Structure-Activity Relationships for Hydroxylated Polychlorinated Biphenyls as Substrates and Inhibitors of Rat Sulfotransferases and Modification of these Relationships by Changes in Thiol Status

Yungang Liu, Jason T. Smart, Yang Song, Hans-Joachim Lehmler, Larry W. Robertson, and Michael W. Duffel

Division of Medicinal and Natural Products Chemistry, College of Pharmacy (Y.L., J.S., M.W.D.); and Department of Occupational and Environmental Health, College of Public Health (Y.S., H.-J.L., L.W.R.), University of Iowa, Iowa City, IA 52242
Running title:

INTERACTIONS OF HYDROXYLATED PCBs WITH RAT SULFOTRANSFERASES

Address correspondence to:

Dr. Michael W. Duffel, Division of Medicinal and Natural Products Chemistry, College of Pharmacy, University of Iowa, Iowa City, IA 52242. Tel: 319-335-8840, Fax: 319-335-8766, E-mail: michael-duffel@uiowa.edu

Number of text pages: 18
Number of tables: 2
Number of figures: 3
Number of references: 40
Number of words in the Abstract: 250
Number of words in Introduction: 708
Number of words in Discussion: 1471

Abbreviations: DTNB, 5,5’-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; GSH: reduced glutathione; GSSG, oxidized glutathione; HPLC, high performance liquid chromatography; OHPCB, hydroxylated polychlorinated biphenyl; CYP, cytochrome P450; PAP, adenosine 3’,5’-diphosphate; PAPS, adenosine 3’-phosphate 5’-phosphosulfate; PCB, polychlorinated biphenyl; SULT, sulfotransferase.
Abstract

Hydroxylated metabolites of polychlorinated biphenyls (OHPCBs) are inhibitors and substrates for various human sulfotransferases (SULTs). Although the rat is often used in toxicological studies on PCBs, the interactions of OHPCBs with rat SULTs are less well understood. In the present study, 15 OHPCBs were investigated as potential substrates or inhibitors of purified recombinant rSULT1A1 and rSULT2A3, the major family 1 and family 2 SULTs present in rat liver, respectively. None of these OHPCBs were substrates for rSULT2A3, 11 weakly inhibited rSULT2A3-catalyzed sulfation of dehydroepiandrosterone, and four had no effect on the reaction. With rSULT1A1, 4-OH PCB 8, 4’-OH PCB 3, 9, 12, 35, and 6’-OH PCB 35 were substrates, while 4’-OH PCB 6, 4-OH PCB 14, 4’-OH PCB 25, 4’-OH PCB 33, 4-OH PCB 34, 4-OH PCB36, 4’-OH PCB 36, 4’-OH PCB 68, and 4-OH PCB 78 inhibited the sulfation of 2-naphthol catalyzed by this enzyme. OHPCBs with a 3,5-dichloro-4-hydroxy substitution were the most potent inhibitors of rSULT1A1, and the placement of chlorine atoms in the ortho- and meta- positions on either ring of para-OH PCBs resulted in significant differences in activity as substrates and inhibitors. The specificity of rSULT1A1 for several inhibitory OHPCBs was altered by pretreatment of the enzyme with oxidized glutathione (GSSG). Four OHPCBs that were inhibitors of rSULT1A1 under reducing conditions became substrates following pretreatment of the enzyme with GSSG. This alteration in specificity of rSULT1A1 for certain OHPCBs suggests that conditions of oxidative stress may significantly alter the sulfation of some OHPCBs in the rat.
Introduction

Although the manufacture of polychlorinated biphenyls (PCBs) has been legally banned in the US for over 30 years, they are persistent in the environment in large quantities and hazardous to public health (ASTDR, 2000; Robertson and Hansen, 2001). The originally released PCBs were predominantly highly chlorinated (6 or more chlorines per molecule). However, PCBs in soils and marine sediments can be de-chlorinated to lower chlorinated congeners (Abramowicz, 1995; Master et al., 2002;). Lower chlorinated PCBs are semi-volatile and present in urban atmospheres of various areas in the U.S. and other countries (Wethington and Hornbuckle, 2005; Ruzickova et al., 2008), and they move in dynamic balance among the atmosphere, water, and soil.

PCBs are biotransformed by cytochrome P450 (CYP) isoforms to hydroxylated PCBs (OHPCBs) (Kaminsky et al., 1981; Safe, 1994; McLean et al., 1996; Ludewig et al., 2007). Lower chlorinated PCBs are often more susceptible than highly chlorinated congeners to biotransformation in CYP-catalyzed reactions to OHPCBs. PCBs administered to rats are initially deposited in the liver and muscles, and then translocated to the skin and adipose tissue (Matthews and Anderson, 1975). The OHPCBs, however, may be selectively concentrated in the liver as compared to adipose tissue, as indicated by a study where the concentration of OHPCBs was about 20 times higher in liver than that in adipose tissues, whereas the concentrations of total PCBs were not significantly different between the two tissues (Guvenius et al., 2002). After CYP-catalyzed hydroxylation of PCBs, the resulting OHPCBs can be conjugated in reactions catalyzed by enzymes such as sulfotransferases (Liu et al., 2006; Wang et al., 2006), UDP-glucuronosyltransferases (Schnellmann et al., 1984; Tampal et al., 2002), and glutathione S-transferases (James, 2001).
The sulfotransferases (SULTs) constitute a superfamily of biotransformation enzymes catalyzing the sulfation of a spectrum of substrates ranging from endogenous hormones and neurotransmitters to xenobiotics. OHPCBs have been observed to be inhibitors and substrates of human cytosolic sulfotransferases (hSULTs), e.g., hSULT1A1 (Wang et al., 2006), hSULT1E1 (Kester et al., 2000), and hSULT2A1 (Liu et al., 2006), major family 1 and family 2 SULTs in humans. While hundreds of toxicological investigations on PCBs have been conducted utilizing rats or tissues/cells derived from rats, relatively little is known about the sulfation of OHPCBs in the rat. Moreover, the specificities of individual SULTs for OHPCBs in that species have not been extensively studied. This gap in our knowledge could potentially cause uncertainty in the extrapolation of studies from rat to human. We hypothesized that OHPCBs interact with rSULT1A1 (also previously known as aryl sulfotransferase IV) and rSULT2A3 (also previously known as sulfotransferase STa), two isoforms of SULT in the rat that are orthologous to human isoforms hSULT1A1 and hSULT2A1, respectively.

rSULT1A1 is also of interest due to its ability to be regulated by the thiol:disulfide status of its environment (Marshall et al., 1997; Marshall et al., 2000; Duffel et al., 2001). There are five cysteine residues, located at positions 66, 82, 232, 283, and 289 in each of the two identical subunits (homodimers) of rSULT1A1. It has been shown that the kinetics, specificity, and pH optima of rSULT1A1 are regulated by the oxidation status of Cys66 (i.e., conversion among the free thiol, a glutathione-protein mixed disulfide, and an intramolecular disulfide between Cys 66 and Cys232) (Marshall et al., 1997; Marshall et al., 2000). These previous studies on rSULT1A1 led to our second hypothesis that interactions of OHPCBs with the purified rSULT1A1 and rSULT2A3 would be modified by changing the oxidative environment of the enzyme with oxidized glutathione. Although the sulfation of dehydroepiandrosterone (DHEA), a known
substrate of rSULT2A3, catalyzed by hepatic cytosol from male rats has been reported to be unaffected by treatment with oxidized glutathione (Maiti et al., 2004), the potential for substrate-specific modulation of a homogeneous preparation of rSULT2A3 by oxidized glutathione has not been investigated.

Thus, in the present study, 15 lower chlorinated congeners of OHPCBs, each bearing one hydroxyl (at the para-position for 14 and the ortho-position for 1 of the congeners) and 1-4 chlorine atoms in different substitution patterns, were investigated for their interaction with homogeneous recombinant rSULT1A1 and rSULT2A3 as substrates and inhibitors. The potential for alteration of the specificity of rSULT1A1 and rSULT2A3 for OHPCBs was explored by pretreatment of each enzyme with oxidized glutathione.
Materials and Methods

Chemicals. The synthesis and characterization of twelve OHPCBs (abbreviations and chemical structures shown in Table 1) has been reported previously (Lehmler and Robertson, 2001). In addition, three new OHPCBs were synthesized by Suzuki coupling of the corresponding chlorinated benzenelboronic acids and a suitable bromo chloro anisole, followed by demethylation with boron tribromide.

3,2’-Dichloro-biphenyl-4-ol (4’-OH PCB 6). White solid; mp=46-47°C (>99% by GC); \(^1\text{H NMR}\) (400 MHz, CDCl\(_3\)): \(\delta/\text{ppm} 7.44-7.42\) (m, 1H), 7.40 (d, J=2.1Hz, 1H), 7.28-7.23 (m, 4H), 7.05 (d, J=8.4Hz, 1H), 5.62 (br s, 1H, -OH); \(^{13}\text{C NMR}\) (100MHz, CDCl\(_3\)): \(\delta/\text{ppm} 151.0, 139.0, 132.9, 132.7, 131.4, 130.2, 130.1, 129.9, 128.9, 127.1, 119.7, 116.0\); MS \(m/z\) (relative intensity): 238 (100, M-H), 139 (80).

2,4’-Dichloro-biphenyl-4-ol (4-OH PCB 8). White solid; mp=107-108°C (>99% by GC); \(^1\text{H NMR}\) (400 MHz, CDCl\(_3\)): \(\delta/\text{ppm} 7.41-7.34\) (m, 4H), 7.19 (d, J=8.4Hz, 1H), 7.00 (d, J=2.6Hz, 1H), 6.82 (dd, J=8.4, 2.6Hz, eH), 5.32 (br s, 1H, -OH); \(^{13}\text{C NMR}\) (100MHz, CDCl\(_3\)): \(\delta/\text{ppm} 155.6, 137.6, 133.5, 133.1, 132.2, 132.1, 131.1, 128.4, 117.0, 114.5\); MS \(m/z\) (relative intensity): 238(100, M-H), 168(36), 139(45).

2,3’,4’-Trichloro-biphenyl-4-ol (4-OH PCB 33). White solid; mp=103-104°C (>99% by GC); \(^1\text{H NMR}\) (400 MHz, CDCl\(_3\)): \(\delta/\text{ppm} 7.51-7.47\) (m, 2H), 7.27-7.25 (m, 1H), 7.18 (d, J=8.4Hz, 1H), 6.99 (d, J=2.6Hz, 1H), 6.81 (dd, J=8.4, 2.6Hz, 1H), 5.03 (br s, 1H, -OH); \(^{13}\text{C NMR}\) (100MHz, CDCl\(_3\)): \(\delta/\text{ppm} 156.0, 139.1, 133.1, 132.3, 132.1, 131.7, 131.6, 131.0, 130.2, 129.2, 117.2, 114.6\); MS \(m/z\) (relative intensity): 272 (100, M-H), 202 (36), 173 (18), 139 (15).

Adenosine 3’-phosphate 5’-phosphosulfate (PAPS) was obtained from Sigma-Aldrich (St. Louis, MO) and further purified by a published procedure (Sekura, 1981) to a purity greater
than 98% as determined by HPLC. 2-Naphthol, dehydroepiandrosterone (DHEA), 1-octylamine, adenosine 3’,5’-diphosphate (PAP), PAP-agarose, reduced glutathione, and oxidized glutathione were used as obtained from Sigma-Aldrich (St. Louis, MO). Dithiothreitol (DTT) was from Research Products International Corp. (Mt. Prospect, IL). All other chemicals and reagents were of the highest purity commercially available.

**Expression and purification of recombinant Rat SULTs.** Recombinant *E. coli* BL 21 (DE3) cells that expressed either rSULT1A1 (Chen et al., 1992) or rSULT2A3 (Sheng and Duffel, 2001) were established using a pET-3c vector as previously described. Cells were grown, cell extract was prepared, and the enzymes were purified using minor modifications of a procedure developed for hSULT2A1 (Liu et al., 2006). Briefly, each *E. coli* cell culture was grown in 3 mL of Luria broth medium (supplemented with 50 μg/mL of ampicillin for cell selection) at 29 °C. After 24 h, 100 μL aliquots of the cell suspension were transferred to each of four 20 mL portions of fresh culture medium (Luria broth medium with 50 μg/mL of ampicillin) and incubated at 29 °C for 24 h. Finally, each 20 mL culture was added to 400 mL of fresh culture medium and incubated with shaking (210 rpm) at 29 °C for 24 h. Isopropyl-1-thio-D-galactopyranoside (1 mM) was present in the final stage of rSULT2A3-expressing cell cultures for 23 h (added 1 h after the start of final stage culture), but was not utilized for rSULT1A1-expressing cell cultures. The cells (weighing about 14 g) were disrupted by sonification in 20 mL of buffer A (10 mM tris-HCL at pH 7.5, 0.25M sucrose, 10% (v/v) glycerol, 1 mM phenylmethylsulfonylfluoride, 1 μM pepstatin, 1 mM DTT, and 2mg/L antipain). The cell homogenate was centrifuged at 24,000 × g for 30 min, and the supernatant fraction was collected as cell extract. Each SULT isoform was purified using PAP-agarose affinity column chromatography (5 mL PAP-agarose in a 1×10 cm column). After the loading of cell extract on
the column, the column was washed with 200 mL buffer B (10 mM tris-HCL buffer at pH 7.5, 0.25 M sucrose, 10 % (v/v) glycerol, 1 μM pepstatin, 1 mM DTT, 2 mg/L antipain, and 0.05% (v/v) Tween 20) to remove non-specifically bound proteins. rSULT1A1 was eluted with a linear gradient formed between 20 mL buffer B and 20 mL buffer B containing 100 μM PAP. The linear gradient for eluting rSULT2A3 was formed between 20 mL buffer B and 20 mL buffer B containing 50 μM PAP. Residual PAP was removed by gel filtration chromatography on a PD-10 column (Amersham Biosciences, Piscataway, NJ) eluted with buffer B. The protein was concentrated by ultrafiltration with a 10-mL Amicon stirred cell and PM-10 membrane (Millipore Corp., Bedford, MA).

Assay of OHPCBs as potential substrates. The sulfation reactions were carried out in a total volume of 30 μL, with 0.25 M potassium phosphate buffer at pH 7.0, 200 μM PAPS, 7.5 mM 2-mercaptoethanol, and each OHPCB in varied concentrations ranging from 3 μM to either its limit of solubility or 400 μM, as appropriate. Acetone was used as co-solvent for each OHPCB and was present in each final reaction mixture at a concentration of 3.3% (v/v). Either rSULT1A1 (0.75 μg) or rSULT2A3 (0.5 μg) was added to initiate each reaction, which was carried out at 37 °C for 6 min and 15 min, respectively. Each reaction was terminated by the addition of 30 μL methanol. The rate of sulfation was determined by substrate-dependent formation of PAP as analyzed by HPLC (Duffel et al., 1989; Sheng et al., 2001).

Inhibition of SULTs by OHPCBs. Those OH-PCBs that were not substrates were investigated for potential inhibition of the sulfation of 2-naphthol (for rSULT1A1) and DHEA (for rSULT2A3). Reactions were conducted under non-saturating substrate concentrations (i.e., with 15 μM 2-naphthol or 10 μM DHEA). Each OHPCB was studied at various concentrations up to full inhibition of the reaction or the limit of solubility, as appropriate, and either 0.75 μg
rSULT1A1 or 0.5 μg rSULT2A3 was used in each assay. Reactions were carried out at 37 °C for 6 min, and the sulfation rate was determined by substrate-dependent formation of PAP as described above.

Effect of oxidized glutathione on the specificities of SULTs for OHPCBs. For studies on the effect of disulfide on the SULTs, DTT was removed from the original enzyme preparations according to a previously reported method (Marshall et al., 1997), with minor modifications. Briefly, either rSULT1A1 or rSULT2A3 in buffer B (0.5-1 mL) was added to a 5 ml PD-10 gel filtration column that had been equilibrated with buffer C (i.e., all components of buffer B except DTT). After elution with buffer C, the protein (3-5 mL) was concentrated to 0.5-1.0 mL by ultrafiltration using a 10-mL Amicon stirred cell with a PM-10 membrane. All manipulations were carried out at 4 °C. The resulting concentration of DTT was less than 0.06 mM as determined by a standard assay for thiols employing 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Jocelyn, 1987).

Stock solutions of reduced and oxidized glutathione (10 mM) were prepared in buffer C, and the pH was adjusted with potassium hydroxide to 7.5. Each enzyme:glutathione mixture was prepared in a 9:1 ratio (v:v), such that the final concentration of either reduced or oxidized glutathione was 1 mM and there was a 10% decrease in protein concentration. Each enzyme in argon-saturated buffer C containing either 1 mM reduced glutathione (GSH) or 1 mM oxidized glutathione (GSSG) was incubated at 25°C for 1 h. Aliquots of these solutions of enzymes were then used in the assay of sulfation reactions as described above, but without addition of any additional reducing agents to the assay mixtures (i.e., 2-mercaptoethanol was absent from the reaction mixtures). Reaction mixtures contained 0.25 M potassium phosphate at pH 7.0, 200 μM PAPS, the OHPCB at either 100 μM or a lower concentration if its limit of solubility was less
than 100 μM, and an aliquot of each enzyme that had been pretreated with either 1 mM GSH or 1 mM GSSG (0.75 μg rSULT1A1 or 0.5 μg rSULT2A3). The volume of the aliquot of enzyme (1.5-2.2 μL) added to each reaction mixture did not affect the pH of the assay. Three replicates were carried out for each treatment. Reaction mixtures were incubated at 37 °C for 6 min (rSULT1A1) and 15 min (rSULT2A3), respectively. An assay containing all components except OHPCB was utilized to determine the OHPCB-dependent formation of PAP with either the reduced or oxidized enzyme. These control experiments in the absence of any OHPCB exhibited less than 18 μM PAP (rSULT1A1) and 13 μM PAP (rSULT2A3) formed in the course of the assay period, and these controls were subtracted from the PAP formed in the presence of OHPCBs to determine the rate of substrate-dependent formation of PAP. For those OHPCBs that were observed to be substrates for oxidized rSULT1A1, multiple concentrations of OHPCBs were then utilized under the same conditions as described above. Two substrates for reduced rSULT1A1, 4’-OH PCB 9 and 6’-OH PCB 35 (representing kinetic profiles both with and without substrate inhibition, respectively) were also examined for differences in sulfation catalyzed by GSH- and GSSG-pretreated rSULT1A1. After each preincubation of enzyme with GSH at 25 °C for 1 h, an aliquot of the mixture was analyzed with DTNB (Jocelyn, 1987) for thiol content in order to determine the stability of the reduced glutathione under the experimental conditions.

**Reversibility of the effect of GSSG on SULT1A1 by reduction.** After removal of reducing agents by PD-10 chromatography, rSULT1A1 was pretreated with either 5 mM DTT or 1 mM GSSG at 25°C for 1 hr as described above. The resulting enzyme preparations were utilized to determine substrate-dependent formation of PAP with either 2-naphthol (250 μM) or 4’-OH PCB 6 (200 μM). An aliquot of 1 mM GSSG-pretreated rSULT1A1 was further treated
with 5 mM DTT (incubation at 25°C for 1 h) to reduce disulfide bonds in the enzyme, and the rate of sulfation of either 2-naphthol or 4’-OH PCB 6 was then determined.

**Solubility of OHPCBs in the SULT assays.** The solubility of each OHPCB was determined at 37°C in 0.25 M potassium phosphate at pH 7.0 containing 3.3% (v/v) acetone and 7.5 mM 2-mercaptoethanol. The previously described use of light scattering at 400 nm (Blomquist et al., 1978; Liu et al., 2006) was employed using a Perkin-Elmer LS-55 Luminescence Spectrometer.
Results

Interaction of OHPCBs with rSULT1A1 and rSULT2A3 as substrates and inhibitors in the presence of reducing agents. Under commonly used SULT assay conditions in the presence of reducing agents (i.e., DTT during enzyme purification and 2-mercaptoethanol in reaction mixtures), six OHPCBs were observed to be substrates for rSULT1A1. As shown in Fig. 1A, four of these six OHPCBs demonstrated substantial substrate inhibition, while two, 4’-OH PCB 35 and 6’-OH PCB 35, did not show substrate inhibition within their limits of solubility. The maximal sulfation rates, and the concentrations at which maximal sulfation rates were observed, varied among the individual OHPCBs. The concentration-velocity curves for the six OHPCBs were complex and not amenable to description by a simple enzyme kinetic model.

Although six of the OHPCBs were substrates, the other nine OHPCBs were found to be inhibitors of rSULT1A1 when 2-naphthol was used as the substrate. Eight of these inhibitors displayed full (100%) inhibition of the enzyme (Fig. 1B), while 4’-OH PCB 36 (data not shown) reached only about 65% inhibition of the enzyme at its limit of solubility (i.e., 50 μM). As shown in Fig. 1B, large variations in the potency of individual OHPCBs in inhibiting rSULT1A1 were seen. The calculated IC50 values for each OHPCB are shown in Table 1, and these ranged from 0.27 to 57.7 μM, with 4-OH PCB 14 as the most potent inhibitor.

Unlike the case with rSULT1A1, none of the fifteen OHPCBs showed any ability to serve as a substrate for rSULT2A3. Moreover, none of the OHPCBs examined were potent inhibitors of the sulfation of DHEA catalyzed by rSULT2A3. Eleven OHPCBs demonstrated weak inhibition of rSULT2A3; eight of them (4’-OH PCB 3, 4’-OH PCB 9, 4’-OH PCB 12, 4-OH PCB 14, 4-OH PCB 34, 4’-OH PCB 36, 4’-OH PCB 68, 4-OH PCB 8) had IC50 values ranging from 32 to 400 μM, and three (4’-OH PCB 6, 4’-OH PCB 33, 4-OH PCB 36) showed
less than 50% inhibition within their solubility limits. 4’-OH PCB 9 was the only one that attained full inhibition of the enzyme. The other four OHPCBs showed neither inhibitory nor substrate activity toward rSULT2A3 within their limits of solubility.

Structure-activity analysis of OHPCBs with rSULT1A1. As shown in Fig. 2A-C, the most potent inhibitors of rSULT1A1 have a 3,5 dichloro-4-hydroxy substitution pattern. For example, a 6-fold difference in IC\textsubscript{50} is observed with the addition of a 5’-chlorine atom to 4’-OH PCB 25 (i.e., 4’-OH PCB 68). The presence or absence of a chlorine atom at the 4-position of the non-phenolic ring has a small effect on the magnitude of the IC\textsubscript{50} value observed (Fig. 2A and 2C). However, a change in chlorine atoms between the 2 and 3 positions on the aromatic ring bearing a 4-OH group (Fig. 2D) results in large changes in the interactions with rSULT1A1, as also the case when chlorine atoms at the 2’ and 3’ positions (on the non-phenolic ring) are altered in a 3-chloro-4-OH PCB (Fig. 2E). Thus, there appears to be a significant role of chlorine atoms in the 2 (or 2’) and 3 (or 3’) positions of 4’-OH PCBs in determining their ability to serve as substrates and inhibitors of rSULT1A1.

Effects of pretreatment of rSULT1A1 and rSULT2A3 with oxidized and reduced glutathione on their abilities to catalyze sulfation of OHPCBs. The nine OHPCBs that were not substrates for either SULT under the assay conditions used for experiments in Table 1, were further examined for their potential to serve as substrates following treatment of the enzymes with oxidized and reduced glutathione. In order to examine the response of these enzymes to the thiol:disulfide ratio in their environment, the DTT present during purification was removed by gel filtration chromatography, and the enzymes were incubated for one hour at 25°C under argon in the presence of either 1 mM GSH or 1 mM GSSG as described in Materials and Methods. At the end of the incubation of the enzyme with 1 mM GSH, the concentration of
GSH was verified by reaction of an aliquot with DTNB. Following the incubation of rSULT1A1 with either GSH or GSSG, the activity of the enzyme was determined as described in Materials and Methods. A final concentration of 100 μM in each assay was utilized for 4’-OH PCB 6, 4-OH PCB 14, 4’-OH PCB 25, and 4-OH PCB 34. The other OHPCBs (i.e., 4’-OH PCB 33, 4-OH PCB 36, 4’-OH PCB 68, and 4-OH PCB 78) were examined at concentrations equal to the limits of their solubility shown in Table 1. None of these nine OHPCBs were substrates for the rSULT1A1 that had been pretreated with 1 mM GSH, a result similar to that obtained under standard assay conditions where the enzyme was in a buffer containing DTT and 2-mercaptoethanol was present in the reaction mixtures (as described above). However, with rSULT1A1 that had been pretreated with 1 mM GSSG, four of the OHPCBs became substrates for the enzyme with the following rates of sulfation (expressed in nmoles product/min/mg protein; n=3): 4’-OH PCB 6 (43.2 ± 9.6), 4-OH PCB 14 (23.2 ± 2.1), 4’-OH PCB 33 (12.1 ± 7.2), and 4’-OH PCB 36 (9.7 ± 1.6). The rSULT2A3 that had been pretreated with either GSH or GSSG did not catalyze sulfation of any of the nine OHPCBs.

Two of the OHPCBs that were substrates for rSULT1A1 under reducing conditions, 4’-OH PCB 9 and 6’-OH PCB 35, were used to investigate the potential modulation of their sulfation by oxidation of rSULT1A1. As shown in Fig. 3A, sulfation of both OHPCBs catalyzed by GSSG-pretreated rSULT1A1 was only slightly increased when compared to that seen with GSH-pretreated enzyme. Furthermore, the absence of substrate inhibition with 6’-OH PCB 35 and the presence of substrate inhibition with 4’-OH PCB 9, were seen in both reduced and oxidized rSULT1A1-catalyzed reactions.

The sulfation of four OHPCBs identified as substrates for oxidized rSULT1A1 was further investigated with varying concentrations of each OHPCB after pretreatment of the
enzyme with 1 mM GSSG. Three of these compounds, namely 4’-OH PCB 6, 4-OH PCB 14 and
4’-OH PCB 33, showed concentration-dependent sulfation (Fig. 3B), whereas 4’-OH PCB 36
showed sulfation only at its limit of solubility, 50 μM. The sulfation of the three OHPCBs
catalyzed by oxidized rSULT1A1 demonstrated a kinetic profile significantly different from that
observed with most of the OHPCBs that were substrates for reduced rSULT1A1.

Reversibility of oxidized rSULT1A1 in its ability to catalyze sulfation of 4’-OH PCB 6

As shown in Table 2, the sulfation of 2-naphthol catalyzed by rSULT1A1 was slightly
enhanced by pretreatment of the enzyme with 1 mM GSSG compared to that with 1 mM GSH
(p<0.05). This effect was similar to that observed for the rates of sulfation of 4’-OH PCB 9 and
6’-OH PCB 35 catalyzed by oxidized versus reduced rSULT1A1. Upon reducing the oxidized
rSULT1A1 by subsequent treatment with 5 mM DTT, the catalytic activity with 2-naphthol as
substrate was reduced to the original level as catalyzed by the enzyme pretreated with only DTT.
For 4’-OH PCB 6, pretreatment of rSULT1A1 with 5 mM DTT or 1mM GSSG led to a more
significant difference in its catalytic activity. Under reducing conditions, 4’-OH PCB 6 was not
a substrate for rSULT1A1, but after pretreatment of the enzyme with 1 mM GSSG, the rate of
sulfation was 37.3 ± 4.0 nmoles of product/mg/min. After reduction of the oxidized enzyme with
5 mM DTT, it no longer catalyzed the sulfation of 4’-OH PCB 6. Thus, the effects of altering the
thiol:disulfide environment of rSULT1A1 were fully reversible for 4’-OH PCB 6.
Discussion

Metabolic hydroxylation of PCBs to OHPCBs in humans and other mammals is catalyzed by various isoforms of CYP, and often represents an initial step in metabolism. Analyses of PCB metabolites in human populations have shown that OHPCBs are persistent in the blood, and hydroxylation has primarily occurred at the para-, and, with a lower frequency, at the meta-positions (Bergman et al., 1994; Sandau et al., 2000). Studies on rats exposed to PCBs have resulted in similar findings; i.e., 4(4’)-hydroxylated and 3(3’)-hydroxylated PCBs were the main metabolites (Chen et al., 1976; Schnellmann et al., 1984; Haraguchi et al., 2004). It is particularly interesting that the concentrations of these OHPCB metabolites in blood may, in some cases, be higher than the parent PCBs, with additional selective concentration of OHPCBs in liver and other tissues (Bergman et al., 1994). For example, one of 13 OHPCBs detected in rat plasma following a dose of Aroclor 1254 was 4-OH-2,3,5,3’,4’-pentachlorobiphenyl, and this OHPCB was observed at concentrations of 0.7-1.8 ng/mg lipid in the liver at various time points (Bergman et al., 1994). Our calculations suggest that this is approximately equivalent to a concentration range of 0.1 – 0.3 μM. Although caution is necessary in comparisons between concentrations of OHPCBs calculated based on tissue lipid content and concentrations utilized with the purified enzyme, a reasonable conclusion is that the tissue concentrations likely to be seen for the OHPCBs examined in our current study would be unlikely to have significant effects on the catalytic activity of rSULT2A3. Thus, our results indicate that the major family 2 SULT in rat liver differs significantly from the major family 2 SULT in human liver, hSULT2A1, in its interactions with OHPCBs. For example, our recent studies indicate that 4-OH PCB 34 and 4’-OH PCB 68 are good substrates for hSULT2A1, and 4’-OH PCB 9 is a potent inhibitor of hSULT2A1 (Liu et al., 2006).
In contrast to rSULT2A3, the major hepatic family 1 SULT in the rat, rSULT1A1, had significant interactions with OHPCBs. For those OHPCBs that were substrates for rSULT1A1, the rate of product formation would depend directly on the concentration of the OHPCB, since they would likely be present at non-saturating concentrations. Some inhibitory OHPCBs, particularly those with IC$_{50}$ values in the sub-micromolar range, may be candidates for examination of in vivo inhibition of rSULT1A1.

These results with rSULT1A1 can be compared with a previous report on human SULT1A1 (Wang et al., 2006), where 18 OHPCBs were found to inhibit the sulfation of 4-nitrophenol catalyzed by recombinant hSULT1A1 and by human liver cytosol, and at least three of these also served as substrates. Several OHPCBs used in that study were also included in our experiments, and there are similarities in the interactions of these OHPCBs with human and rat SULT1A1. However, there are also some notable differences. Unlike rSULT1A1 which was most potently inhibited by OHPCBs bearing the 3,5-dichloro-4-hydroxy substitution pattern (e.g., 4-OH PCB 14, 4-OH PCB 34, and 4-OH PCB 36), OHPCBs with a 3,5-dichloro-4-hydroxy substitution pattern showed slightly weaker inhibition of hSULT1A1 than 3-chloro-4-hydroxy substituted PCBs (Wang et al., 2005). In this regard, the rSULT1A1 is more similar to hSULT1E1 (human estrogen sulfotransferase), where the 3,5-dichloro-4-hydroxy substitution pattern in OHPCBs provided the most potent inhibition of hSULT1E1 (Kester et al., 2000).

Taken together, the above findings with OHPCBs indicate that the major hepatic family 2 SULTs in the rat and human (rSULT2A3 and hSULT2A1, respectively) are distinctly different in their interactions with OHPCBs, while the interactions of OHPCBs with rSULT1A1 have more similarities to the human family 1 SULTs than differences. It should be noted that this does not necessarily mean that all family 2 SULTs in the rat do not interact strongly with OHPCBs, as the
possibility for other rat SULT2 isoforms to interact with OHPCBs cannot be excluded in the present study.

The concentrations of OHPCBs present in blood and tissues are important determinants of their interactions with SULTs. It is becoming increasingly apparent that concentrations of OHPCBs vary significantly with species and tissue. Moreover, the roles of protein-binding of OHPCBs are receiving increasing attention as factors influencing these in vivo concentrations. For example, recent studies on placental transfer of PCBs and OHPCBs in humans (Park, et al., 2008) show that OHPCBs are more efficiently transferred than the parent PCBs. This increased placental transfer to the fetus was proposed to be due to higher protein-binding of OHPCBs as opposed to the greater lipid distribution of the parent PCBs (Park, et al., 2008). Further elaboration of differences in the transport and tissue concentrations of OHPCBs will undoubtedly facilitate analysis of potential in vivo interactions with SULTs.

The kinetic characteristics of various OHPCBs as substrates for rSULT1A1 under standard assay (reduced) conditions demonstrated significant variations, with most showing substrate inhibition. The kinetic data for OHPCBs with substrate inhibition, however, were not described well by a simple Michaelis-Menten model. 4-Nitrophenol, a prototype substrate for rSULT1A1 that has been extensively studied, displays pronounced substrate inhibition in reactions catalyzed by reduced rSULT1A1, and this has been classified as a form of uncompetitive substrate inhibition (Marshall et al., 2000). The underlying mechanism for this kinetic behavior is due to the formation of a dead end ternary complex: E-PAP-ROH (where E is the rSULT1A1, PAP is the reaction product derived from PAPS, and ROH is a phenolic substrate) (Marshall et al., 2000). The binary complex of PAP bound to reduced rSULT1A1 (i.e., E-PAP) is relatively stable, and the binding of different phenolic
substrates (e.g., 4-nitrophenol, OHPCBs, and others) to E-PAP may have differential effects on the stability of the inhibitory ternary complex (Marshall et al., 2000; Duffel et al., 2001).

Compared to reduced rSULT1A1, oxidized rSULT1A1 exhibits profoundly altered kinetic interactions with its substrates. As exemplified with 4-nitrophenol and 2-naphthol, changes in pH optima (from 5.2 to 6.3 and 5.4 to 7.2, respectively), and disappearance of substrate inhibition have been observed upon oxidation of rSULT1A1 with GSSG (Marshall et al., 1997; Marshall et al., 1998; Marshall et al., 2000). Additional changes in substrate specificity were seen upon oxidation of the enzyme with GSSG (Marshall et al., 2000). Upon treatment of rSULT1A1 with GSSG in the present study, we observed the conversion of four of the nine inhibitory OHPCBs to substrates. The underlying structural modification responsible for the altered kinetic behavior upon treatment of rSULT1A1 with GSSG for short time periods has been shown to be the formation of a glutathione-protein mixed disulfide at Cys66 followed by the formation of an intramolecular disulfide between Cys66 and Cys232 (Marshall et al., 1997). As rSULT1A1 is oxidized further, e.g., treatment with 1 mM GSSG at 25 °C for more than 1 h, additional cysteines form disulfide bonds and the specific activity of the enzyme is decreased; after treatment for 12-24 h, all five cysteines are oxidized, and the enzyme is completely inactivated (Marshall et al., 1997; Marshall et al., 2000). Homology modeling studies indicate that oxidation of Cys66 and Cys232 affects the conformation of the protein in the vicinity of the PAPS/PAP binding site, and therefore changes the dissociation of the E-PAP-ROH dead-end complex (Duffel et al., 2001). Thus, under oxidative conditions, the ternary complex E-PAP-ROH may undergo nucleotide exchange with PAPS and the catalytic cycle can proceed (Marshall et al., 2000). As noted in the previous studies on the mechanism of rSULT1A1 under reduced and oxidized conditions, the changes due to initial disulfide formation are reversible (Marshall et al., 1997; Marshall et al., 2000). Indeed, our results also indicated that GSSG-
dependent changes in the kinetic behavior of rSULT1A1 with 4’-OH PCB 6 were reversible by reduction with DTT (Table 2).

As described in the original studies on the effect of oxidation on mechanism of the enzyme (Marshall et al., 1997; Marshall et al., 2000), these effects of partial oxidation of cysteines in rSULT1A1 suggest an important regulatory mechanism whereby the specificity and kinetics of the enzyme can be altered by oxidative stress. This is particularly intriguing in the case of the OHPCBs examined in the current study, since, for some OHPCBs, cellular oxidative stress may determine whether the molecule inhibits rSULT1A1 or is sulfated in a reaction catalyzed by the enzyme. In addition to oxidative stress created by disease states or exposure to other xenobiotics, recent work on the potential role of quinone metabolites of PCBs and their involvement in creation of oxidative stress within cells (Amaro et al., 1996; Srinivasan et al., 2002) suggests that, by inducing oxidative stress, some OHPCBs may influence sulfation of other OHPCBs or interfere with other sulfation reactions. Finally, the recent report of disulfide-mediated regulation of hSULT1E1 (Maiti et al., 2007) and the presence of cysteine residues homologous to Cys66 in other SULTs suggest that the substrate-dependent nature of the effects seen with the OHPCBs in our current results may also be seen in the redox regulation of other SULTs.
References


Park JS, Bergman A, Linderholm L, Athanasiadou M, Kocan A, Petrik J, Drobna B, Trnovec T
Charles MJ and Hertz-Picciotto I (2008) Placental transfer of polychlorinated biphenyls,
their hydroxylated metabolites and pentachlorophenol in pregnant women from eastern

Robertson LW and Hansen LG Eds (2001) PCBs: Recent Advances in Environmental Toxicology
and Health Effects, 461 pp, University of Kentucky Press, Lexington.

exchange of polychlorinated biphenyls and organochlorine pesticides across central and

Safe SH (1994) Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic

metabolites of PCBs (OH-PCBs) and other chlorinated phenolic compounds in whole
blood from Canadian Inuit. Environ Health Perspect 108:611-616.

Schnellmann RG, Volp RF, Putnam CW and Sipes IG (1984) The hydroxylation, dechlorination,
and glucuronidation of 4,4’-dichlorobiphenyl (4-DCB) by human hepatic microsomes.
Biochem Pharmacol 33:3503-3509.


New York.

Sheng JJ and Duffel MW (2001) Bacterial expression, purification, and characterization of rat


Footnotes

This study was supported by the National Institutes of Health through grants P42 ES013661 (to L.W.R., M.W.D. and H.J.L.) and K25 ES012475 (to H.J.L.) from the National Institute of Environmental Health Sciences, and research grant R01 CA038683 (to M.W.D.) from the National Cancer Institute. We also acknowledge programmatic support through the University of Iowa Environmental Health Sciences Research Center (NIEHS/NIH P30 ES05605). The contents of this paper are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.

Portions of this work were presented at the 5th PCB Workshop, Iowa City, Iowa, May 18-22, 2008.
Figure Legends

Fig. 1. OHPCBs were either substrates or inhibitors of rSULT1A1. (A) Six OHPCBs used in the present study were substrates for rSULT1A1. Data are the means ± standard errors of triplicate (4’-OH PCB 12 and 4’-OH PCB 35) or duplicate (4’-OH PCB 3, 4-OH PCB 8, 4’-OH PCB 9, and 6’-OH PCB 35) determinations. (B) Eight OHPCBs were inhibitors of rSULT1A1 with full inhibition of sulfation of 2-naphthol. Duplicate assays were utilized at each OHPCB concentration to determine IC50 values; the mean values for each concentration of OHPCB are shown.

Fig. 2. Structure-activity relationships for OHPCB-inhibition of rSULT1A1. Panels A-C show relationships between IC50 values and the 3,5-dichloro-4-hydroxy substitution pattern. Panel D illustrates the importance of chlorine atoms in the 2 and 3 positions of the aromatic ring of a PCB bearing a 4-OH group. Panel E shows the effects of a chlorine atom in the 2’ or 3’ position of the non-phenolic ring of a chloro-4-OH PCB.

Fig. 3. Sulfation of OHPCBs catalyzed by oxidized and reduced rSULT1A1. (A) rSULT1A1 that had been either oxidized by pretreatment with 1 mM GSSG (solid lines; closed symbols) or reduced by pretreatment with 1 mM GSH (dashed lines; open symbols) catalyzed the sulfation of 6’-OH PCB 35 (● or ○) and 4’-OH PCB 9 (■ or □). (B) rSULT1A1 pretreated with 1 mM GSSG catalyzed the sulfation of 4’-OH PCB 33 (△), 4’-OH PCB 6 (◊), and 4-OH PCB 14 (▼). Data are the means ± standard errors of duplicate (panel A) or triplicate (panel B) determinations.
<table>
<thead>
<tr>
<th>OH PCB</th>
<th>Chem. structure</th>
<th>Solubility$^a$ (μM)</th>
<th>IC$_{50}$ (μM)</th>
<th>Rat SULT1A1</th>
<th>Rat SULT2A3</th>
</tr>
</thead>
<tbody>
<tr>
<td>4’-OH PCB 3</td>
<td></td>
<td>300</td>
<td>b</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td>4’-OH PCB 6</td>
<td></td>
<td>250</td>
<td>22.7</td>
<td>d</td>
<td></td>
</tr>
<tr>
<td>4-OH PCB 8</td>
<td></td>
<td>≥500</td>
<td>b</td>
<td>400$^c$</td>
<td></td>
</tr>
<tr>
<td>4’-OH PCB 9</td>
<td></td>
<td>500</td>
<td>b</td>
<td>190</td>
<td></td>
</tr>
<tr>
<td>4’-OH PCB 12</td>
<td></td>
<td>≥500</td>
<td>b</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td>4-OH PCB 14</td>
<td></td>
<td>≥1000</td>
<td>0.27</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>4’-OH PCB 25</td>
<td></td>
<td>100</td>
<td>57.7</td>
<td>e</td>
<td></td>
</tr>
<tr>
<td>4’-OH PCB 33</td>
<td></td>
<td>50</td>
<td>26.2</td>
<td>d</td>
<td></td>
</tr>
<tr>
<td>4-OH PCB 34</td>
<td></td>
<td>300</td>
<td>0.34</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td>4’-OH PCB 35</td>
<td></td>
<td>50</td>
<td>b</td>
<td>e</td>
<td></td>
</tr>
<tr>
<td>4’-OH PCB 36</td>
<td></td>
<td>50</td>
<td>0.54</td>
<td>d</td>
<td></td>
</tr>
<tr>
<td>4’-OH PCB 36</td>
<td></td>
<td>50</td>
<td>40</td>
<td>50$^c$</td>
<td></td>
</tr>
<tr>
<td>4’-OH PCB 68</td>
<td></td>
<td>50</td>
<td>0.85</td>
<td>32$^c$</td>
<td></td>
</tr>
<tr>
<td>4-OH PCB 78</td>
<td></td>
<td>33</td>
<td>4.4</td>
<td>e</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Solubility of each OHPCB was detected according to the light scattering intensity in the same solution as in the reactions.

$^b$This OHPCB was a substrate for rSULT1A1: see Figure 1.
cThis is an approximate value due to 50% inhibition at the limit of solubility.
dLess than 50% inhibition was reached within the limit of solubility.
eThis OHPCB was neither a substrate nor an inhibitor for rSULT2A3.
Table 2. Reversibility of catalytic changes in rSULT1A1 following oxidative modification of the enzyme.

<table>
<thead>
<tr>
<th>Pretreatment of rSULT1A1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>5 mM DTT</th>
<th>1 mM GSSG</th>
<th>DTT after GSSG&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Naphthol, 250 µM</td>
<td>38.3±4.7</td>
<td>50.2±2.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>37.4±1.5</td>
</tr>
<tr>
<td>4’-OH PCB 6, 100 µM</td>
<td>0</td>
<td>37.3±4.0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup>5 mM DTT (dithiothreitol) and/or 1 mM GSSG (oxidized glutathione) were incubated with the enzyme at 25°C for 1 hr before use in an assay for sulfotransferase activity. Data are the means and standard deviations of three determinations.

<sup>b</sup>One aliquot of GSSG-treated enzyme was further treated with DTT at 25 °C for 1h.

<sup>c</sup>This value is statistically different from the group treated with 5 mM DTT alone (p<0.05 by t-test).
Fig. 1.

A

Rate of sulfation, nmol/mg/min

0 30 60 90 120 150

OHPCBs, μM

4'-OH PCB 3
4-OH PCB 8
4'-OH PCB 9
4'-OH PCB 12
4'-OH PCB 35
6'-OH PCB 35

B

Sulfation of 2-naphthol, % of control

0 20 40 60 80 100 120

OHPCBs, μM

0.001 0.01 0.1 1 10 100 1000

4-OH PCB 14
4-OH PCB 34
4-OH PCB 36
4-OH PCB 68
4-OH PCB 78
4-OH PCB 6
4-OH PCB 33
4'-OH PCB 25
Fig. 2.

A

IC$_{50}$ = 23 μM

IC$_{50}$ = 58 μM

IC$_{50}$ = 0.8 μM

B

IC$_{50}$ = 0.3 μM

IC$_{50}$ = 0.3 μM

IC$_{50}$ = 0.5 μM

C

IC$_{50}$ = 0.3 μM

IC$_{50}$ = 0.8 μM

Substrate

D

IC$_{50}$ = 26 μM

Substrate

E

IC$_{50}$ = 58 μM
Fig. 3.