Roles of Human CYP2A6 and Monkey CYP2A24 and 2A26 Cytochrome P450 Enzymes in the Oxidation of 2,5,2',5'-Tetrachlorobiphenyl

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2,5,2',5'-tetrachlorobiphenyl by human and monkey CYP2A enzymes

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Number of Text Pages: 39
Number of Tables: 1
Number of Figures: 10
Number of References: 55
Number of Words in Abstract: 236
Number of Words in Introduction: 528
Number of Words in Discussion: 1479

ABBREVIATIONS: P450 or CYP, cytochrome P450; b5, cytochrome b5; PCB, polychlorinated biphenyl; TCB, tetrachlorobiphenyl; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.
ABSTRACT.

2,5,2’,5’-Tetrachlorobiphenyl (TCB) induced Type I binding spectra with cytochrome P450 (P450 or CYP) 2A6 and 2A13, having $K_s$ values of 9.4 and 0.51 µM, respectively. However, CYP2A6 oxidized 2,5,2’,5’-TCB to form 4-hydroxylated products at a much higher rate (~1.0 min$^{-1}$) than CYP2A13 (~0.02 min$^{-1}$), based on analysis by LC-MS/MS. Formation of 4-hydroxy-2,5,2’,5’-TCB by CYP2A6 was greater than that of 3-hydroxy-2,5,2’,5’-TCB and three other hydroxylated products. Several human P450 enzymes, including CYP1A1, 1A2, 1B1, 2B6, 2D6, 2E1, 2C9, and 3A4, did not show any detectable activities in oxidizing 2,5,2’,5’-TCB. Cynomolgus monkey CYP2A24, which shows 95% amino acid identity to human CYP2A6, catalyzed 4-hydroxylation of 2,5,2’,5’-TCB at a higher rate (~0.3 min$^{-1}$) than CYP2A26 (93% identity to CYP2A6, ~0.13 min$^{-1}$) and CYP2A23 (94% identity to CYP2A13, ~0.008 min$^{-1}$). None of these human and monkey CYP2A enzymes were catalytically active in oxidizing other TCB congeners, such as 2,4,3’,4’-, 3,4,3’,4’-, and 3,5,3’,5’-TCB. Molecular docking analysis suggested that there are different orientations of interaction of 2,5,2’,5’-TCB with the active sites (over the heme) of human and monkey CYP2A enzymes and that ligand interaction energies ($U$ values) of bound protein-ligand complexes show structural relationships of interaction of TCBs and other ligands with active sites of CYP2A enzymes. Catalytic differences in human and monkey CYP2A enzymes in the oxidation of 2,5,2’,5’-TCB are suggested to be due to amino acid changes at substrate recognition sites, i.e., V110L, I209S, I300F, V365M, S369G, and R372H, based on the comparison of primary sequences.
Introduction

Commercial polychlorinated biphenyl (PCB) mixtures have been widely used in electrical transformers, electrical switches, sealants, plasticizers, rubbers, adhesives, carbonless copy papers, paints, inks, and dust control agents (IARC Monograph, 2015; Mills et al., 2007; Victor et al., 1989). Yusho disease occurred in western Japan in 1968 following the ingestion of rice oil contaminated with PCBs and polychlorinated dibenzofurans. Also a similar incident in Taiwan was referred to as Yu-cheng disease in 1979 (Kuratsune et al., 1972; Kunita et al., 1984). After cessation of production and use of commercial PCBs after 1970, the levels of PCBs in human tissues and milk have decreased yearly in Japan (Yakushiji et al., 1979; 1984). However, due to their strong persistent nature, PCBs have been shown to be present at significant levels in environmental and biological samples (Kunita et al., 1984; Yakushiji et al., 1984). Konishi et al. (2006) determined the levels of PCB congeners in the human milk in Osaka and found that 12 major PCBs including three hepta-, two hexa-, four penta-, and four tetra-chlorobiphenyl congeners in milk decreased between 1973 and 2000, but the rate of elimination depends on the individual PCB congeners determined, probably due to their susceptibilities to be chemically degraded and enzymatically metabolized (Todaka et al., 2008; Arnich et al., 2009; Akutsu et al., 2005).

2,5,2′,5′-Tetrachlorobiphenyl (#PCB52) (IARC Monograph, 2015; Mills et al., 2007) (Figure 1) is one of the PCB congeners identified in human milk and in foodstuffs at significant levels and has been extensively studied in its biological activities and metabolism
by xenobiotic-metabolizing enzymes in experimental animal models (Safe, 1993; Yoshimura et al., 1975; Ishida et al., 1991; Koga et al., 1995; Borlakoglu et al., 1991; Preston et al., 1983). Cytochrome P450 (P450 or CYP) has been shown to be the major enzyme responsible for the oxidation of this TCB, and there are species-related differences in the metabolism by different forms of P450s in rats, mice, guinea pigs, hamsters, and rabbits (Borlakoglu et al., 1991; Preston et al., 1983; Koga et al., 1996). However, little is known about the roles of human P450 enzymes in the metabolism of 2,5,2′,5′-TCB. Our previous studies have shown that 2,5,2′,5′-TCB induces Type I binding spectra with CYP2A13 and 2A6 and is able to inhibit coumarin 7-hydroxylation activities catalyzed by CYP2A6 and 2A13, indicating that these P450 enzymes may participate in the metabolism of this TCB congener (Shimada et al., 2013).

In this study, we determined whether human CYP2A6 and 2A13 and other human P450 enzymes are able to oxidize 2,5,2′,5′-TCB to oxygenated products and how homologous monkey CYP2A enzymes oxidize this TCB. We used recombinant human and monkey P450 enzymes expressed in *Escherichia coli* and in microsomes of *Trichoplusia ni* cells and analyzed product formation with GC-MS and LC-MS-MS analyses. Molecular docking simulation was used to probe the interaction of 2,5,2′,5′-TCB with active sites (over the heme) of these CYP2A enzymes. Other TCB isomers including 2,4,3′,4′-, 3,4,3′,4′-, and 3,5,3′,5′-TCBs, which have been shown to be contaminants in the environment (Konishi et al., 2006; Todaka et al., 2008; Akutsu et al., 2005), were also studied for their oxidation by these P450 enzymes.
Materials and Methods

Chemicals. 2,5,2',5'-TCB (Figure 1) and 4-hydroxy-2,5,2',5'-TCB were synthesized as reported previously (Yoshimura et al., 1975; Ishida et al., 1991; Koga et al., 1995). 3-Hydroxy-2,5,2',5'-TCB was isolated from the feces of rats treated i. p. with 2,5,2',5'-TCB at a single dose of 200 mg/kg of body weight according to a method reported previously (Hanioka et al., 1991). 2,4,3',4'-, 3,4,3',4'-, and 3,5,3',5'-TCB (Figure 1) and the metabolites 3-hydroxy-2,5,3',4'-TCB, a mixture of 3-hydroxy- and 4-hydroxy-2,5,3',4'-TCB, 5-hydroxy-2,4,3',4'-TCB, 4-hydroxy-2,3,3',4'-TCB (a metabolite of 2,4,3',4'-TCB), and 4-hydroxy-3,5,3',5'-TCB, were obtained from the sources described previously (Koga et al., 1989; 1992; 1994).

Other chemicals and reagents used in this study were obtained from the sources described previously or were of the highest quality commercially available (Shimada et al., 2013; 2015; 2016a).

Enzymes. Expression in E. coli and purification of human P450 enzymes were described previously (Parikh et al., 1997; Shimada et al., 2009; 2011; 2013). Bacterial bicistronic CYP1A2, 1B1, 2A6, 2A13, 2C9, and 3A4 membranes in which human NADPH-P450 reductase was co-expressed were prepared, and the E. coli membranes were suspended in 10 mM Tris-HCl buffer (pH 7.4) containing 1.0 mM EDTA and 20% glycerol (v/v) as described (Sandhu et al., 1993; 1994; Guengerich, 2015). CYP1A1, 2A6, 2A13, 2C9, 3A4, NADPH-P450 reductase, and cytochrome bs (bs) were purified from membranes of
recombinant *E. coli* as described elsewhere (Sandhu et al., 1993; 1994; Guengerich, 2015; Shimada et al., 2013).

Expression of cynomolgus monkey CYP2A23, 2A24, and 2A26 in *E. coli* together with human NADPH-P450 reductase was done as described previously (Uehara et al., 2014; 2015).

Recombinant CYP2B6, 2E1, and 2D6, expressed in microsomes of *T. ni* cells infected with a baculovirus containing human P450 and NADPH-P450 reductase cDNA inserts, were obtained from GENTEST (Woburn, MA). P450 contents in these microsomes were based on the values in the data sheets provided by the manufacturer.

**Spectral Binding Titrations.** Purified CYP2A6 and 2A13 enzymes were diluted to 1.0 µM in 0.10 M potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v), and binding spectra were recorded with subsequent additions of 2,5,2′,5′-TCB and other TCB congeners in a JASCO V-550 or an OLIS-Aminco DW2a spectrophotometer (On-Line Instrument Systems, Bogart, GA) as described previously (Shimada et al., 2009; 2011; 2013). Spectral dissociation constants (*K_s*) were estimated using GraphPad Prism software (GraphPad Software, San Diego, CA), either using hyperbolic plots or quadratic fits for tight binding.

**Oxidation of 2,5,2′,5′-TCB and other TCB isomers.** Oxidative metabolism of 2,5,2′,5′-TCB and other TCB isomers by P450 enzymes was determined in a standard incubation mixture (0.25 mL) containing bicistronic P450s (50 pmol of P450 in *E. coli* membranes or 10 pmol of P450s in microsomes of *T. ni* cells (co-expressing human NADPH-P450 reductase)), 50 µM of each TCB isomers, and an NADPH-generating system
consisting of 0.5 mM NADP+, 5 mM glucose 6-phosphate, and 0.5 unit of yeast glucose 6-phosphate dehydrogenase/mL (Shimada et al., 2015; 2016a). (The PCBs and other chemicals were dissolved in (CH₃)₂SO as 10 mM stock solutions and diluted into aqueous solution, with the final solvent concentration ≤0.5%, v/v.).

In reconstitution experiments, P450 membranes were replaced by purified P450 (50 pmol), NADPH-P450 reductase (100 pmol), bs (100 pmol) (when required), and L-α-dilauroyl-syn-glycero-3-phosphocholine (50 µg) as described previously (Shimada et al., 2015; 2016a). Our previous studies have suggested that more than 2-fold excess of NADPH-P450 reductase over P450 is required to account for full catalytic activities for drug oxidations in reconstituted systems, although the ratio of expression of the reductase and P450 is not always as high in bicistronic systems (Shimada et al., 2000; Yamazaki et al., 2001). Incubations were carried out at 37 °C, following a preincubation time of 1 min. Reactions were terminated by adding 0.25 mL of cold CH₃OH and then extracted with 0.5 ml of a CHCl₃-ethyl acetate mixture (1:1, v/v) twice. The organic layers were subjected to filtration using Disposable Syringe Filters (Iwaki, Osaka, Japan; Cellulose Acetate Membrane, 3 mm x, 0.20 µm; Code 2012-003) and the filtrates (10 µL) were used for analysis with GC-MS and LC-tandem mass spectrometry (LC-MS/MS).

**LC/MS/MS Analysis of 2,5,2′,5′-TCB Metabolites.** LC-MS/MS analyses were performed using a liquid chromatograph (ACQUITY UPLC I-Class system, Waters, MA, USA) coupled to a tandem quadruple mass spectrometer (Xevo TQ-S, Waters) (Kakimoto et al.,
Chromatographic separation was performed on an octadecylsilane (C$_{18}$) column (CORTECS C$_{18}$, 100 mm × 2.1 mm i.d., 1.6 μm, Waters) or an RP-amide column (Ascentis Express, 100 mm × 2.1 mm i.d., 2 μm, Sigma-Aldrich, MO, USA). The temperature of the C$_{18}$ column was maintained at 45 °C and gradient elution (CH$_3$CN; 35% in water to 75% (v/v) over 10 min) was done at a flow rate of 0.25 mL/min. The temperature of the RP-amide column was maintained at 7 °C and gradient elution (acetonitrile; 75% in water to 95% (v/v) over 10 min; hold for 2 min) was used at a flow rate of 0.15 mL/min.

MS/MS analysis was performed on a negative electrospray ionization mode with a capillary voltage of 3000 V, cone voltage of -10 V, and collision energy of 20 eV. The selected reaction-monitoring (SRM) mode was used to quantify the m/z 306.9 to 270.9 transitions for identification of mono-hydroxylated metabolites and for further verification (the m/z 306.9 to 234.8 transition was also determined). The product ion m/z 306.9 was scanned following elution from a C$_{18}$ column; the same collision energy of SRM mode was used for obtaining the product ion mass spectrum.

**GC/MS Analysis of TCB isomers.** GC-MS was done with a gas chromatograph (7890A series, Agilent Technologies, CA, USA) coupled with a mass spectrometer (Quattro micro GC, Waters, MA, USA) using a capillary column (VF-5ms, 30 m × 0.25 mm i.d., 0.25 μm film thickness, Agilent Technologies, CA, USA); column temperature program: 60 ºC (held for 1 min) to 140 ºC at 20 ºC/min, and to 260 ºC at 8 ºC/min, then to 300 ºC (held for 5 min) at 40 ºC/min; carrier gas, helium at 1.0 mL/min. The MS conditions used were: electron
ionization mode; electron energy, 70 eV; and ion source temperature, 250 °C. The selected ion-monitoring (SIM) mode of $m/z$ 292 was used to quantify TCB concentration and for further verification, the $m/z$ 290 was also used. Mass Lynx (version 4.1) was used to control the LC/MS/MS and GC/MS/MS systems and to acquire and process the data.

**Other Assays.** P450 and protein contents and coumarin 7-hydroxylation activities (CYP2A6 and 2A13) were determined by methods described previously (Omura and Sato, 1964; Brown et al., 1989).

**Docking Simulations into Human P450 Enzymes.** Crystal structures of CYP2A6 bound to coumarin (PBD 1Z10), pilocarpine (PDB 3T3R), and nicotine (PDB 4EJJ) and CYP2A13 bound to nicotine (PDB 4EJG), NNK (PDB 4EJH), indole (PDB 2P85), and pilocarpine (PDB 3T3S) have been reported and were used in this study (Yano et al., 2005; DeVore et al., 2009; 2012a; 2012b; Smith et al., 2007; Shimada et al., 2016b). Simulations were carried out after removing each ligand from these P450 structures using the MMFF94x force field described in the MOE software (ver. 2015.10, Computing Group, Montreal, Canada) as described (Shimada et al., 2010; Shimada et al., 2011; Shimada 2013). Crystal structures of monkey CYP2A23, 2A24, and 2A26 have not yet been reported, and these P450 primary sequences were aligned with human CYP2A6 1Z10 for CYP2A24 and with CYP2A13 4EJG for CYP2A23 and 2A26 using MDE software for modeling of three dimensional structures. Ligand-interaction energies ($U$ values) were obtained by use of ASE dock program in MOE software. Lower $U$ value is an indication of higher interaction between a
chemical and the enzyme.

**Kinetic Analysis.** Kinetic parameters were estimated by nonlinear regression analysis of hyperbolic plots using the program Kaleida-Graph (Synergy Software, Reading, PA) or Graphpad Prism (Graphpad, La Jolla, CA).
Results

Spectral interactions of 2,5,2´,5´-TCB with purified CYP2A6 and 2A13. We first examined the spectral interaction of 2,5,2´,5´-TCB with purified CYP2A6 and 2A13 and found that CYP2A13 interacted more strongly with TCB to produce Type I binding spectra than CYP2A6 did (Figure 2). The $K_s$ value was 0.51 $\mu$M for CYP2A13, while that with CYP2A6 was 9.4 $\mu$M. The Type I spectral changes ($K_s$ values) of interaction of CYP2A13 with 2,4,3´,4´-, 3,4,3´,4´-, and 3,5,3´,5´-TCB were >80, 54, and 13.6 $\mu$M, respectively, and such spectral interactions of these TCBs with CYP2A6 were not found.

GC-MS analysis of metabolism of 2,5,2´,5´-TCB by CYP2A6 and 2A13. 2,5,2´,5´-TCB was incubated with CYP2A6 and 2A13 in the presence of an NADPH-generating system, and the parent compound and its metabolites were extracted with CH$_3$OH and then with CHCl$_3$-ethyl acetate mixture (1:1, v/v). The organic layer, after the centrifugation, was submitted directly to GC-MS to determine the changes in TCB concentration during the metabolism (note that TCB levels were decreased when the extracts were evaporated dryness under a nitrogen atmosphere). Incubation with CYP2A6, but not CYP2A13, caused decreases in TCB concentration in a time-dependent manner and the decreases were more markedly at lower substrate concentrations, resulting in 50% decreases in TCB during 60-min incubation (Figure 3A and 3B).

Metabolism of 2,5,2´,5´-TCB by CYP2A6. Since the above results suggested that CYP2A6 was more active than CYP2A13 in metabolizing 2,5,2´,5´-TCB, LC-MS/MS analysis
(with a CORTECS C18 column) was carried out to identify the oxidative metabolites (Figure 4). We first searched the products having molecular mass between m/z 250 and m/z 350 and found several peaks of which four oxidative metabolites, namely m1, m2, m3, and m4 were suggested to be mono-oxygenated products having (molecular ions) m/z 307 (Figure 4A and 4B). We also searched whether or not di-oxygenated products were formed during metabolism of TCB with CYP2A6 and found that none of the molecular mass of m/z 323 were formed (results not shown).

Four peaks—m1, m2, m3, and m4—having m/z 307 were further analyzed with product ion scanning to determine the fragmentation ion spectrum (Figure 4C, 4D, 4E, and 4F, respectively). Elimination of HCl from the products m1, m2, m3, and m4 gave new peaks of m/z 271 in these cases (Figure 4C-4F).

Time-dependent formation of four oxygenated products (m1, m2, m3, and m4) was analyzed on incubation of TCB with CYP2A6 and 2A13 (Figure 5A and 5B); in the case of CYP2A13 we detected only two products, m2 and m4. With both P450 enzymes examined, m2 was the most abundant and we found that CYP2A6 was about 40-fold more active in forming m2 than CYP2A13. (Product m2 was later identified to be a mixture of 3- and 4-hydroxylated TCB products.). Kinetic analysis indicated that Km values for the formation of m1, m2, m3, and m4 by CYP2A6 were 21, 22, 26, and 40 µM, the Km values for m2 and m4 by CYP2A13 were 16 and 18 µM, respectively (Figure 5C and 5D).

The effects of bs on CYP2A6- and 2A13-dependent oxidation of 2,5,2′,5′-TCB in
reconstituted monooxygenase systems containing human NADPH-P450 reductase and
L-α-1,2-dilauroyl-sn-glycero-3-phosphocholine were examined, and none of the activities
were enhanced with $b_5$ were observed in our assay conditions (results not shown).

**Oxidation of 2,5,2',5'-TCB by Human P450 Enzymes and Monkey CYP2A**

**Enzymes.** We compared product formation of 2,5,2',5'-TCB by human CYP2A6 and 2A13
and monkey CYP2A23, 2A24, and 2A26 by using two LC columns, a CORTECS C$_{18}$ column
(Figure 6A, 6C, 6E, 6G, and 6I) and an RP-amide column (Figure 6B, 6D, 6F, 6H, and 6J).
The former column did not separate 3- and 4-hydroxylated products (showing a single peak at
the position of m2) (Figure 6A, 6C, 6E, 6G, and 6I). However, the RP-amide column was
used to successfully identify the 4- and 3-hydroxy products; the formation of 4-hydroxy
product was greater than the 3-OH product with all of the human and monkey CYP2A
enzymes examined (Figure 6B, 6D, 6F, 6H, and 6J). CYP2A6 produced 4-hydroxy-TCB at
the highest rate and CYP2A24 and 2A26 produced metabolite m3 at higher rates than
CYP2A6. Both human CYP2A13 and monkey CYP2A23 had low catalytic activities for
oxidizing this TCB.

Ten human P450 enzymes (CYP1A1, 1A2, 1B1, 2A6, 2A13, 2B6, 2D6, 2E1, 2C9, and
3A4) and three monkey CYP2A enzymes were compared with regard to their catalytic
activities in oxidizing 2,5,2',5'-TCB (Table 1). Separate experiments with GC-MS were also
done to investigate the disappearance of the substrate 2,5,2',5'-TCB after metabolism by
P450s (incubating for 60 min), and it was found that CYP2A6, 2A24, and 2A26 (but not other
P450s) caused decreases in TCB concentration by about 42, 24, and 24%, respectively (Table 1). LC-MS/MS analysis showed that of ten human P450s examined, only CYP2A6 and 2A13 catalyzed the oxidation of 2,5,2’,5’-TCB at significant levels. Formation of 4-hydroxy-2,5,2’,5’-TCB by CYP2A6 and 2A13 was 34- and 15-fold higher, respectively, than that of 3-hydroxy product (Table 1) and the former enzyme was more active than CYP2A13. Other human P450 enzymes did not show any detectable activities in oxidizing 2,5,2´,5-TCB. Among the three monkey CYP2A enzymes, CYP2A24 was the most active followed by CYP2A26, and CYP2A23 was least active in catalyzing the oxidation of 2,5,2´,5´-TCB (Table 1). The ratio of formation of 4- to 3-hydroxylated products with monkey P450 2A enzymes was lower than those with human CYP2A6 and 2A13 (Table 1).

Oxidation of 2,4,3’,4´-, 3,4,3´,4´-, and 3,5,3´,5´-TCB by Human and Monkey P450s.

Because these results suggest that human and monkey CYP2A enzymes are active in catalyzing 2,5,2´,5´-TCB, we re-examined metabolism of 2,4,3’,4´-, 3,4,3´,4´-, and 3,5,3´,5´-TCB by these enzymes. Neither human CYP2A6 and 2A13 nor monkey CYP2A23, 2A24, or 2A26 oxidized these three TCB isomers when the chromatographic profiles of incubates were compared in the presence and absence of an NADPH-generating system (results not shown). We also studied the metabolism of 2,4,3´,4´-TCB by human CYP1A1, 1A2, 1B1, and 3A4, of 3,4,3´,4´-TCB by human CYP1A1, 1A2, and 1B1, and of 3,5,3´,5´-TCB by human CYP1A1, 1A2, 1B1, 2B6, and 3A4 and found that these three TCB isomers were not oxidized by these P450 enzymes (results not shown).
Docking Simulations of Interactions of 2,5,2',5'-TCB with Human and Monkey CYP2A enzymes. We compared the molecular interactions of 2,5,2',5'-TCB with human and monkey CYP2A enzymes using docking simulation as described in Materials and Methods (Figures 7). Several amino acid residues in substrate-recognition sites (SRSs) (Gotoh, 1992) were indicated in the figure. Crystal structures of CYP2A6 1Z10 and CYP2A13 4EJG were used to see the interaction of 2,5,2',5'-TCB with human CYP2A6 and 2A13, respectively and were employed for alignment of monkey CYP2A23, 2A24, and 2A26. Ligand-interaction energies (\(U\) values) of bound protein-ligand complexes obtained for the interaction of 2,5,2',5'-TCB with CYP2A6 and 2A13 were 33.0 and -6.9, respectively, and those with CYP2A24, 2A23, and 2A26 were 43.1, -22.8, and -9.29, respectively. The possible interaction of Asn297 with the C5' atom of 2,5,2',5'-TCB was found in CYP2A6 and 2A13 and the distance between C3 and C4 of the TCB and the Fe in the heme of CYP2A6 and 2A13 were calculated to be 2.97 and 4.67 Å and 6.06 and 3.91 Å, respectively (Figure 7). The distance between the C6 atom of TCB and the Fe in the heme of CYP2A13 was 4.83 Å. The position of Gly-301 in CYP2A6 was somewhat different from that of Ala-301 in CYP2A13 around the TCB molecule.

Our results showed that C3 and C4 positions of 2,5,2',5'-TCB interacted with (the heme of) CYP2A6, while C4 and C6 were interacting with (the heme of) CYP2A24 (Figures 7A and 7C). The distribution of amino acids surrounding the TCB was not so different in CYP2A6 and 2A24, except that a possible hydrogen bond interaction between Asn297 and TCB was found in a case of CYP2A26. Molecular docking analyses of CYP2A23 and 2A26
were aligned using CYP2A13 4EJG as a model structure, and it was found that C3 and C4 of TCB interacted with (the heme of) CYP2A24, but interaction with C4 position was found in CYP2A26.

**Correlation of U-Values in Interaction of Human and Monkey CYP2A Enzymes with CYP2A Ligands and Four TCBs.** We found that there were different orientations in molecular interaction of 2,5,2´,5´-TCB over the heme of CYP2A6 and 2A13 when examined with CYP2A6 1Z10 and 4EJJ for CYP2A6 (Fig. 8A) and CYP2A13 4EJH and 4EJG for CYP2A13 (Fig. 8B). We compared ligand-interaction energies (U values) with the molecular structures of CYP2A6 4EJJ and 1Z10 in the cases of CYP2A6 and 2A24 and CYP2A13 4EJG and 4EJH in cases of CYP2A13, 2A23, and 2A26 (Fig. 9). Ligands used for the comparison were four TCBs (2,4,3´,4´-, 2,5,2´,5´-, 3,4,3´,4´-, and 3,5,3´,5´-TCBs), five known CYP2A ligands (coumarin, nicotine, NNK, indole, and pilocarpine), and three chemicals (naphthalene, phenanthrene, and biphenyl) that we recently found to be good substrates of CYP2A13 and 2A6 (Shimada et al., 2016b). There were good correlations of interaction for a total of 12 chemicals with CYP2A6 and 2A13 using two reported molecular structures, except that the correlation coefficient was not as high (r = 0.66) in CYP2A24 when we used CYP2A6 1Z10 and 4EJJ for alignment of molecular structure of the enzyme.

By comparing interaction of human CYP2A6 and 2A13 with four TCB congeners used in this study, the lowest U values obtained in these cases were determined when interactions of CYP2A13, 2A23, and 2A26 with 2,5,2´,5´-TCB was examined, and with the latter two P450s
the $U$ values were low in interaction with 2,4,3',4'-TCB (Fig. 9). Correlation coefficients of $U$ values obtained with CYP2A6, 2A13, 2A23, 2A24, and 2A26 using crystal structures of 1Z10 and 4EJJ for CYP2A6 and 2A24 and of 4EJG and 4EJH for CYP2A13, 2A23, and 2A26 with twelve ligands were also determined (Supplemental Material Table 1).
Discussion

Our present results showed that CYP2A6 is an important enzyme in the oxidation of 2,5,2',5'-TCB to form 4-hydroxy-2,5,2',5'-TCB as a major metabolite and three to four metabolites as minor ones, including 3-hydroxy-2,5,2',5'-TCB, and that CYP2A13 had very low activities in oxidizing 2,5,2',5'-TCB as compared with CYP2A6. We also found decreases in the concentration of 2,5,2',5'-TCB in the reaction mixture on GC-MS analysis when it was incubated with CYP2A6 but not CYP2A13. These two human CYP2A enzymes were not active in oxidizing 2,4,3',4', 3,4,3',4' and 3,5,3',5'-TCB, as judged by the results obtained with our LC-MS/MS system. Other human P450 enzymes (including CYP1A1, 1A2, 1B1, 2B6, 2C9, and 3A4) did not catalyze the oxidation of 2,4,3',4', 3,4,3',4' and 3,5,3',5'-TCB, as well as 2,5,2',5'-TCB. In addition, we found that monkey CYP2A24, which shows 95% amino acid identity to CYP2A6, catalyzed the oxidation of 2,5,2',5'-TCB to form 4-hydroxy- and 3-hydroxy-2,5,2',5'-TCB at rates of ~0.3 min⁻¹ and ~0.04 min⁻¹, respectively. Another monkey P450, CYP2A26 (93% amino acid identity to CYP2A6) (Emoto et al., 2013), catalyzed the 4-hydroxylation of 2,5,2',5'-TCB at a rate about one-half that catalyzed by CYP2A24. The above two monkey P450 2A enzymes showed substrate disappearance on analysis with GC-MS (Table 1). Monkey CYP2A23, whose amino acid identity to CYP2A13 is 94% (Emoto et al., 2013), was not very active; the activities were comparable to those catalyzed by human CYP2A13. When we compared ligand-interaction energies (\(U\) values) with human and monkey CYP2A enzymes using the reported crystal structures of CYP2A6 and 2A13 (Yano et al., 2005; Smith et al., 2007; DeVore and Scott,
2012), we obtained good correlation coefficients with the 12 chemicals used in this study (Fig. 9 and Supplemental Table 1), indicating that the structures of human and monkey CYP2A enzymes are similar and that small amino acid changes in the molecular structures may define how these P450 2A enzymes catalyze the oxidation of 2,5,2´,5´-TCB. Phylogenetic trees of human and monkey CYP2A enzymes revealed changes in catalytic specificity of oxidation of ligands such as 2,5,2´,5´-TCB (Supplemental Fig. 1).

Because human CYP2A6 and monkey CYP2A24 (and also CYP2A26) were more active in catalyzing the oxidation of 2,5,2´,5´-TCB than CYP2A13 and 2A23, we compared amino acid differences in substrate recognition sites (SRSs) (Gotoh, 1992) in these human and monkey CYP2A enzymes (Fig. 10). The amino acid differences V110L in SRS1, I208S in SRS2, Q239K, and L240E in SRS3, I300F in SRS4, V365M, I366L, and S369G in SRS5, and R372H in SRS6 were noted between CYP2A6/2A24 and CYP2A13/2A24, indicating that these residues are important in catalyzing 2,5,2´,5´-TCB, as has been reported previously using human CYP2A variants and their various ligands (DeVore et al., 2009; 2012a; 2012b; DeVore and Scott 2012; He et al., 2004; Sansen et al., 2007). It is also interesting to note that there is a difference in G207A in CYP2A6 and 2A24; 4-hydroxylation of 2,5,2´,5´-TCB by the former P450 was about 3-fold higher than CYP2A24. The importance of Gly-207 has been reported in mouse coumarin hydroxylation catalyzed by P450coh (Juvonen et al., 1993). Small differences in amino acid sequences in monkey CYP2A24 and 2A26 were also noted (Fig. 10) and are suggested to cause catalytic differences in these P450s.
In rats, 2,5,2',5'-TCB has been shown to be oxidized to 3-hydroxy-2,5,2',5'-TCB as a major metabolite in vivo (Yoshimura et al., 1975; Ishida et al., 1991; Koga et al., 1995), and this reaction is catalyzed by CYP2B1 and 2B2 in vitro (Ishida et al., 1991; Preston et al., 1983; Matsusue et al., 1996). Guinea pig CYP2B18, hamster P450 HPB-1, and rabbit CYP2B4 have also been suggested to participate in the 3-hydroxylation of 2,5,2',5'-TCB in liver microsomes in vitro (Koga et al., 1995a; 1995b; 1998; Ohta et al., 2009). Interestingly, hamster CYP2A8 catalyzes the oxidation of 2,5,2',5'-TCB to form 4-hydroxy-2,5,2',5'-TCB at a rate of 0.022 min^{-1} (Koga et al., 1996); the turnover number is similar to that catalyzed by human CYP2A13 (0.017 min^{-1}) but not that by CYP2A6 (~1.0 min^{-1}) found in this study. In fact, the amino acid identity of CYP2A8 to CYP2A13 and 2A6 has been found to be only 76 and 74%, respectively (Emoto et al., 2013), and the 2A Subfamily comparisons have been shown to be different in human and hamster CYP2A enzymes (Supplemental Fig. 1). In addition, there are differences in amino acid sequences in the SRS regions between human and hamster CYP2A enzymes (Fig. 10).

2,5,2',5'-TCB induced Type I binding spectra with CYP2A6 and 2A13, having $K_s$ values of 9.4 and 0.51 $\mu$M, respectively, although the former enzyme catalyzed oxidation of 2,5,2',5'-TCB at much higher rate than CYP2A13. Molecular docking simulation studies showed that ligand-interaction energies ($U$ values) for interaction of 2,5,2',5'-TCB with CYP2A13 4EJH (NNK-type), CYP2A13 2P85 (indole-type), CYP2A13 3T3S (pilocarpine-type), and CYP2A13 4EJG (nicotine-type), were -22.2, -7.1, -2.9, and -5.0, respectively, and with CYP2A6 3T3R (pilocarpine-type), CYP2A6 4EJJ (nicotine-type), and
CYP2A6 1Z10 (coumarin-type), were 16.4, 62, and 39, respectively. These results suggest that 2,5,2',5'-TCB more readily interacts with CYP2A13 than CYP2A6 in inducing Type I binding spectra, having lower values of ligand-interaction energies, and that these interaction affinities are not consistent with the rates of 2,5,2',5'-TCB oxidation by these P450 enzymes.

McGraw et al. (2006) reported that 2,4,5,2',5'-pentachlorobiphenyl is oxidized by CYP2A6 to a 4-hydroxylated metabolite at a rate of ~0.5 nmol/min/nmol P450, with a $K_m$ value of 34 $\mu$M. The activity reported by them is comparable to the rate of metabolism of 2,5,2',5'-TCB by CYP2A6 found in this study, suggesting that CYP2A6 is preferentially responsible for the metabolism of PCB congeners having chlorine at the 2- and 5-positions and that it shows attenuated oxidation of PCB congeners when they have 2,4,5-chlorine atom substitution on one of the benzene rings. Schnellmann et al. (1993) found that 2,4,5,2',4',5'-hexachlorobiphenyl is not metabolized by human liver microsomes, although this congener has been shown to be oxidized by human CYP2B6 to a 3-hydroxyl product at very low rate (0.0064 nmol/min/nmol P450) (Ariyoshi et al., 1995). In comparing three PCB congeners, Ghiasuddin et al. (1976) reported that 2,5,2'-trichlorobiphenyl is extensively metabolized by rat liver microsomes, followed by 2,5,2',5'-TCB and then 2,4,5,2',5'-pentachlorobiphenyl.

PCBs have been identified in air, soil, water, and other environmental samples and also detected in human blood and tissue samples (IARC Monograph, 2015; Victor et al., 1989; Konishi et al., 2006). Non-Aroclor or non-legacy PCB contaminants have been determined
in the environments of homes and cities and accumulated in the bodies of exposed populations in various cities in the United States (Martinez et al., 2009; Hu et al., 2012; Sun et al., 2006; Koh et al., 2015). 2,4,3′,4′-TCB has been reported to be one of the most persistent TCB isomers in biological samples and human milk and can be detected in food samples such as fish and other meats, eggs, and bovine milk (Konishi et al., 2006; Todaka et al., 2008; Akutsu et al., 2005). Yoshimura et al. reported that 2,4,3′,4′-TCB is metabolized in vivo in rats to 4-hydroxy-2,5,3′,4′-TCB which is formed, possibly through a 4,5-oxide followed by an NIH-shift of a chlorine atom from the 4-position (Koga et al., 1992; 1973), indicating the possible role of P450 enzymes in the reaction. However, little is known about the in vitro metabolism of 2,4,3′,4′-TCB in humans as well as in laboratory animals, probably due to its slow oxidation by P450 enzymes. In fact, our present studies showed that none of the oxygenated metabolites of 2,4,3′,4′-TCB was detected using human and monkey P450 enzymes determined. We also found that 3,4,3′,4′-TCB, one of the coplanar PCB congeners present in human milk (Konishi et al., 2006), was not oxidized by human CYP1A1 and other P450s to oxidative metabolites, although rat CYP1A1 has been shown to catalyze 3,4,3′,4′-TCB to form 4- and 5-hydroxylated products at a molar ratio of 2.2:1 (Ishida et al., 1991). There are species-related differences in the metabolism of 3,4,5,3′,4′-pentachlorobiphenyl in rat and human CYP1A1, in that the former enzyme catalyzes 4-hydroxylation of this compound but human CYP1A1 does not (Inui et al. (2014); Yamazaki et al., 2011).

In conclusion, these studies showed that 2,5,2′,5′-TCB oxidation is catalyzed by
CYP2A6 to form 4-hydroxy-2,5,2',5'-TCB as a major metabolite and at least four minor products, including 3-hydroxy-2,5,2',5'-TCB, in humans. CYP2A13 oxidized 2,5,2',5'-TCB at a very slow rate compared with CYP2A6 and other human P450 enzymes, including CYP1A1, 1A2, 1B1, 2B6, 2D6, 2E1, 2C9, and 3A4, which did not oxidize 2,5,2',5'-TCB. The orthologous monkey CYP2A24 (95% identity to CYP2A6) was found to oxidize 2,5,2',5'-TCB to form 4- and 3-hydroxy-2,5,2',5'-TCB at rates of 0.29 min\(^{-1}\) and 0.042 min\(^{-1}\), respectively. Monkey CYP2A26 (93% identity to CYP2A6) catalyzed the oxidation of 2,5,2',5'-TCB at lower rates than CYP2A24 but at much higher rates than CYP2A23 (94% identity to CYP2A13). Small amino acid changes in the SRS regions of human and monkey CYP2A enzymes are suggested to determine how these P450 enzymes catalyze the oxidation of 2,5,2',5'-TCB. Ligand-interaction energies (\(U\) values) in molecular docking analysis were suggested to be useful markers to predict the molecular interaction of 12 chemicals (four TCBs, five known P450 2A substrates, and naphthalene, phenanthrene, and biphenyl) with human and monkey CYP2A enzymes.
Authorship contribution

Participated in research design: Shimada, Kakimoto, Murayama, and Takenaka

Conducted experiments: Shimada, Kakimoto, and Murayama

Performed data analysis: Shimada, Kakimoto, Takenaka, Murayama, Uehara, and Yamazaki

Contributed new reagents or analytical tools: Koga, Uehara, and Kim

Wrote or contributed to the writing of the manuscript: Shimada, Yamazaki, Guengerich, and Komori
References


He XY, Shen J, Hu WY, Ding X, Lu AY, and Hong JY (2004) Identification of Val117 and


women in Sapporo City, Japan. *Chemosphere* 73: 923-931.


Footnotes

Tsutomu Shimada, Kensaku Kakimoto, Shigeo Takenaka, and Masayuki Komori were supported in part by Grants from the Ministry of Education, Science, and Culture of Japan and the Ministry of Health and Welfare of Japan. Nobuyuki Koga, Shotaro Uehara, Hiroshi Yamazaki, and F. Peter Guengerich were also supported partly by a Health and Labor Scientific Research Grant [H27-food-designated-017], the Japan Society for the Promotion of Science Grant-in-Aid for Young Scientists B [15K18934] and Scientific Research [26460206], and by United States Public Health Service grant [R01 GM118122], respectively.

This article has supplemental material available at dmd.aspetjournals.org.

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Figure legends

**Fig. 1.** Structure of chemicals used in this study

**Fig. 2.** Spectral interaction of 2,5,2’,5’-TCB with CYP2A6 and 2A13. The absolute spectra were first obtained and then this was repeated after adding 2,5,2’,5’-TCB to a 1 µM concentration of each of P450 enzymes (upper part of the Figs. 2A and 2B). Difference spectra were then obtained by subtracting the P450 spectra (in the absence of 2,5,2’,5’-TCB) from the P450 spectra (in the presence of 2,5,2’,5’-TCB) (lower part of the figures). Spectral dissociation constants ($K_s$) were estimated from hyperbolic plots using GraphPad Prism software (GraphPad Software, San Diego, CA) (Figs. 2a and 2b).

**Fig. 3.** Effects of incubation time on the levels of 2,5,2’,5’-TCB after the metabolism with CYP2A6 (A) and 2A13 (B). TCB at 12.5 µM (closed circles), 25 µM (open squares), and 50 µM (closed squares) were incubated with 0.05 µM CYP2A6 (A) or 2A13 (B) for different periods of time and after incubation the remaining TCB was extracted with CH$_3$OH and then with a CHCl$_3$–ethyl acetate mixture (1:1, v/v). The organic layer, after the centrifugation, was submitted directly GC-MS to determine the levels of TCB. Results are expressed as means of duplicate determinations.

**Fig. 4.** LC-MS/MS analysis of metabolism of 2,5,2’,5’-TCB by CYP2A6. Product formation
was determined by LC (CORTECS C_{18} column) on analysis of total ion scans between \textit{m/z} 250 and 350 (A) and SRM (\textit{m/z} 306.9 \rightarrow 270.9) (B). Major metabolites were tentatively termed m1, m2, m3, and m4 in Figure 4A and 4B. The product ion mass spectra of metabolites m1, m2, m3, and m4 were also determined (C, D, E, and F, respectively).

**Fig. 5.** Effects of incubation time (A and B) and kinetic analysis (C and D) of oxidation of 2,5,2',5'-TCB by CYP2A6 (A and C) and 2A13 (B and D). 2,5,2',5'-TCB (at 25 \( \mu \text{M} \)) was incubated with CYP2A6 (A) and 2A13 (B) for indicated periods of time and the metabolites formed were analyzed with LC-MS/MS. Note that the values in vertical lines are different in Figure A and B (10-fold scale difference) since CYP2A6 gave higher rates formation of TCB metabolites than CYP2A13 did. Lineweaver-Burke plots of oxidation of 2,5,2',5'-TCB by CYP2A6 (C) and 2A13 (D) and \( K_m \) values of formation of m1, m2 (mixture of 3- and 4-hydroxy products), m3, and m4 by CYP2A6 and those of m2 and m4 by CYP2A13 were indicated in the figures. Results are expressed as means of 2-3 experiments.

**Fig. 6.** Oxidation of 2,5,2',5'-TCB by CYP2A6 (A and B), CYP2A24 (C and D), CYP2A26 (E and F), CYP2A13 (G and H), and CYP2A23 (I and J) on analysis with LC-MS/MS using CORTECS C_{18} column (A, C, E, G, and I) and RP-amide column (B, D, F, H, and J). P450 and 2,5,2',5'-TCB concentrations used were 0.1 and 50 \( \mu \text{M} \), respectively, and incubation time was 60 min. The asterisk (*) in Fig. 6B, 6D, and 6F indicates minor metabolites (structures not determined) corresponding to peaks in Fig. 6A, 6C and 6F.
Fig. 7. Molecular docking simulation of interaction of 2,5,2',5'-TCB with human CYP2A6 (A) and 2A13 (B) and monkey CYP2A24 (C), 2A23 (D), and 2A26 (E). Distance between chemical sites of TCB and the centers (Fe) of the heme of CYP2A enzymes are indicated in red. Positions of carbon atoms in 2,5,2',5'-TCB were shown in black.

Fig. 8. Docking simulation of 2,5,2',5'-TCB (shown in green and blue) to CYP2A6 1Z10 (coumarin-bound form) and CYP2A6 4EJJ (nicotine-bound form), respectively (A) and 2,5,2',5'-TCB (shown in yellow and red) to CYP2A13 4EJH (NNK-bound form) and CYP2A13 4EJG (nicotine-bound form), respectively (B).

Fig. 9. Correlation of ligand–interaction energies ($U$ values) using molecular docking with CYP2A6 1Z10 (coumarin-bound form) and CYP2A6 4EJJ (nicotine-bound form) (A) and CYP2A13 4EJG (nicotine-bound form) and CYP2A13 4EJH (NNK-bound form) (B). Typical CYP2A substrates (coumarin, nicotine, pilocarpine, indole, and NNK) and naphthalene, phenanthrene, and biphenyl derivatives used in our recent paper (43) were included for correlative comparison. The correlation coefficient ($r$) was determined with all of these chemicals, including four TCB congeners. Similarly, correlation of $U$ values in monkey CYP2A24, 2A23, and 2A26 enzymes and these chemicals are also shown.

Fig. 10. Differences in amino acid sequences in SRS-1 through -6 regions of CYP2A6, 2A24,
2A26, 2A13, 2A23, and 2A8. Amino acid residues of P450s that differed from those of CYP2A6 are indicated in black characters.
### TABLE 1

**Oxidation of 2,5,2',5'-TCB by human and monkey P450s**

<table>
<thead>
<tr>
<th>Species</th>
<th>P450</th>
<th>Substrate disappearance* (%) in 60 min</th>
<th>Product formation (nmol/min/nmol P450)</th>
<th>Ratio of 4-OH/3-OH</th>
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<td></td>
<td></td>
<td>4-OH-TCB</td>
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<td>42 ± 3.1</td>
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<td>24 ± 3.1</td>
<td>0.13 ± 0.021</td>
<td>0.021 ± 0.0031</td>
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</table>

*a* A reconstituted system containing CYP1A1 and NADPH-P450 reductase; *b* *E. coli* membranes co-expressing P450 and NADPH-P450 reductase; *c* Baculo enzyme system expressing P450 and NADPH-P450 reductase.

*GC-MS analysis of 2,5,2',5'-TCB after metabolism for 60 min. Results are expressed as means ± SD (n=3-5) (these values were not analyzed for statistical significance because a large number of different comparisons were made).*
Fig. 1

2,5,2’,5’-TCB  2,4,3’,4’-TCB  3,4,3’,4’-TCB  3,5,3’,5’-TCB
Fig. 2

- **A**: Graph showing the absorbance of CYP2A6. The inset includes the equation $K_a (\mu M) = 9.4 \pm 1.9$ and $A_{max} = 0.011 \pm 0.001$.

- **B**: Graph showing the absorbance of CYP2A13. The inset includes the equation $K_a (\mu M) = 0.51 \pm 0.07$ and $A_{max} = 0.051 \pm 0.001$. 

Wavelength (nm)

Absorbance

2,5,2',5'-TCB (µM)

350 450 550 650

-0.04 0.00 0.04 0.08 0.12
Fig. 3

A

GC response (%)

100

50

0

0 20 40 60

Incubation time (min)

B

GC response (%)

100

50

0

0 20 40 60
Fig. 4

(A) Chromatogram showing peaks m1, m2, m3, and m4. The retention time ranges from 6 to 9 minutes.

(B) Enlarged view of the chromatogram highlighting peaks m1, m2, and m3. The retention time ranges from 7 to 9 minutes.

(C) Mass spectrum with peaks at m/z 306.67, 306.57, and 306.95.

(D) Mass spectrum with peaks at m/z 306.79.

(E) Mass spectrum with peaks at m/z 270.90 and 271.18.

(F) Mass spectrum with peaks at m/z 270.52, 270.90, and 306.89.
Fig. 5
Fig. 6
Fig. 7

CYP2A6 (1Z10)  
Ile208  
Phe107  
Phe209  
Phe480  
Asn297  
Val117  
Var365  
(U = 33.0)

CYP2A13 (4EJG)  
Ser208  
Phe107  
Phe209  
Phe480  
Asn297  
Leu366  
Met365  
(U = -6.9)

CYP2A24 (model: 1Z10)  
Ile208  
Phe107  
Phe209  
Phe480  
Asn297  
Val117  
Ile300  
Ala301  
(U = 43.1)

CYP2A23 (model: 4EJG)  
Ser208  
Phe107  
Phe209  
Phe480  
Asn297  
Leu366  
Val117  
Ile300  
(U = -22.8)

CYP2A26 (model: 4EJG)  
Ser208  
Phe107  
Phe209  
Phe480  
Asn297  
Val117  
Ile300  
Val365  
(U = -9.28)
Fig. 8

A

2,5,2',5'-TCB

CYP2A6

B

2,5,2',5'-TCB

CYP2A13
Fig. 9

![Graphs](image)

Legend:
- CYP2A6
- CYP2A13
- CYP2A24
- CYP2A23
- CYP2A26

Ligand-interaction energy (U value)

- Phenanthrene
- 2,5,2',5'-TCB
- 3,5,3',5'-TCB
- 2,4,3',4'-TCB
- Naphthalene
- Biphenyl
- Indole
- NNK
- Pilocarpine
- Coumarin
- Nicotine

Correlation coefficients:
- r = 0.93
- r = 0.92
- r = 0.65
- r = 0.97
- r = 0.94
### Fig. 10

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<td>CYP2A6</td>
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<td>AFQVL QGL</td>
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<td>208</td>
<td>239</td>
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