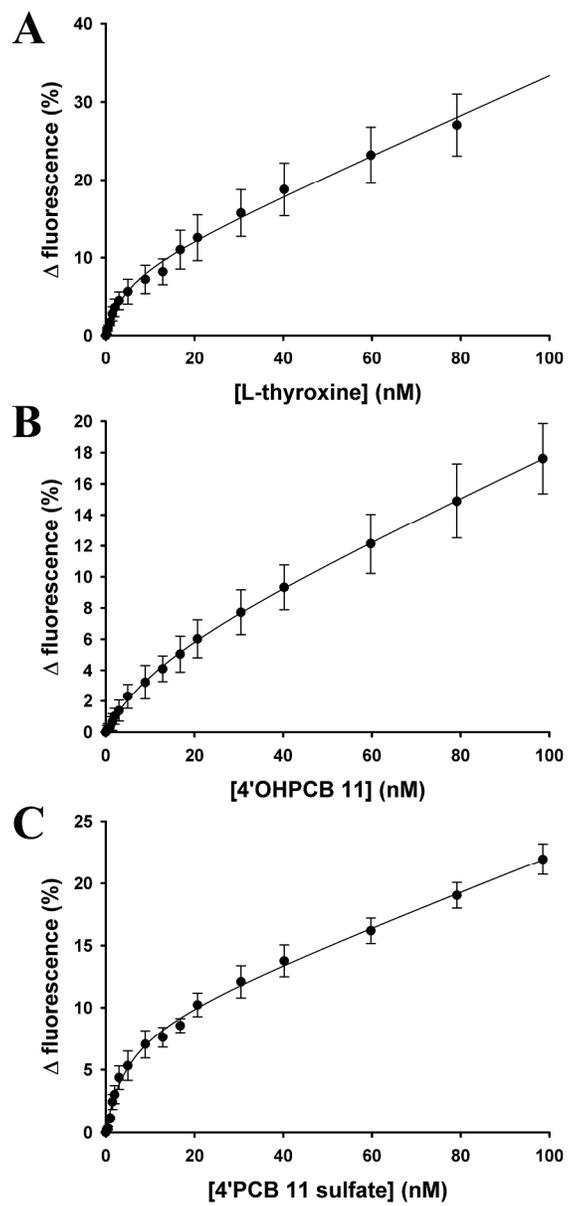
Figure 2: Binding of L-thyroxine, 4'OHPCB11, 4'PCB 11 sulfate, and T4 to the high-affinity site on TTR. Data for the binding of T4 (A), 4OHPCB 11 (B), and 4'PCB 11 sulfate (C) were determined by ANS displacement using ligand concentrations between 0 and 100 nM. Data were fit to a one-site plus nonspecific binding equation, and data points represent means of at least three experiments (Table 1). Error bars indicate standard deviations.

Figure 3: Molecular docking of PCB sulfates into the T4 binding site on TTR. (A) Comparison between the original crystal structure (PDB # 2F7I, blue) and docked 2',6'-difluorobiphenyl-4-carboxylic acid/ TTR (red) complex. Close-up views of the TTR binding site are shown following docking with 4'PCB 11 sulfate (B), 4'PCB 12 sulfate (C), 2'PCB 3 sulfate (D), 3'PCB 3 sulfate (E), and 4'PCB 3 sulfate (F).

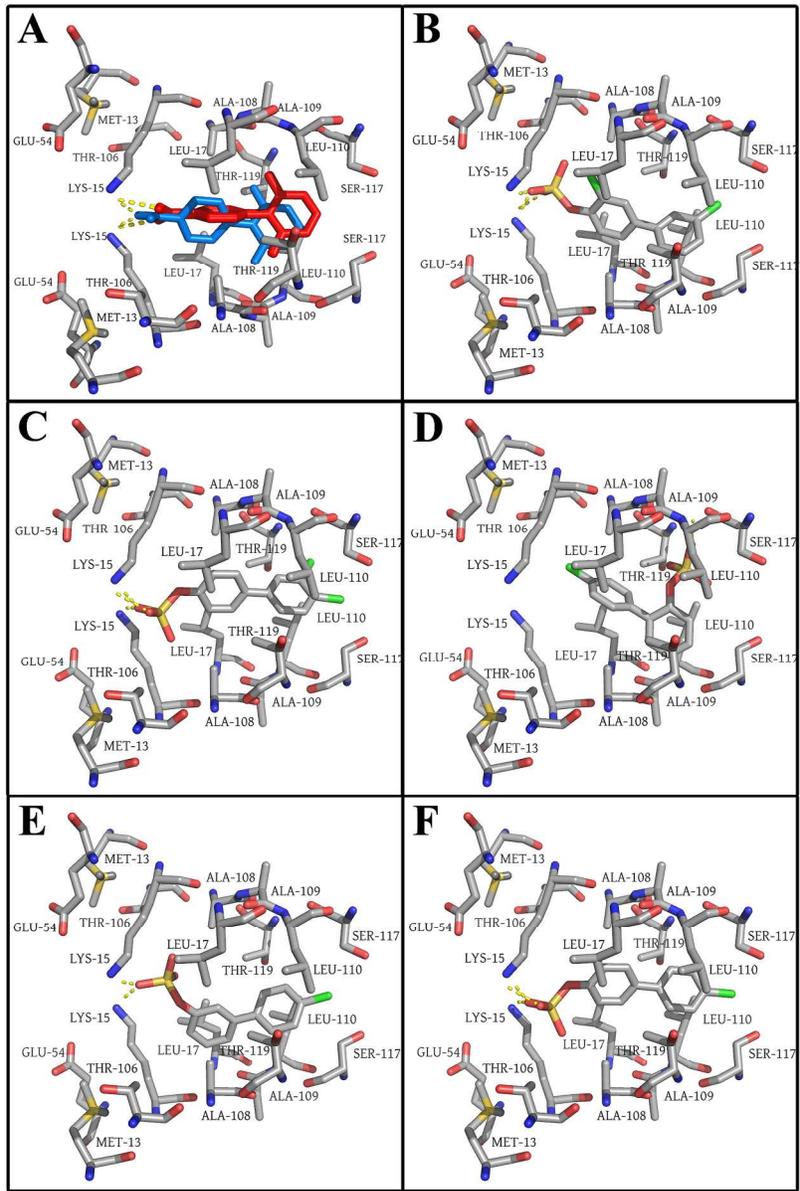
Figure 4: OHPCBs are substrates for human SULT1A1. The sulfation of 2'OHPCB 3, 3'OHPCB 3, 4'OHPCB 3, 4'OHPCB 11, and 4'OHPCB 12 is catalyzed by a cytosolic extract of *E. coli* expressing recombinant human SULT1A1. Significance levels of $p < 0.05$ (*) and $p < 0.001$ (**), as compared to 0 μ M OHPCB, are indicated.



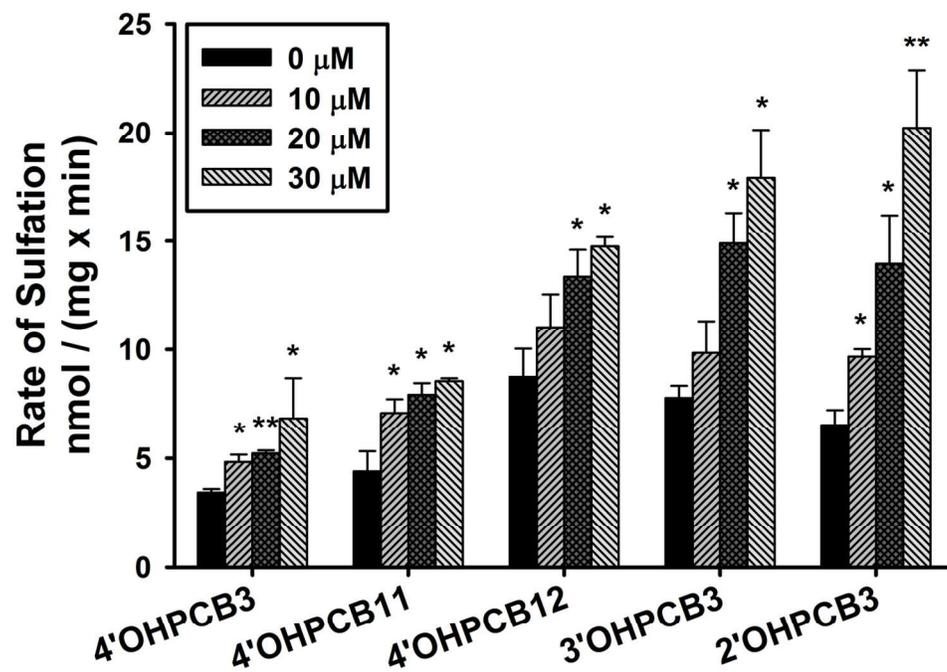
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