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Determination of the human cytochrome P450 monooxygenase catalyzing the enantioselective oxidation of 2,2′,3,5′,6-pentachlorobiphenyl (PCB 95) and 2,2′,3,4,4′,5′,6-heptachlorobiphenyl (PCB 183)

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Abstract 2,2′,3,5′,6-Pentachlorobiphenyl (PCB 95) and 2,2′,3,4,4′,5′,6-heptachlorobiphenyl (PCB 183) possess axial chirality and form the aS and aR enantiomers. The enantiomers of these congeners have been reported to accumulate in the human body enantioselectively via unknown mechanisms. In this study, we determined the cytochrome P450 (CYP) monooxygenase responsible for the enantioselective oxidization of PCB 95 and PCB 183, using a recombinant human CYP monooxygenase. We evaluated 13 CYP monooxygenases, namely CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C19, CYP2E1, CYP2J2, CYP3A4, CYP3A5, CYP4F2, and aromatase (CYP19), and revealed that CYP2A6 preferably oxidizes aS-PCB 95 enantioselectively; however, it did not oxidize PCB 183. The enantiomer composition was elevated from 0.5 (racemate) to 0.54. In addition, following incubation with CYP2A6, the enantiomer fraction (EF) of PCB 95 demonstrated a time-dependent increase.

Keywords Enantioselective oxidation · 2,2′,3,5′,6-pentachlorobiphenyl · 2,2′,3,4,4′,5′,6-heptachlorobiphenyl · Cytochrome P450 2A6 · Enantioselective analysis · Enantiomer

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Introduction

Polychlorinated biphenyls (PCBs) are a class of persistent organic pollutants, theoretically consisting of 209 possible congeners (Mills et al. 2007). They were mass produced and used in items such as transformers, capacitors, and dielectric fluids, but were banned globally in the early 1980s. Theoretically, 78 of the 209 PCB congeners have rotational isomers. Under environmental and physiological conditions, 19 congeners that are chlorine-substituted in 3- or 4-ortho position are chiral and have configurational aS and aR forms called enantiomers (Lehmler and Robertson 2001; Toda et al. 2012). The physical properties of enantiomers, such as density, melting and boiling points, refractive index, and thermal conductivity, are identical. However, the physiological behavior of enantiomers is known to be different, since functional biomolecules (such as enzymes) can distinguish between their three-dimensional (3-D) structures precisely, leading to differences in pharmacological effects and toxicity. This results in the phenomenon of enantiomeric enrichment. The enantiomer composition ratio is expressed as the enantiomer fraction (EF) value, which is calculated as the ratio of one enantiomer to the sum of both enantiomers (Hoekstra et al. 2002). An EF value of 0.5 indicates that the enantiomers exist as racemates (Harner et al. 2000).

PCBs are present in technical products such as Kanechlor and Aroclor, which contain these enantiomers in the same proportion, and hence, their EF value is 0.5. Enantiomeric enrichment occurs in biota through the process of environmental distribution. In fact, Kania-Korwel et al. proposed that

enantioselective accumulation occurred in various environmental media such as sediments, fish, birds, and mammals (Kania-Korwel and Lehmler 2015). We previously reported that in human breast milk, the time-dependent change in the enantiomer composition of chiral PCB congeners was a good biomarker (Konishi et al. 2016). In addition, we also studied the time-dependent changes in the EF value in fish, which is the major source of human exposure to PCBs. We successfully performed an enantioselective analysis for the highest accumulated chiral congener, namely 2,2′,3,4,4′,5′,6-heptachlorobiphenyl (PCB 183), without any interference. From 1982 to 2012, although the EF value of PCB 183 in the fish samples remained close to that in the racemate (0.5), the composition of aR-(+)-PCB 183 increased in the human breast milk (Konishi et al. 2016). The same trend was also observed in breast milk samples from Spain (Bordajandi et al. 2008). Therefore, enantiomeric enrichment may be potentially attributed to certain physiological processes, including biotransformation, transportation, protein binding, absorption, and excretion, occurring in the human body. Previously, it was believed that PCB 183 could not be metabolized and persisted in organisms because of its high lipophilicity (Warner et al. 2009). However, our results indicated that PCB 183 was enantioselectively metabolized and eliminated via specific biological pathways.

2,2′,3,5′,6-Pentachlorobiphenyl (PCB 95) is also reported to be one of the chiral congeners. In the 1970s, Sundström and Jansson compared the metabolic pathway for PCB 95 among the rats, mice, and quail. It was observed that meta oxidation preferentially occurred in rodents, but in birds, para oxidation was preferred. Thus, the nature of metabolism was diverse and depended on the class of organism (Sundström and Jansson 1975). Recently, the metabolism and effects of these metabolites on the nervous system, depending on their chirality, are actively being studied. Stamou et al. showed that PCB 95 altered hepatic cytochrome P450 (CYPs) monooxygenases properties, but did not change the enantiomer composition of PCB 95 in the rat brain (Stamou et al. 2015). Warner et al. oxidized several chiral PCBs, including PCB 95 and 183, using rat CYP2B1 and human CYP2B6. It was observed that PCB 95 was hydroxylated by the rat CYP2B1, but not by the human CYP2B6. Further, the enantiomeric composition of PCB 183 was not changed after reaction with both the CYPs (Warner et al. 2009). Lu et al. showed that PCB 95 was enantioselectively oxidized by the rat CYP2B1 and formed 4-OH-PCB 95 (Lu et al. 2013). Upon incubation with the human liver microsomes, PCB 95 also formed 4′-OH-PCB 95 (Uwimana et al. 2016). These observations indicated that the metabolic reactions catalyzed by CYPs are different between rats and humans. Additionally, in humans, the CYP isoform that catalyzes PCB 95 oxidation has not been determined.

PCB metabolites are known to have adverse effects on the human nervous system, especially when members of the non-coplanar class of PCBs are metabolized and biotransformed to form hydroxylated or methoxylated PCBs (Soechitram et al. 2004; Park et al. 2006; Kania-Korwel and Lehmler 2016). PCBs which are chlorine-substituted at the 2,3,6- positions in one ring affect the calcium channels in brain and disrupt the functioning of the ryanodine receptor (RyR) (Pessah et al. 2006). Both PCB 95 and PCB 183 are the congeners that meet the above criterion, and hence, they have the potential to act as neurotoxic agents. Generally, different receptors recognize different enantiomers. Consequently, the disrupting effect of different PCB enantiomers on RyR is thought to be different. Therefore, it is necessary to understand the detailed mechanisms underlying enantiomeric enrichment mediated by metabolic processes, and the toxic effects caused by biased EF values. In particular, the role of CYPs in enantioselective metabolism is important because they catalyze the first step (oxidization) in xenobiotic metabolism. Herein, we determined the CYP isoform that catalyzes the enantioselective oxidization of PCB 95 and PCB 183.

Materials and methods

Materials

PCB 95 and PCB 183 were purchased from AccuStandard (CT, USA). The pooled human S9 fraction, CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C19, CYP2E1, CYP2J2, CYP3A4, CYP3A5, CYP4F2, aromatase (CYP19), rat CYP2B1, and control microsomes (as the negative control) were purchased from Corning (NY, USA). Nicotinamide adenine dinucleotide phosphate (NADPH) regeneration system [solution A: 26.1 mM NADP+, 66 mM glucose-6-phosphate, and 66 mM magnesium chloride ($MgCl_2$); solution B: 40 U/mL glucose-6-phosphate dehydrogenase and 5 mM sodium citrate; final concentration used was as per the manufacturer's instructions] was also purchased from Corning (NY, USA). The rat S9 fraction was obtained from Kikkoman (Tokyo, Japan). Other reagents used were of the highest grade available and obtained from Wako Pure Chemical (Osaka, Japan).

Oxidation of PCBs by human CYP isoforms and S9

PCB oxidation by human CYP isoforms and S9 was evaluated according to the method described by Shimada et al. (2016). Briefly, the reaction mixture containing 0.1 M potassium phosphate buffer (pH 7.4), NADPH regeneration system, and 40 nM PCB 95 or 0.4 nM PCB 183 dissolved in dimethyl sulfoxide (DMSO) was pre-incubated at 37 °C. Oxidation was initiated by adding the recombinant P450 enzyme. After

incubation at 37 °C, the reaction was terminated by adding an equal volume of ice-cold methanol. The residual PCBs were extracted twice with two volumes of an ethyl acetate–chloroform mixture (1:1, *v*/v). The supernatants were directly analyzed by gas chromatography coupled with tandem mass spectrometry (GC–MS/MS). Additionally, oxidation with control microsomes was also performed. Each reaction was conducted in triplicates, except those involving S9 s with PCB 183 ($n = 1$).

GC–MS/MS analysis

The analysis was performed according to the methods reported previously (Toda et al. 2012; Konishi et al. 2016) with slight modifications. The GC–MS/MS analysis was performed using a gas chromatography system, coupled to a triple-stage quadrupole mass spectrometer (Agilent 7010; Agilent Technologies, CA, USA), and connected to a capillary column. PCB 95 was analyzed using the CP-Chirasil-DEX CB capillary column (i.d., 25 m × 0.25 mm; film thickness, 0.25 μm; Agilent). The BGB-172 column (i.d., 30 m × 0.25 mm; film thickness, 0.25 μm; BGB Analytik AG, Switzerland) was used for analyzing PCB 183. Helium was used as the carrier gas (flow rate, 1 mL/min). The injection temperature and volume were 280 °C and 1 μL, respectively. For mass analysis of the pentachlorobiphenyls (*m/z*, 323.9 > 253.7) and heptachlorobiphenyls (*m/z*, 393.8 > 323.9), electron ionization and selected reaction monitoring (SRM) mode (positive) were used. The ion source temperature and ionization voltage were 250 °C and 38 eV, respectively. The temperature program for PCB 95 analysis was 140 °C for 1 min, 140 to 180 °C at 20 °C/min, and hold at 180 °C for 35 min (total run time, 38 min) and that for PCB 183 analysis was 120 °C for 2 min, 120 to 250 °C at 20 °C/min, and hold at 250 °C for 5 min (total run time, 72 min).

Data analysis

Determination of enantioselective oxidation was performed by calculating the EF value using the following formula: EF = area of first eluent peak/sum of areas of both enantiomers. Stasistical analysis was conducted by Student's *t*-test.

Results and discussion

To determine the CYP isoforms that catalyzed the enantioselective oxidation of PCB 95 and PCB 183, the following 13 CYPs were evaluated: CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C19, CYP2E1, CYP2J2, CYP3A4, CYP3A5, CYP4F2, and CYP19. The representative chromatographs for PCB 95 analysis after a 3-h incubation with CYPs (40 pmol/mL each) are shown in Fig. 1. Under the aforementioned conditions, we identified the first and second eluents as the aR and aS forms, respectively (data not shown). The EF values obtained after a 3-h oxidization by the human CYP isoforms, rat CYP2B1, and S9 fractions are shown in Fig. 2.

As shown in Fig. 2, we observed that human CYP2A6 significantly increased the EF value of PCB 95 ($p < 0.01$), similar to that observed in the study on human breast milk

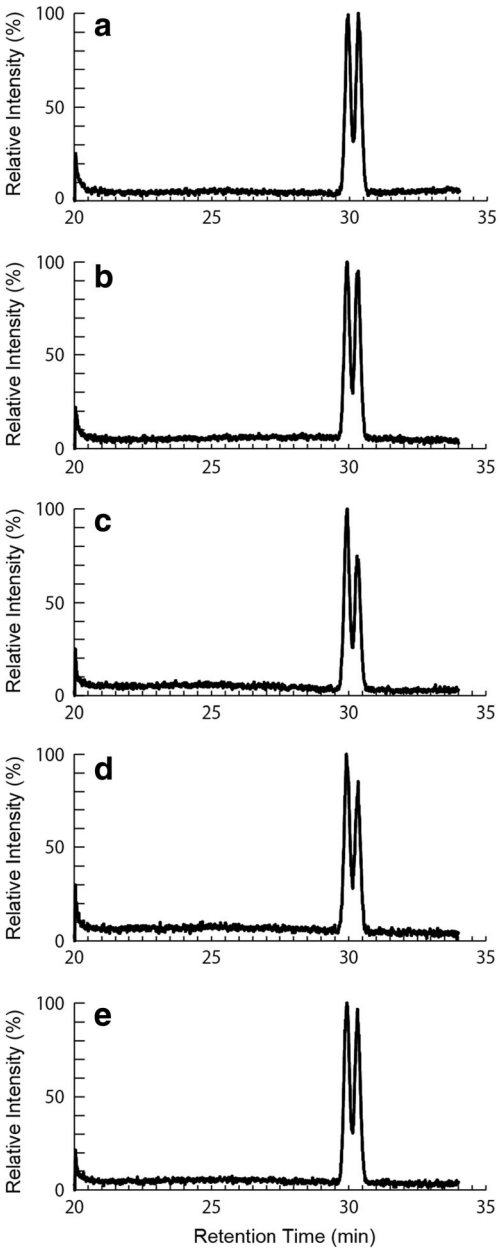

Fig. 1 Representative chromatographs for PCB 95 after treatment with control microsomes [lacking cytochrome P450s (CYPs)] (**a**), human S9 (**b**), human CYP2A6 (**c**), rat S9 (**d**), and rat CYP2B1 (**e**). The first and second peaks were determined to be aR-PCB 95 and aS-PCB 95, respectively. Relative peak height was reported (where the height of the highest peak was considered to be 100%)

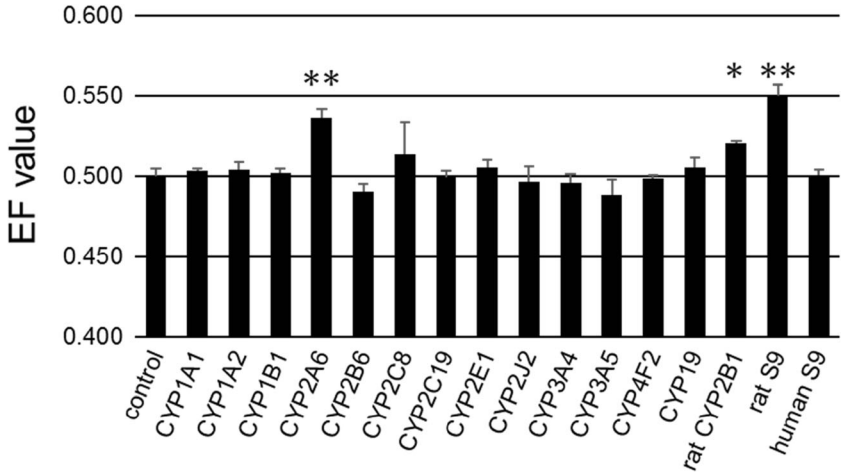

Fig. 2 Changes in enantiomer fraction (EF) value of PCB 95 after a 3-h treatment with 13 human CYP isoforms, rat CYP2B1 (40 pmol/mL each), or S9s (1 mg/mL). EF values were calculated using the following formula: EF = area of first eluent peak/sum of areas of both enantiomers. When the EF value was equal to 0.5, both enantiomers were considered to be present at the same concentration in the solution (racemate). All experiments were conducted in triplicates. Error bars indicate standard deviations. Asterisk indicates statistical significance calculated using the Student's *t* test (*indicates $p < 0.05$ and **indicates $p < 0.01$)

(Bordajandi et al. 2008; Konishi et al. 2016). Likewise, similar to previous studies (Warner et al. 2009; Lu et al. 2013), rat CYP2B1 also significantly increased the EF value of PCB 95 ($p < 0.05$); however, this increase was not more than that observed for human CYP2A6. In case of the S9 fraction, only rat S9 increased the EF value of PCB 95 ($p < 0.01$). Rat S9 was prepared from the rat liver pretreated with a carcinogenic polycyclic aromatic hydrocarbon, benzo[a]pyrene, and a representative pharmaceutical compound, phenobarbital, to induce the liver enzymes that in turn induced all drug-metabolizing enzymes including those belonging to the CYP1, CYP2, and CYP3 families. Although no data was available for the CYP isoforms, the CYP2 family may potentially catalyze the oxidation of PCB 95. Although human CYP2A6 catalyzed the oxidation of PCB 95, no change was observed in the EF values of PCB 95 exposed to human S9. We compared the enzyme activities between recombinant CYP2A6 and human S9 by the data sheet obtained from the manufacture. The coumarin-7-hydroxylation activity of CYP2A6 in human S9 is reported to be 250 pmol/(mg protein × min). Compared to the recombinant CYP2A6 (38 pmol/[pmol CYP2A6 × min]) used in this study, the activity of CYP2A6 in human S9 was approximately 6 times lower. Thus, human S9 did not express enough CYP2A6 activity to alter the EF value of PCB 95. The CYP2A6 concentration- and time-dependent changes in the EF value of PCB 95 metabolized by CYP2A6 are shown in Fig. 3a, b, respectively. While the final concentration of P450 ranged from 10 to 80 pmol/mL, the EF values remained stable at ~ 0.56. The EF values gradually increased in a time-dependent manner (up to 3 h), but the increase was not significant. We also measured the absolute amount of residual PCB 95, and observed that it gradually decreased with time. If the rate of oxidation between the enantiomers was different, the EF value might continue to increase and the difference in residual amount of each enantiomer might also increase in a time-dependent manner. In this study, the EF value of PCB 95

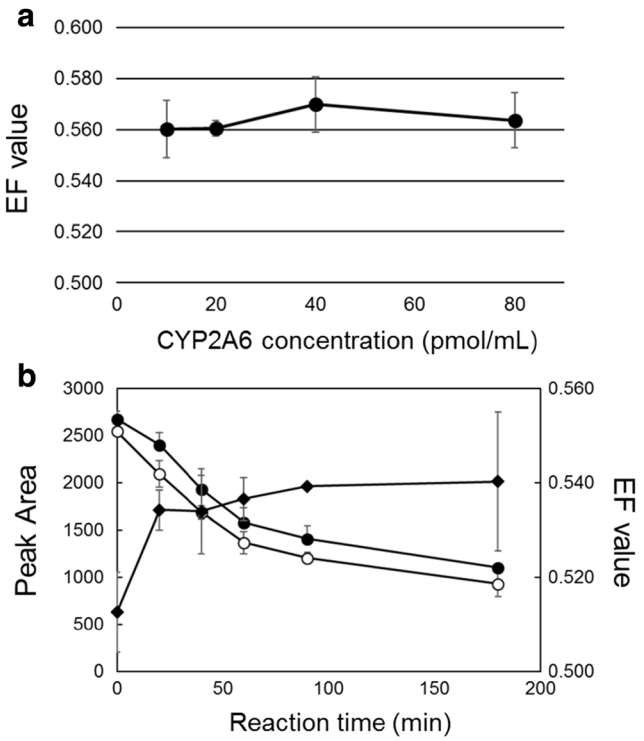

Fig. 3 P450 concentration-dependent (**a**) and time-dependent (**b**) changes in the enantiomer fraction (EF) values, after treatment of PCB 95 with human cytochrome P450 (CYP) 2A6. **b** Each plot indicates the peak area of aR-PCB 95 (black circle), aS-PCB 95 (white circle), and EF value (black diamond shape). All experiments were conducted in triplicates and error bar shows standard deviation

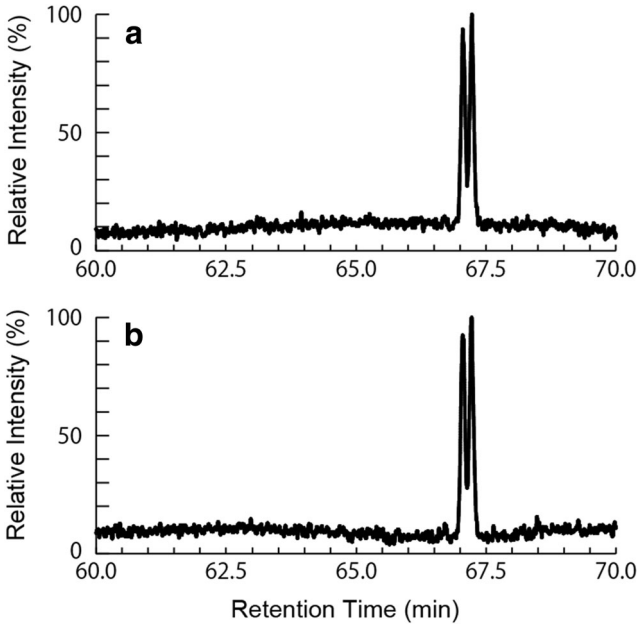

Fig. 4 Representative chromatograph for PCB 183 after treatment with human CYP2B6 (**a**) and human liver S9 fraction (**b**). The first and second peaks were determined to be aS-(+) PCB 183 and aR-(+)-PCB 183, respectively

reached a plateau phase after 20 min, and further oxidization was continued at the same rate. Further, the differences in the binding ability to the CYP2A6 cavity and the rate of oxidization among the PCB 95 enantiomers need to be elucidated. We observed that aS-PCB 95 was preferentially oxidized both in the rat and human samples, and the main subtype mediating this effect in human samples was CYP2A6. To the best of our knowledge, this is the first report which identified the specific CYP isoform responsible for causing enantiomeric enrichment of PCB 95 in the human body.

The representative chromatographs for PCB 183 analysis after a 3-h incubation with CYPs (40 pmol/mL each) are shown in Fig. 4. Under the aforementioned conditions, the first and second eluents were identified as the aS-(+) PCB 183 and the aR-(−)-PCB 183 forms, respectively (Toda et al. 2012). The EF values after 3-h oxidization by the human CYP isoforms and S9 s are shown in Fig. 5. Under these experimental conditions, we observed no changes in the EF value or degradation of the residual PCB 183, indicating that the tested isoforms did not induce oxidation. The EF value of CYP1B1 was observed to decrease. However, the peak area and peak height data indicated that the enantioselective degradation of PCB 183 was not mediated by CYP1B1. Since PCB 183 is a highly lipophilic congener, we hypothesized that it would not dissolve in the water-based reaction mixture. Therefore, we maintained its concentration as low as possible, at which peaks could be determined by GC–MS/MS; however, no changes in peak areas were observed. PCB 183 accumulation in the human body is limited (Hirai et al. 2005), and therefore, reports focusing on this molecule are also less. Furthermore, due to its high lipophilicity, PCB 183 is believed to remain stable in the human body and not get metabolized. Thus, reports related to the metabolism and toxicity of PCB 183 are not available. In our previous study, we observed that specific biological processes may cause enantiomeric enrichment of PCBs in human breast milk (Konishi et al. 2016). Hence, alternate mechanisms for biotransformation, such as binding to transporter proteins and cell membrane permeability, should also be considered. Furthermore, using computer simulations to elucidate the CYP isoforms with potential binding capacity for PCB 183 might also be useful.

Lower chlorinated PCBs are considered to be oxidized by the CYP2B and CYP3A isoforms in rodents, and the non-ortho PCBs (co-planar PCBs) are known to be metabolized by the CYP1 subtypes and dioxins (Grimm et al. 2015). In this study, we identified CYP2A6 as the enzyme responsible for catalyzing the oxidization of PCB 95. CYP2A6 is a well-known monooxygenase associated with the activation of precarcinogens, such as nitrosamine in tobacco smoke

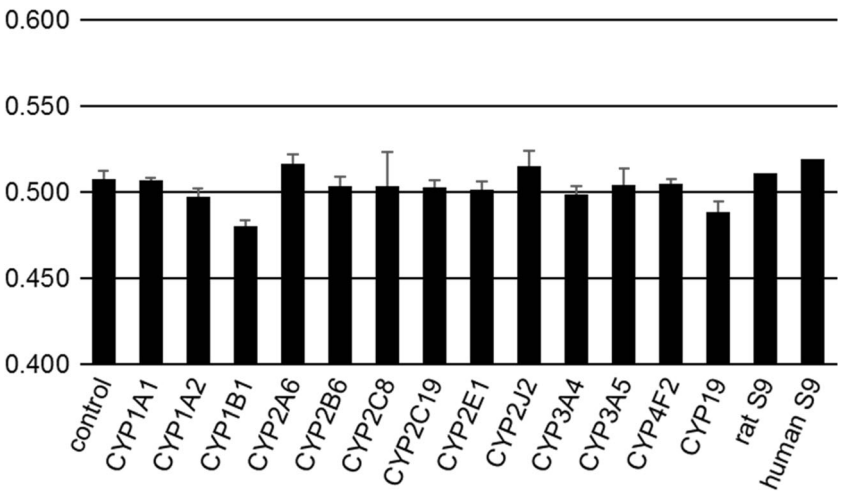

Fig. 5 Changes in the enantiomer fraction (EF) values of PCB 183 after a 3-h treatment with human CYP isoforms (40 pmol/mL, respectively) or S9s (1 mg/mL). The EF values were calculated using the following formula: EF = area of first eluent peak/sum of areas of both enantiomers. An EF value equal to 0.5 indicated that both enantiomers were present at the same concentration in the solution (racemate). All experiments were conducted in triplicates, except those involving S9s (n = 1) and error bar shows standard deviation

(Raunio and Rahnasto-Rilla 2012). Furthermore, it has been reported that it also oxidizes 2,2′,5,5′-tetrachlorobiphenyl (PCB 52) (Shimada et al. 2016) and 2,2′,4,5,5′-pentachlorobiphenyl (PCB 101) (McGraw and Waller 2006). Shimada reported that CYP2A6 preferentially catalyzes the PCB congeners with chlorine at the 2- and 5-positions on one benzene ring. One benzene ring of PCB 95 is also substituted by chlorine at the 2- and 5-positions. Thus, PCB 95 was metabolized by CYP2A6 (Shimada et al. 2016). In rats, the CYPs that are homologous to the human CYP2A6 have not been identified, indicating that the CYPs catalyzing PCB 95 oxidation could be different in rats. According to some reports, human CYP2A6 and rat CYP2B1 catalyze the oxidation of some terpenes such as verbenone, a component of the essential oil from rosemary species, by a similar mechanism (Miyazawa et al. 2003; Miyazawa and Gyoubu 2007). Thus, despite lacking sequence homology, human CYP2A6 and rat CYP2B1 recognize some common substrates.

PCB 95 mainly causes neurotoxicity in humans. When the fetus is exposed during its development to PCB 95, an immature nervous system and behavioral deficiencies are observed (Pessah et al. 2010). High levels of PCB 95 have been observed in the brains of individuals with immature nervous systems (Mitchell et al. 2012). PCB 95 and its hydroxylated metabolites are good ligands for RyR 1 and 2, which control the homeostasis of calcium ions in the brain and muscle cells, and induce unintended calcium release in these tissues (Pessah et al. 2010). It has been reported that only the (+) form of another chiral congener, PCB 136 (2,2′,3,3′,6,6′-hexachlorobiphenyl), functions as a ligand for RyR (Pessah et al. 2009). For the PCB 95 enantiomers, further investigations are necessary to reveal the different metabolic products formed by post-catalysis, their reactivity with CYP2A6, and the mechanism by which they affect RyR activation and the nervous system in humans.

In conclusion, we attempted to elucidate the critical CYP enzymes that catalyze the enantiomeric enrichment of PCB 95 and PCB 183. We observed that CYP2A6 predominantly oxidized aS-PCB 95. In future, we shall attempt to understand the metabolites produced by CYP2A6-mediated catalysis of PCBs and the enantiomer-specific toxic effects induced on the human nervous system.

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