

# Roles of Human CYP2A6 and Monkey CYP2A24 and 2A26 Cytochrome P450 Enzymes in the Oxidation of 2,5,2',5'-Tetrachlorobiphenyl<sup>§</sup>

Tsutomu Shimada, Kensaku Kakimoto, Shigeo Takenaka, Nobuyuki Koga, Shotaro Uehara, Norie Murayama, Hiroshi Yamazaki, Donghak Kim, F. Peter Guengerich, and Masayuki Komori

Laboratory of Cellular and Molecular Biology, Osaka Prefecture University, Izumisano, Osaka, Japan (T.S., S.T., M.K.); Osaka Prefectural Institute of Public Health, Higashinari-ku, Osaka, Japan (K.K.); Faculty of Nutritional Sciences, Nakamura Gakuen University, Johnan-ku, Fukuoka, Japan (N.K.); Laboratory of Drug Metabolism and Pharmacokinetics, Showa Pharmaceutical University, Machida, Tokyo, Japan (S.U., N.M., H.Y.); Department of Biological Sciences, Konkuk University, Seoul, South Korea (D.K.); and Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee (F.P.G.)

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## ABSTRACT

2,5,2',5'-Tetrachlorobiphenyl (TCB) induced type I binding spectra with cytochrome P450 (P450) 2A6 and 2A13, with  $K_s$  values of 9.4 and 0.51  $\mu\text{M}$ , respectively. However, CYP2A6 oxidized 2,5,2',5'-TCB to form 4-hydroxylated products at a much higher rate ( $\sim 1.0 \text{ minute}^{-1}$ ) than CYP2A13 ( $\sim 0.02 \text{ minute}^{-1}$ ) based on analysis by liquid chromatography–tandem mass spectrometry. 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 ( $\sim 0.3 \text{ minute}^{-1}$ ) than CYP2A26 (93% identity to CYP2A6,  $\sim 0.13 \text{ minute}^{-1}$ ) and

CYP2A23 (94% identity to CYP2A13,  $\sim 0.008 \text{ minute}^{-1}$ ). 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 (McFarland and Clarke, 1989; Mills et al., 2007; International Agency for Research on Cancer, 2015). 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 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 biologic samples (Kunita et al., 1984; Yakushiji et al., 1984). Konishi et al. (2006) determined the levels of PCB congeners in human milk in Osaka and found that 12 major PCBs, including three hepta-, two hexa-, four penta-, and four tetrachlorobiphenyl congeners, decreased between 1973 and 2000, but the rate of elimination depends on the individual PCB congeners determined, probably due to their susceptibilities to being chemically degraded and enzymatically metabolized (Akutsu et al., 2005; Todaka et al., 2008; Armich et al., 2009).

2,5,2',5'-Tetrachlorobiphenyl (#PCB52) (Mills et al., 2007; International Agency for Research on Cancer, 2015) (Fig. 1) is one of the PCB congeners identified in human milk and in foodstuffs at significant levels, and its biologic activities and metabolism by xenobiotic-metabolizing enzymes has been extensively studied in experimental animal models (Yoshimura et al., 1975; Preston et al., 1983; Borlakoglu et al., 1991; Ishida et al., 1991; Safe, 1993; Koga et al., 1995a). Cytochrome P450 (P450) has been shown to be

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**ABBREVIATIONS:**  $b_5$ , cytochrome  $b_5$ ; GC-MS, gas chromatography–mass spectrometry; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; LC-MS/MS, liquid chromatography–tandem mass spectrometry; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; P450, cytochrome P450; PCB, polychlorinated biphenyl; PDB, Protein Data Bank; SRS, substrate-recognition site; TCB, tetrachlorobiphenyl.

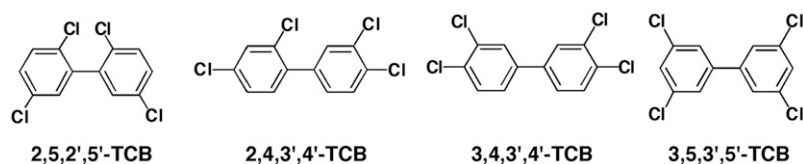


Fig. 1. Structure of chemicals used in this study.

the major enzyme responsible for the oxidation of this tetrachlorobiphenyl (TCB), and there are species-related differences in the metabolism by different forms of P450s in rats, mice, guinea pigs, hamsters, and rabbits (Preston et al., 1983; Borlakoglu et al., 1991; 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 gas chromatography–mass spectrometry (GC-MS) and liquid chromatography–tandem mass spectrometry (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 (Akutsu et al., 2005; Konishi et al., 2006; Todaka et al., 2008), were also studied for their oxidation by these P450 enzymes.

### Materials and Methods

**Chemicals.** 2,5,2',5'-TCB (Fig. 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., 1995a). 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 (Fig. 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, 2016b).

**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 coexpressed 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 previously described (Sandhu et al., 1993, 1994; Guengerich, 2015). CYP1A1, 2A6, 2A13, 2C9, 3A4, NADPH-P450 reductase, and cytochrome *b<sub>5</sub>* (*b<sub>5</sub>*) were purified from membranes of recombinant *E. coli* as described elsewhere (Sandhu et al., 1993, 1994; Shimada et al., 2013; Guengerich, 2015).

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  $\mu$ M 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 ( $K_d$ ) were estimated using GraphPad Prism software (GraphPad Software, San Diego, CA), using either 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 P450 in *E. coli* membranes or 10 pmol P450s in microsomes of *T. ni* cells coexpressing human NADPH-P450 reductase), 50  $\mu$ M each TCB isomer, and an NADPH-generating system consisting of 0.5 mM NADP<sup>+</sup>, 5 mM glucose 6-phosphate, and 0.5 units of yeast glucose 6-phosphate dehydrogenase/ml (Shimada et al., 2015, 2016b). [The PCBs and other chemicals were dissolved in (CH<sub>3</sub>)<sub>2</sub>SO as 10 mM stock solutions and diluted into aqueous solution, with the final solvent concentration  $\leq$ 0.5% (v/v).]

In reconstitution experiments, P450 membranes were replaced by purified P450 (50 pmol), NADPH-P450 reductase (100 pmol), *b<sub>5</sub>* (100 pmol) (when required), and *L*- $\alpha$ -dilauroyl-*sn*-glycero-3-phosphocholine (50  $\mu$ g) as described previously (Shimada et al., 2015, 2016b). Our previous studies have suggested that more than 2-fold excess 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., 2002). Incubations were carried out at 37°C, following a preincubation time of 1 minute. Reactions were terminated by adding 0.25 ml of cold CH<sub>3</sub>OH and then extracted with 0.5 ml of a CHCl<sub>3</sub>-ethyl acetate mixture [1:1 (v/v)] twice. The organic layers were subjected to filtration using Disposable Syringe Filters (Cellulose Acetate Membrane, 3 mm  $\times$  0.20  $\mu$ m, code 2012-003; Iwaki, Osaka, Japan), and the filtrates (10  $\mu$ l) were used for analysis with GC-MS and 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, Milford, MA) coupled to a tandem quadrupole mass spectrometer (Xevo TQ-S; Waters) (Kakimoto et al., 2015). Chromatographic separation was performed on an octadecylsilane (C<sub>18</sub>) column (CORTECS C<sub>18</sub>, 100  $\times$  2.1-mm i.d., 1.6  $\mu$ m; Waters) or an RP-amide column (Ascentis Express, 100  $\times$  2.1-mm i.d., 2  $\mu$ m; Sigma-Aldrich, St. Louis, MO). The temperature of the C<sub>18</sub> column was maintained at 45°C, and gradient elution [CH<sub>3</sub>CN; 35% in water to 75% (v/v) over 10 minutes] was performed 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 minutes; hold for 2 minutes] 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 mode was used to quantify the *m/z* 306.9 to 270.9 transitions for identification of monohydroxylated 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<sub>18</sub> column; the same collision energy of the selected reaction-monitoring mode was used for obtaining the product ion mass spectrum.

**GC/MS Analysis of TCB Isomers.** GC-MS was performed with a gas chromatograph (7890A series; Agilent Technologies, Santa Clara, CA) coupled with a mass spectrometer (Quattro micro GC; Waters) using a capillary column (VF-5ms, 30 m  $\times$  0.25-mm i.d., 0.25- $\mu$ m film thickness; Agilent Technologies); the column temperature program was as follows: 60°C (held for 1 minute) to 140°C at 20°C/min, and to 260°C at 8°C/min, then to 300°C (held for 5 minutes)

at 40°C/min; carrier gas, helium was set at 1.0 ml/min. The MS conditions used were as follows: electron ionization mode; electron energy, 70 eV; and ion source temperature, 250°C. The selected ion-monitoring 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 in Human P450 Enzymes.** Crystal structures of CYP2A6 bound to coumarin [Protein Data Bank (PDB) 1Z10], pilocarpine (PDB 3T3R), and nicotine (PDB 4EJ), and CYP2A13 bound to nicotine (PDB 4EJG), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (PDB 4EJH), indole (PDB 2P85), and pilocarpine (PDB 3T3S) have been reported and were used in this study (Yano et al., 2005; Smith et al., 2007; DeVore et al., 2009, 2012; DeVore and Scott, 2012a; Shimada et al., 2016a). Simulations were carried out after removing each ligand from these P450 structures using the MMFF94x force field described in the MOE software (version 2015.10; Computing Group, Montreal, Canada) as previously described (Shimada et al., 2010, 2011, 2013). Crystal structures of monkey CYP2A23, 2A24, and 2A26 have not yet been reported, and these P450 primary sequences were aligned with human CYP2A6 (PDB 1Z10) for CYP2A24, and with CYP2A13 (PDB 4EJG) for CYP2A23 and 2A26 using MOE software for modeling of three-dimensional structures (Chemical Computing Group, Inc., Montreal, Canada). Ligand-interaction energies ( $U$  values) were obtained by use of the ASE dock program in the MOE software. A lower  $U$  value is an indication of a higher interaction between a chemical and the enzyme.

**Kinetic Analysis.** Kinetic parameters were estimated by nonlinear regression analysis of hyperbolic plots using the program KaleidaGraph (Synergy Software, Reading, PA) or GraphPad Prism (GraphPad Software, 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 (Fig. 2). The  $K_s$  value was 0.51  $\mu$ M for CYP2A13, whereas that for 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<sub>3</sub>OH and then with CHCl<sub>3</sub>-

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 to 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 marked at lower substrate concentrations, resulting in 50% decreases in TCB during 60-minute incubation (Fig. 3, A and B).

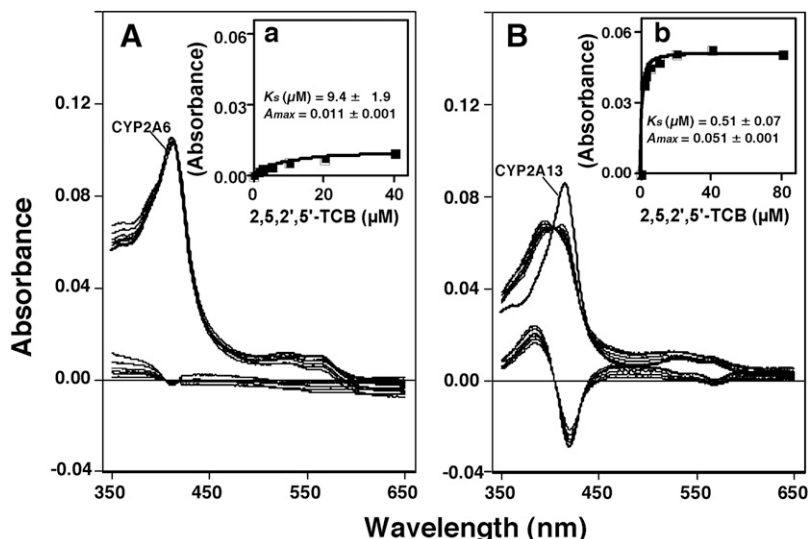
**Metabolism of 2,5,2',5'-TCB by CYP2A6.** Since the aforementioned results suggested that CYP2A6 was more active than CYP2A13 in metabolizing 2,5,2',5'-TCB, LC-MS/MS analysis (with a CORTECS C<sub>18</sub> column) was carried out to identify the oxidative metabolites (Fig. 4). We first searched the products with a molecular mass between  $m/z$  250 and  $m/z$  350 and found several peaks in which four oxidative metabolites (namely, m1, m2, m3, and m4) were suggested to be monooxygenated products with (molecular ions)  $m/z$  307 (Fig. 4, A and B). We also searched whether dioxygenated products were formed during metabolism of TCB with CYP2A6 and found that none with a molecular mass of  $m/z$  323 were formed (results not shown).

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

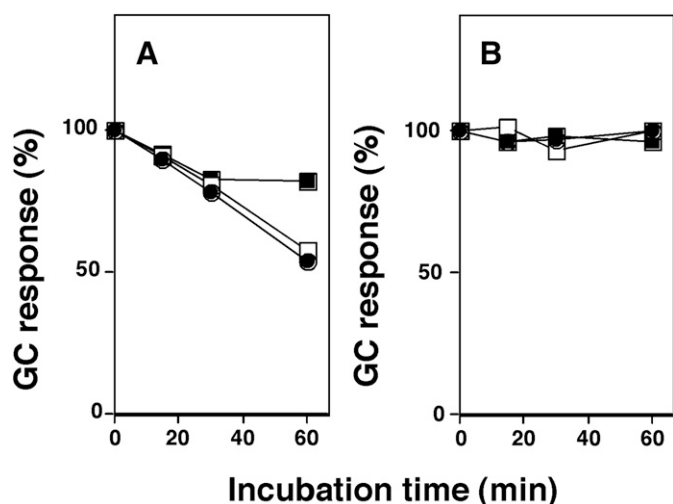
Time-dependent formation of four oxygenated products (m1, m2, m3, and m4) was analyzed on incubation of TCB with CYP2A6 and 2A13 (Fig. 5, A and B); in the case of CYP2A13, we detected only two products, m2 and m4. With both P450 enzymes examined, m2 was the most abundant, and we found that CYP2A6 was 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  $K_m$  values for the formation of m1, m2, m3, and m4 by CYP2A6 were 21, 22, 26, and 40  $\mu$ M, and the  $K_m$  values for m2 and m4 by CYP2A13 were 16 and 18  $\mu$ M, respectively (Fig. 5, C and D).

The effects of  $b_5$  on CYP2A6- and 2A13-dependent oxidation of 2,5,2',5'-TCB in reconstituted monooxygenase systems containing human NADPH-P450 reductase and L- $\alpha$ -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,



**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  $\mu$ M concentration of each P450 enzyme (A and B, top). 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 (bottom part of the figures). Spectral dissociation constants ( $K_s$ ) were estimated from hyperbolic plots using GraphPad Prism software (GraphPad Software) (a and b).



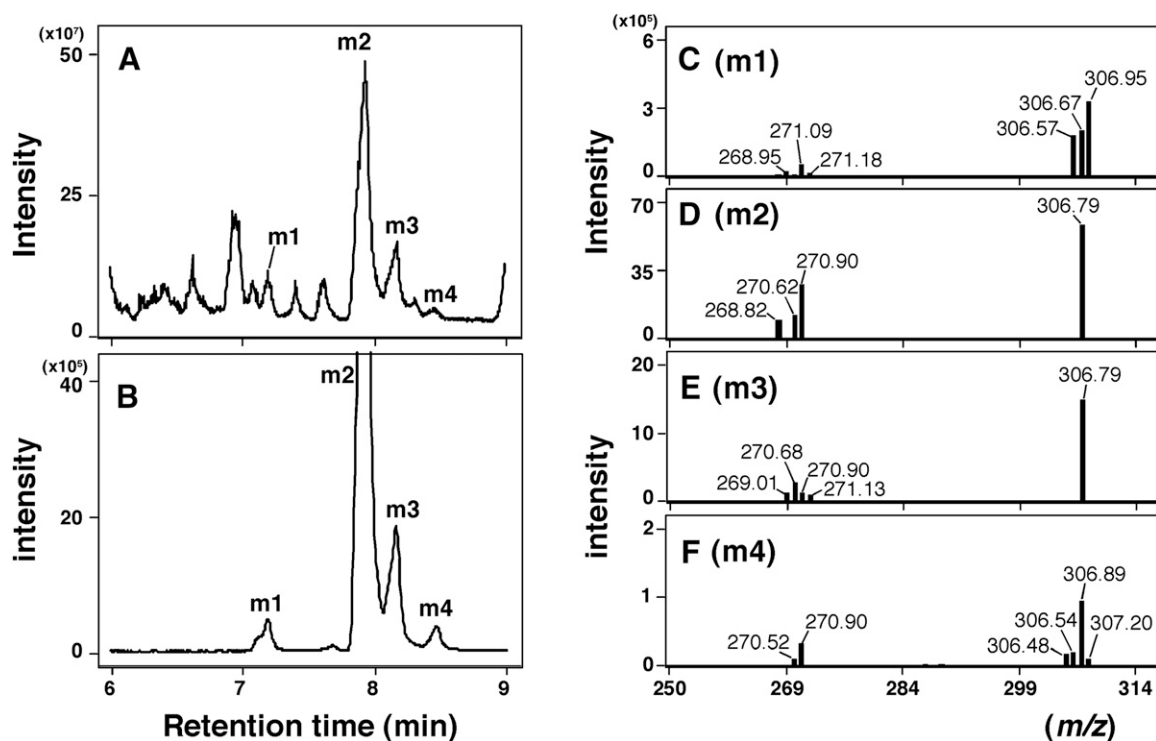
**Fig. 3.** Effects of incubation time on the levels of 2,5,2',5'-TCB after metabolism with CYP2A6 (A) and 2A13 (B). TCB at 12.5 μM (closed circles), 25 μM (open squares), and 50 μM (closed squares) were incubated with 0.05 μM CYP2A6 (A) or 2A13 (B) for different periods of time, and after incubation,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

2A24, and 2A26 by using two LC columns, a CORTECS C<sub>18</sub> column (Fig. 6, A, C, E, G, and I) and an RP-amide column (Fig. 6, B, D, F, H, and J). The former column did not separate 3- and 4-hydroxylated products (showing a single peak at the position of m2) (Fig. 6, A, C, E, G, and I). 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

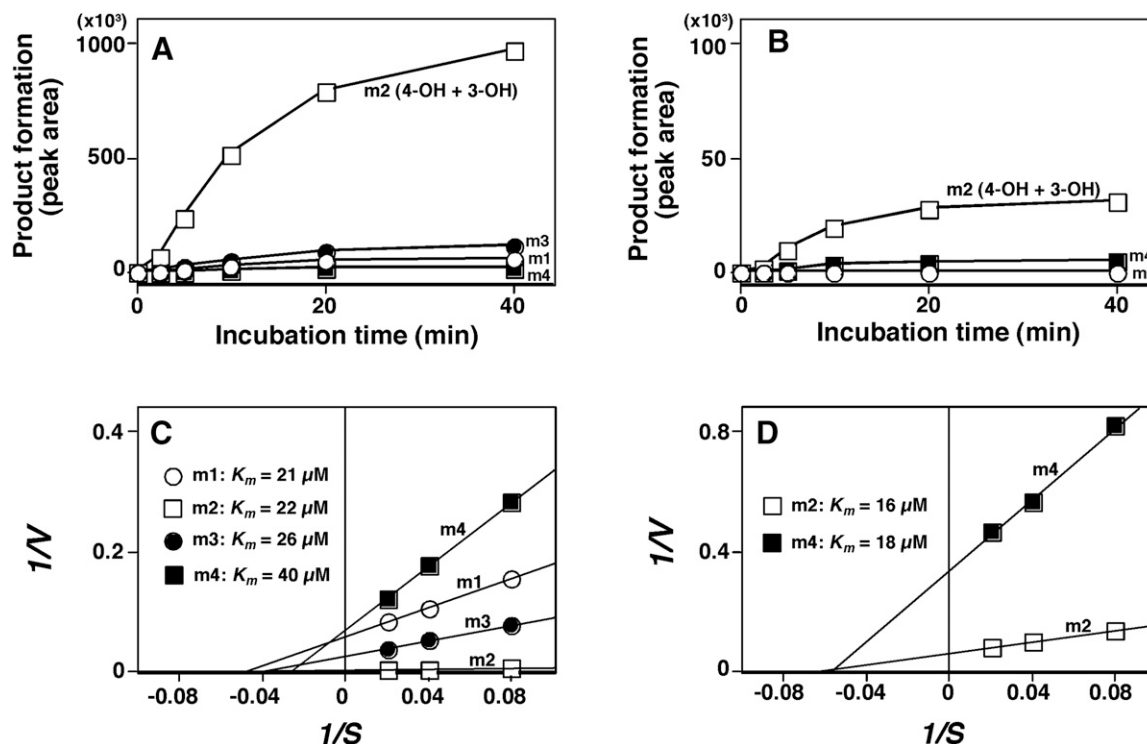
monkey CYP2A enzymes examined (Fig. 6, B, D, F, H, and J). CYP2A6 produced 4-hydroxy-TCB at the highest rate, and CYP2A24 and 2A26 produced metabolite m3 at higher rates than CYP2A6. Both human CYP2A13 and monkey CYP2A23 had low catalytic activities for oxidizing this TCB.

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 performed to investigate the disappearance of the substrate 2,5,2',5'-TCB after metabolism by P450s (incubating for 60 minutes), 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 10 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



**Fig. 4.** LC-MS/MS analysis of metabolism of 2,5,2',5'-TCB by CYP2A6. Product formation was determined by LC (CORTECS C<sub>18</sub> column) on analysis of total ion scans between *m/z* 250 and 350 (A) and selected reaction-monitoring (*m/z* 306.9 > 270.9) (B). Major metabolites were tentatively termed m1, m2, m3, and m4 in (A) and (B). The product ion mass spectra of metabolites m1 (C), m2 (D), m3 (E), and m4 (F) were also determined.



**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 of vertical lines are different in (A) and (B) (10-fold scale difference) since CYP2A6 gave higher rates of formation of TCB metabolites than CYP2A13. Lineweaver-Burk 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 are indicated in the figures. Results are expressed as means of two to three experiments.

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; 3,4,3',4'-TCB by human CYP1A1, 1A2, and 1B1; and 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).

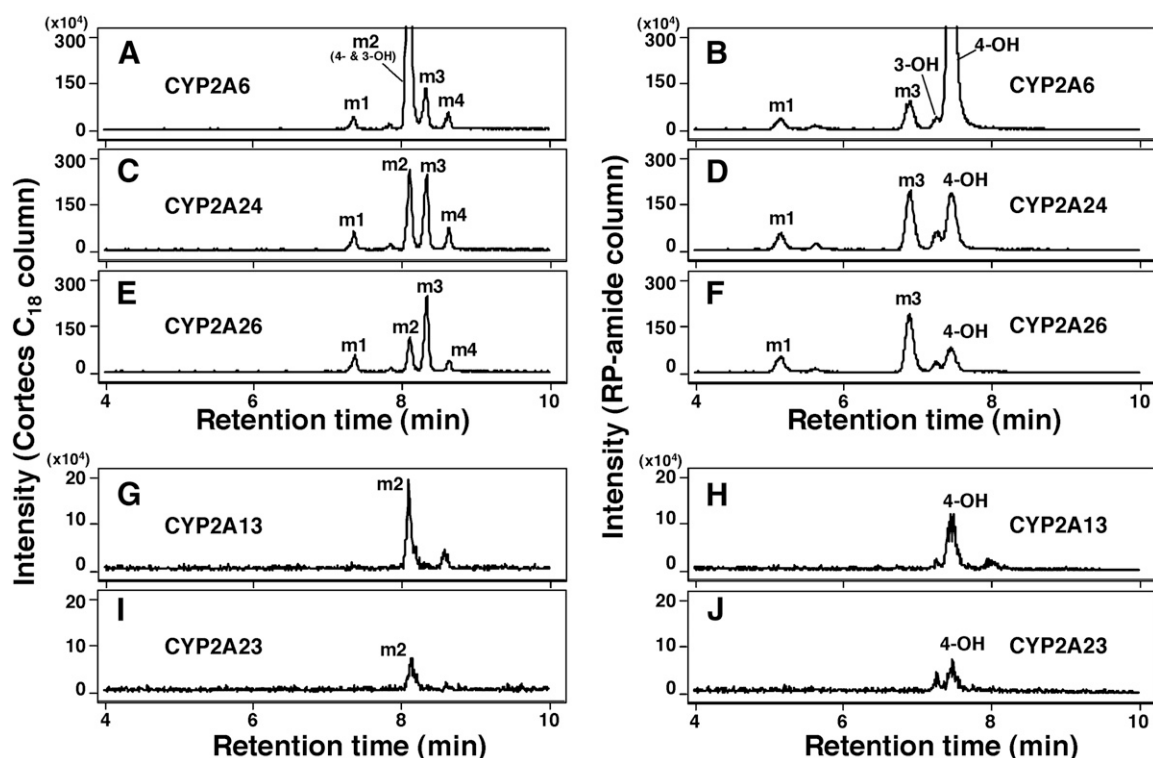
**Docking Simulations of Interactions of 2,5,2',5'-TCB with Human and Monkey CYP2A Enzymes.** We compared the molecular interactions of 2,5,2',5'-TCB with human and monkey CYP2A enzymes using docking simulation as described in *Materials and Methods* (Figs. 7). Several amino acid residues in substrate-recognition 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 used 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 was calculated to be 2.97 and 4.67  $\text{\AA}$  and 6.06 and 3.91  $\text{\AA}$ , respectively (Fig. 7). The distance between the C6 atom of TCB and the Fe in the heme of CYP2A13 was 4.83  $\text{\AA}$ . The position of Gly301 in CYP2A6 was somewhat different from that of Ala301 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, whereas C4 and C6 interacted with (the heme of) CYP2A24 (Fig. 7). 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 the 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 the 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 the 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 the 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., 2016a). 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 the 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 were 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,



**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 a CORTECS C<sub>18</sub> 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  $\mu$ M, respectively, and incubation time was 60 minutes.

and 2A26 using crystal structures of 1Z10 and 4EJJ for CYP2A6 and 2A24 and of 4EJG and 4EJH for CYP2A13, 2A23, and 2A26 with 12 ligands were also determined (Supplemental 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

TABLE 1  
Oxidation of 2,5,2',5'-TCB by human and monkey P450s

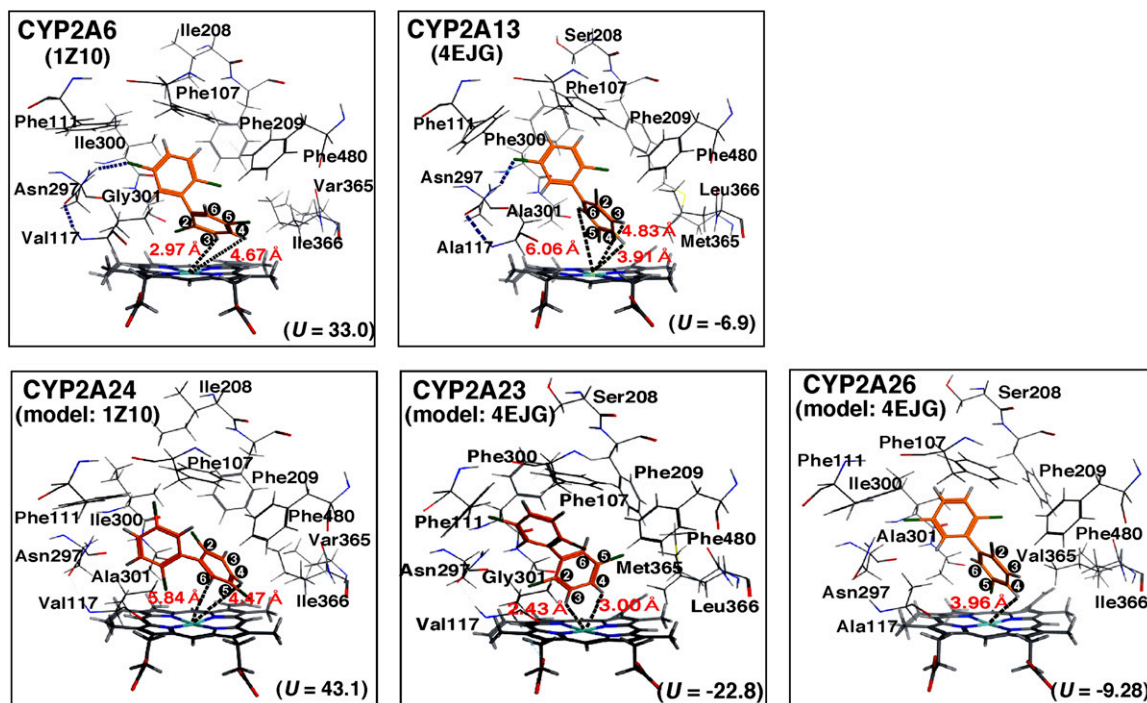
P450	Substrate Disappearance <sup>a</sup>	Product Formation		Ratio of 4-OH/3-OH
		4-OH-TCB	3-OH-TCB	
Human	% in 60 min	nmol/min/nmol P450	nmol/min/nmol P450	
CYP1A1 <sup>b</sup>	<1.0	<0.001	<0.0001	—
CYP1A2 <sup>c</sup>	<1.0	<0.001	<0.0001	—
CYP1B1*3 <sup>c</sup>	<1.0	<0.001	<0.0001	—
CYP2A6 <sup>c</sup>	42 ± 3.1	0.98 ± 0.11	0.029 ± 0.0032	34
CYP2A13 <sup>c</sup>	<1.0	0.017 ± 0.0041	0.0011 ± 0.003	15
CYP2B6 <sup>d</sup>	<1.0	<0.001	<0.0001	—
CYP2D6 <sup>d</sup>	<1.0	<0.001	<0.0001	—
CYP2E1 <sup>d</sup>	<1.0	<0.001	<0.0001	—
CYP2C9 <sup>c</sup>	<1.0	<0.001	<0.0001	—
CYP3A4 <sup>c</sup>	<1.0	<0.001	<0.0001	—
Monkey				
CYP2A23 <sup>c</sup>	<1.0	0.0076 ± 0.0011	0.0020 ± 0.0005	3.8
CYP2A24 <sup>c</sup>	24 ± 4.4	0.29 ± 0.032	0.042 ± 0.0048	6.9
CYP2A26 <sup>c</sup>	24 ± 3.1	0.13 ± 0.021	0.021 ± 0.0031	6.2

<sup>a</sup>GC-MS analysis of 2,5,2',5'-TCB after metabolism for 60 minutes. Results are expressed as the means ± S.D. (*n* = 3–5) (these values were not analyzed for statistical significance because a large number of different comparisons were made).

<sup>b</sup>A reconstituted system containing CYP1A1 and NADPH-P450 reductase.

<sup>c</sup>*E. coli* membranes coexpressing P450 and NADPH-P450 reductase.

<sup>d</sup>Baculo enzyme system expressing P450 and NADPH-P450 reductase.

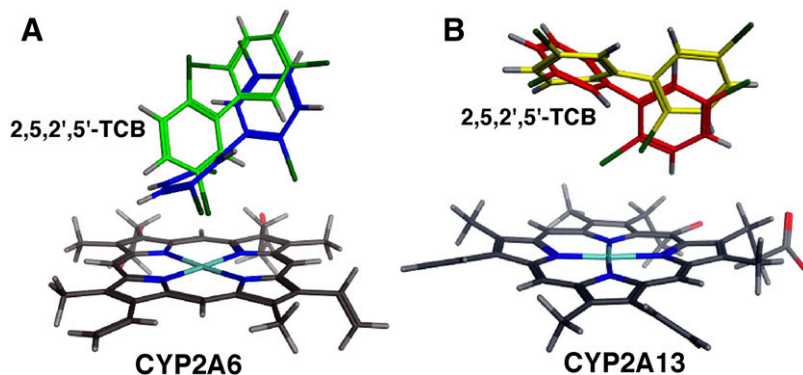


**Fig. 7.** Molecular docking simulation of interaction of 2,5,2',5'-TCB with human CYP2A6 and 2A13 and monkey CYP2A24, 2A23, and 2A26. Distance between chemical sites of TCB and the centers (Fe) of the heme of CYP2A enzymes are indicated in red. Positions of carbon atoms in 2,5,2',5'-TCB are shown in black.

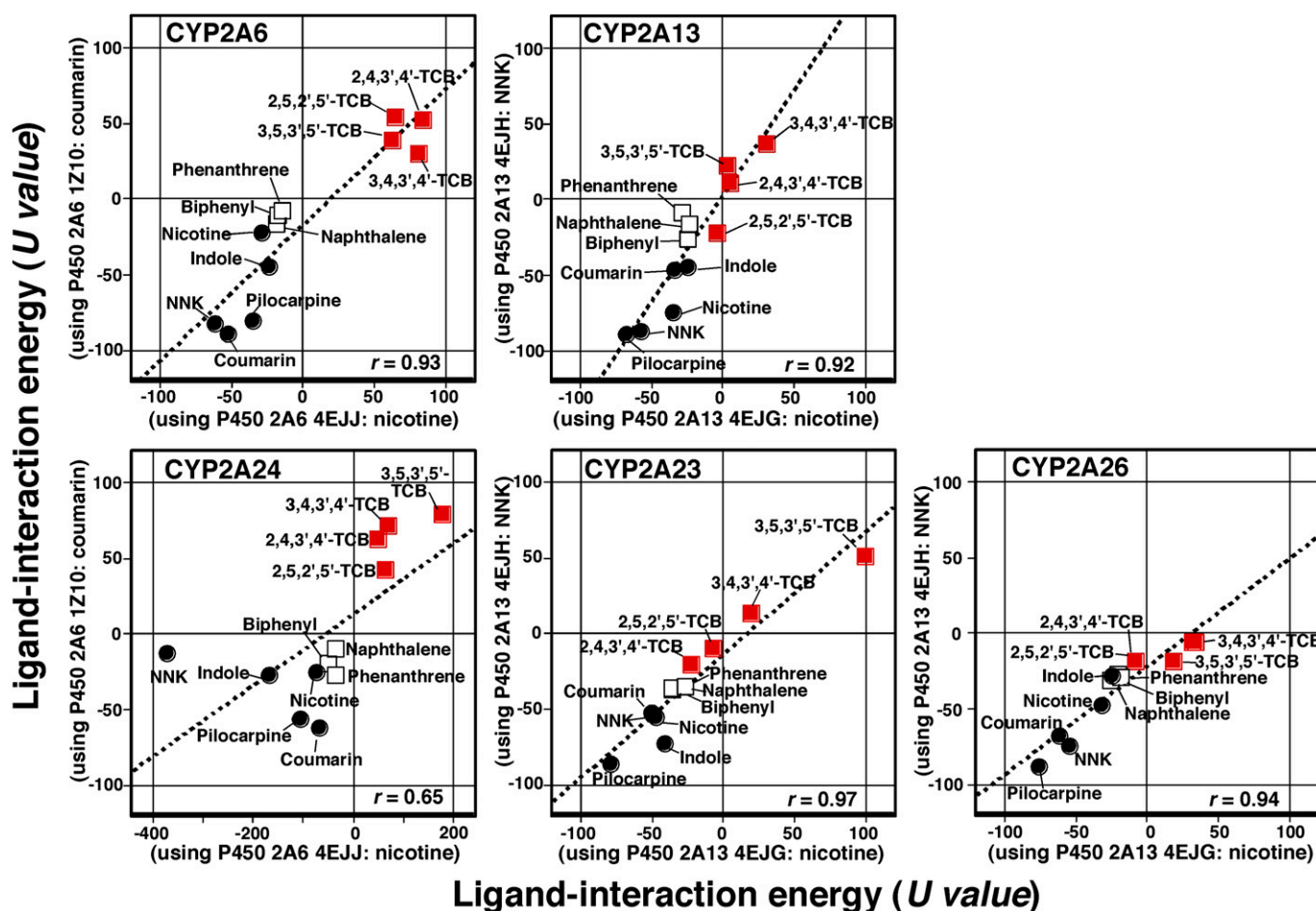
well as 2,5,2',5'-TCB. In addition, we found that monkey CYP2A24, which shows 95% amino acid identity to CYP2A6, catalyzed the oxidation of 2,5,2',5'-TCB to form 4-hydroxy- and 3-hydroxy-2,5,2',5'-TCB at rates of  $\sim 0.3 \text{ minute}^{-1}$  and  $\sim 0.04 \text{ minute}^{-1}$ , respectively. Another 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 previous 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; 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 the catalytic specificity of oxidation of ligands such as 2,5,2',5'-TCB (Supplemental Fig. 1).

Because human CYP2A6 and monkey CYP2A24 (and CYP2A26) were more active in catalyzing the oxidation of 2,5,2',5'-TCB than CYP2A13 and 2A23, we compared amino acid differences in SRSs (Gotoh, 1992) in these human and monkey CYP2A enzymes (Fig. 10). The differences in amino acids 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 CYP2A13/2A23, 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 (He et al., 2004; Sansen et al., 2007; DeVore et al., 2009, 2012; DeVore and Scott, 2012). 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 Gly207 has been



**Fig. 8.** Docking simulation of 2,5,2',5'-TCB (shown in green and blue) to CYP2A6 1Z10 (coumarin-bound form) and CYP2A6 4EJG (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) and CYP2A13 4EJG (nicotine-bound form) and CYP2A13 4EJH (NNK-bound form). Typical CYP2A substrates (coumarin, nicotine, pilocarpine, indole, and NNK) and naphthalene, phenanthrene, and biphenyl derivatives used in our recent paper (Shimada et al., 2016a) 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.

reported in mouse coumarin hydroxylation catalyzed by P450<sub>coh</sub> (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., 1995a,b), and this reaction is catalyzed by CYP2B1 and 2B2 in vitro (Preston et al., 1983; Ishida et al., 1991; Matsusue et al., 1996). Guinea pig CYP2B18, hamster P450 4-hydroxy-1-(3-pyridyl)-1-butanone-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,b, 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 minute<sup>-1</sup> (Koga et al., 1996); this study found that the turnover number is similar to that catalyzed by human CYP2A13 (0.017 minute<sup>-1</sup>) but not that catalyzed by CYP2A6 (~1.0 minute<sup>-1</sup>). 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, with  $K_s$  values of 9.4 and 0.51  $\mu$ M, respectively, although the former enzyme catalyzed oxidation of 2,5,2',5'-TCB at a 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 and Waller (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. (1983) found that



SRS Amino acid P450	SRS-1					Dif	SRS-2			Dif	SRS-3			Dif
	107	110	111	117			208	209			238	239	240	
CYP2A6	GRGEQ	ATFDW	VFKGY	GVVFS	NG	0/22	LRMML	GIF	0/8	AFQLL	QGL	0/8		
CYP2A24	GRGEQ	ATFDW	VFKGY	GVVFS	NG	0/22	LGMML	AIF	2/8	AFKEL	QGL	2/8		
CYP2A26	GRGEQ	ATFNW	VFKGY	GVAFS	NG	2/22	LRMML	GSF	1/8	AFKEL	QGL	2/8		
CYP2A13	GRGEQ	ATFDW	LFKGY	GVAFS	NG	2/22	LRMML	GSF	1/8	AFKEL	QGL	2/8		
CYP2A23	GRGEQ	ATFDW	LFKGY	GVVFS	NG	1/22	LRMML	GSF	1/8	AFKEL	QGL	2/8		
CYP2A8	DRGEQ	AFFDW	FFKGY	GVVFS	SG	4/22	LGMIM	RSF	5/8	AYKEM	QGL	4/8		

SRS Amino acid P450	SRS-4					Dif	SRS-5					Dif	SRS-6			Dif
	297	299	300	301	305		365	366	369	370	372		478	480		
CYP2A6	NLVMT	TLNLF	IGGTE	TVST		0/19	GDVIP	MSLAR	R	0/11	PKHVG	FAT	0/8			
CYP2A24	NLMMT	TLNLF	IAGTE	TVST		2/19	GDVIP	MSLAR	R	0/11	PKHAG	FAT	1/8			
CYP2A26	NLLMT	ALNLF	IAGTE	TVST		3/19	GNVIP	MSLAR	R	1/11	PKHVG	FAT	0/8			
CYP2A13	NLVMT	TLNLF	FAGTE	TVST		2/19	GDMLP	MGLAH	R	4/11	PKHVG	FAT	0/8			
CYP2A23	NLVL T	SLNLF	FGGTE	TVST		3/19	GDMLP	FGVAH	R	5/11	PKHMG	FAT	1/8			
CYP2A8	NLLMT	TLNLF	FAGTE	TVST		3/19	VDVVP	LGLPR	S	6/11	PQYFS	FAA	5/8			

**Fig. 10.** Differences in amino acid sequences in SRS-1 through SRS-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.

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 a 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 detected in human blood and tissue samples (McFarland and Clarke, 1989; Konishi et al., 2006; International Agency for Research on Cancer, 2015). Non-Aroclor or nonlegacy PCB contaminants have been determined in the environments of homes and cities and have accumulated in the bodies of exposed populations in various cities in the United States (Sun et al., 2006; Martinez et al., 2010; Hu et al., 2012; Koh et al., 2015). 2,4,3',4'-TCB has been reported to be one of the most persistent TCB isomers in biologic samples and human milk and can be detected in food samples such as fish and other meats, eggs, and bovine milk (Akutsu et al., 2005; Konishi et al., 2006; Todaka et al., 2008). Yoshimura et al. (1973) reported that 2,4,3',4'-TCB is metabolized in vivo in rats to 4-hydroxy-2,5,3',4'-TCB, which is possibly formed through a 4,5-oxide followed by an NIH-shift of a chlorine atom from the 4 position (Koga et al., 1992), 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 were detected using the determined human and monkey P450 enzymes. 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 (Yamazaki et al., 2011; Inui et al. 2014).

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 and 0.042 minute<sup>-1</sup>, 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 Contributions

*Participated in research design:* Shimada, Kakimoto, Murayama, Takenaka.

*Conducted experiments:* Shimada, Kakimoto, Murayama.

*Contributed new reagents or analytic tools:* Koga, Uehara, Kim.

*Performed data analysis:* Shimada, Kakimoto, Takenaka, Murayama, Uehara, Yamazaki.

*Wrote or contributed to the writing of the manuscript:* Shimada, Yamazaki, Guengerich, Komori.

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**Address correspondence to:** Dr. Tsutomu Shimada, Laboratory of Cellular and Molecular Biology, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-58 Rinku-Orai-Kita, Izumisano, Osaka 598-8531, Japan.  
E-mail: t.shimada@vet.osakafu-u.ac.jp

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