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Enantioselective metabolism of chiral polychlorinated biphenyl 2,2',3,4,4',5',6-Heptachlorobiphenyl (CB183) by human and rat CYP2B subfamilies

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1 ABSTRACT

2 Chiral polychlorinated biphenyls (PCBs) have atropisomers that have different axial 3 chiralities and exist as racemic mixtures. However, biochemical processes often result in 4 the unequal accumulation of these atropisomers in organisms. This phenomenon leads to 5 enantiospecific toxicity enhancement or reduction because either of the atropisomers 6 mainly affects toxicity expression. Enantioselective accumulation is caused by 7 cytochrome P450 (CYP, P450) monooxygenases, especially the CYP2B subfamilies. 8 Therefore, this study investigates the metabolism of a chiral PCB in vitro. Both 9 atropisomers isolated from racemic 2,2',3,4,4',5',6-heptachlorobiphenyl (CB183) were 10 metabolized by human CYP2B6, but not rat CYP2B1. This may be due to the difference 11 in the size of the substrate-binding cavities of CYP2B6 and CYP2B1. The stable 12 accommodation of (-)-CB183 in the cavity without any steric hindrance explained the 13 preferential metabolism of (-)-CB183 compared to (+)-CB183. Two hydroxylated 14 metabolites, 3'-OH-CB183 and 5-OH-CB183, were identified. The docking study showed 15 that the 3'-position of the trichlorophenyl ring closely approaches the heme of CYP2B6. 16 To our knowledge, this is the first study to elucidate the structural basis of chiral PCB 17 metabolism by P450 isozymes. These results will help promote the precise toxicity 18 evaluation of chiral PCBs and provide an explanation of the structural basis of chiral PCB 19 metabolism.

20

21 Keywords: atropisomer, chiral polychlorinated biphenyl, cytochrome P450
22 monooxygenase, enantioselective metabolism, CB183

23

24 1. Introduction

25 Polychlorinated biphenyls (PCBs) have been widely used in industrial products like 26 insulating oils for electrical machinery, transmitters, and condensers owing to their 27 incombustibility and insulation properties. PCBs are known as legacy persistent organic 28 pollutants (POPs) and continuous efforts are being made to eliminate their production and 29 usage. Yet, environmental contamination (Zhu et al., 2022) due to PCBs and instances of 30 their bioaccumulation in humans and wildlife are frequently reported (Quinete et al., 31 2014). Multiple adverse effects of PCBs on human health are known, such as 32 carcinogenicity, reproductive impairment, and developmental neurotoxicity. Of the 209 33 PCB congeners, 19 PCB congeners that have either three or four ortho chlorine 34 substituents display axial chirality and are called chiral PCBs. Generally, atropisomers of 35 chiral PCBs that are non-superimposable mirror images of each other exist as racemic 36 mixtures in the environment and have identical physical and chemical properties. 37 However, they show different metabolism, accumulation, and toxicity in vivo because of 38 their different three-dimensional structures. 2,2',3,4,4',5',6-Heptachlorobiphenyl 39 (CB183) was reported to accumulate enantioselectively in human breast milk and adipose 40 tissue, suggesting that the concentration of one atropisomer is lower in vivo (Bordajandi 41 et al., 2008; Konishi et al., 2016; Toda et al., 2012). Mammalian cytochrome P450 (CYP 42 or P450) monooxygenases are known to metabolize various PCB congeners. Rat 43 CYP1A1 metabolizes the most toxic PCB congener 3,3',4,4',5-pentachlorobiphenyl 44 (CB126) to hydroxylated metabolites (Yamazaki et al., 2011). Docking models of CB126 45 with CYP1A1 revealed that the 4-OH-3,3',4',5-tetrachlorobiphenyl metabolite is formed 46 because CB126 occupies the active site of CYP1A1 in such a manner that its 4-position is closest to the heme iron. In contrast, 2,3',4,4',5-pentachlorobiphenyl (CB118) is 47 48 metabolized by human and rat CYP2B subfamilies (Mise et al., 2016). Docking studies

49 showed that CB118 is positioned closer to the heme iron of human CYP2B6 compared to 50 that of rat CYP2B1. Therefore, human CYP2B6 produces higher amounts of hydroxylated metabolites. These studies suggest that the stable accommodation of PCBs 51 52 in the substrate-binding cavities of P450s and their vicinity to the heme iron are 53 responsible for the high PCB metabolism. The CYP2B subfamily has also been employed 54 for the enantioselective metabolism of chiral PCBs. While both rat CYP2B1 and human 55 CYP2B6 biotransformed 2,2',3,6-tetrachlorobiphenyl (CB45) to hydroxylated 56 metabolites, they showed a different atropisomer preference that reflected in the opposite 57 enantiomer fractions obtained for the two isozymes (Warner et al., 2009). Furthermore, 58 CYP2B-induced rat liver microsomes preferentially metabolized (+)-CB136 to (+)-5-59 OH-CB136 (Wu et al., 2011).

60 Enantioselective binding activities of 2,2',3,5',6-pentachlorobiphenyl (CB95) 61 atropisomers toward a ryanodine receptor (RyR) were also observed (Feng et al., 2017). 62 (-)-CB95 showed a higher binding activity toward RyR than its racemate, leading to 63 enantiomer-specific toxicity. The same phenomenon was observed in the case of (-)-64 2,2',3,3',6,6'-hexachlorobiphenyl (CB136), which acts as a sensitizer toward RyR and 65 causes neurodevelopmental toxicity (Yang et al., 2014). Therefore, it is important to 66 further explore the enantioselective metabolism of chiral PCBs by P450 isozymes, 67 especially by the CYP2B subfamily, since PCB toxicity is dependent on metabolism. Chiral CB183 is one of the most potent congeners to activate the RyR-Ca²⁺ channel 68 69 complex type 1 (Holland et al., 2017; Pessah et al., 2006). This receptor is regulating a 70 cellular signaling through calcium release. Therefore, clarification of its metabolic fate is 71 of great interest. It was reported that the serum, feces, and liver microsomes from CYP2B-72 induced rats showed the production of 3'-OH-CB183 and 5-OH-CB183 (Ohta et al., 2007, 73 2006). However, the enantioselective metabolism of CB183 and its structural basis have74 not been explored.

In this study, we show the enantioselective metabolism of CB183 atropisomers by human and rat CYP2B subfamilies *in vitro*. Separated atropisomers (Figure 1) were used as substrates of P450 isozymes in metabolism experiments. This study reveals several important aspects of the enantioselective metabolism of CB183, which will allow an adequate evaluation of its toxicity. Furthermore, docking models of CB183 atropisomers and the human CYP2B6 reveal the structural basis underlying its enantioselective metabolism.

82

83 2. Materials and methods

84 2.1. Chemicals

85 The two atropisomers were separated from racemic CB183 (AccuStandard, New 86 Haven, CT) by chiral HPLC on a CHIRALCEL® OJ-H column (Daicel Co., Osaka, 87 Japan) under the conditions reported previously (Toda et al., 2012). Each of the 88 atropisomers were dissolved in dimethyl sulfoxide to achieve a final concentration of 0.25 mM. ¹³C-labeled OH-PCBs (MHPCB-MXA: [¹³C₁₂]-4-Hydroxy-3',4'-dichlorobiphenyl, 89 ¹³C₁₂]-4-hydroxy-2',4',5'-trichlorobiphenyl, ¹³C₁₂]-4-hydroxy-2',3',4',5'-90 ¹³C₁₂]-4-hydroxy-2',3,4',5,5'-pentachlorobiphenyl, 91 tetrachlorobiphenyl, $[^{13}C_{12}]-4-$ ^{[13}C₁₂]-4-hydroxy-2,2',3,3',4',5,5'-92 hydroxy-2',3,3',4',5,5'-hexachlorobiphenyl, heptachlorobiphenyl, and [¹³C₁₂]-4-hydroxy-2,2',3,4',5,5',6-heptachlorobiphenyl), used 93 as internal standards, and $[^{13}C_{12}]$ -2,3',4',5-tetrachlorobiphenyl, used as the syringe spike, 94 95 were purchased from Wellington Laboratories (Guelph, Canada). The 3'-OH-CB183 and 96 5-OH-CB183 standards were kindly provided by Dr. T. Okumura (Environmental

97 Pollution Control Center, Osaka Prefecture, Osaka, Japan). Microsomes containing
98 NADPH-cytochrome P450 oxidoreductase (CPR) and cytochrome b₅, along with human
99 CYP2B6 or rat CYP2B1 that are heterologously produced in recombinant insect cells
100 were purchased from BD Biosciences (San Jose, CA, USA).

101 2.2. Measurement of hydroxylation activity of CYP2B subfamilies toward CB183

102 The reaction solution contained 2.5 µM (+)- or (-)-CB183, 80 nM human CYP2B6 or 103 rat CYP2B1, 3.3 mM MgCl₂, 100 mM potassium phosphate buffer (pH 7.4), NADPH 104 regenerating system (5 mM NADPH, 5 mM glucose-6-phosphate, 1 unit glucose-6-105 phosphate dehydrogenase) in a total volume of 0.5 mL. The reaction was initiated by 106 adding a microsomal fraction and was incubated for 120 min at 37 °C with continuous 107 shaking. Control experiments were performed using the same procedure without the 108 addition of NADPH. The reaction was terminated moving the tubes containing the reaction mixture on ice, and 20 µL of 50 ng/mL ¹³C-OH-PCB was added as an internal 109 110 standard. The metabolites were extracted twice with 2 mL of hexane and derivatized by 111 methylation as described previously (Sakiyama et al., 2007). Quantification and 112 identification of the metabolites (Table S1-S3 of Supplementary Information) were 113 performed by high-resolution gas chromatography and high-resolution mass 114 spectrometry (HRGC/HRMS: GC, 6890N [Agilent Technologies, Tokyo, Japan]; MS, 115 JMS-800D [JEOL Ltd., Tokyo, Japan]) equipped with an HT8-PCB column (Kanto 116 Chemical Co. Inc., Tokyo, Japan) using SIM modes under the conditions described 117 previously (Goto et al., 2018). The identification of retention times between metabolites and the authentic standards, and isotope ratios ([M+2]+:[M+4]+) of MeO-118 119 heptachlorobiphenyls were employed to identify OH-PCBs. Relative calibration curves 120 that are prepared by the division of the peak areas of different concentrations of 5-MeO-

121 CB183 by that of $[^{13}C_{12}]$ -4-MeO-2,2',3,4',5,5',6-heptachlorobiphenyl were used to 122 quantify M1 and M2. The recovery rates for metabolites were calculated by using an 123 internal standard and syringe spikes as 57–125% for hydroxylated heptachlorobiphenyls. 124 2.3. Molecular docking of CB183 in the substrate-binding site of human CYP2B6 125 Both atropisomers of CB183 were docked in the substrate-binding site of human 126 CYP2B6 using Surflex Dock in SYBYL 8.0 (Tripos, St Louis, MO) (Gay et al., 2010; 127 Ruppert et al., 1997). To determine a reasonable conformation of CB183, 13 crystal 128 structures of human CYP2B6 stored in the Protein Data Bank (PDB IDs: 3IBD, 3QOA, 129 3QU8, 3UA5, 4I91, 4RQL, 4RRT, 4ZV8, 5EM4, 5UAP, 5UDA, 5UEC, and 5UFG) were 130 superimposed.

- 131
- 132 3. Results and discussion

133 3.1. Detection and identification of CB183 metabolites

134 In microsomes containing human CYP2B6, two different NADPH-dependent 135 hydroxylated heptachloro metabolites (M1 and M2) were detected for both the 136 atropisomers by HRGC/HRMS (Figure 2B-E). In contrast, no metabolites were detected 137 in rat CYP2B1 for both isomers (Figure 2F-I). A possible reason for this could be the 138 different volumes of the substrate-binding cavities of rat CYP2B1 (472 Å) and human 139 CYP2B6 (559 Å) (Mise et al., 2016). This is an important factor when considering the 140 stable accommodation of substrates in the binding site, which is responsible for the 141 varying metabolizing activities of the different P450 isozymes. This suggests that the 142 binding cavity of rat CYP2B1 is too small to accommodate the heptachlorinated CB183. 143 However, rat CYP2B1 has been reported to metabolize hexachlorinated PCB congeners, 144 such as 2,2',3,3',4,6'-hexachlorobiphenyl (CB132) and CB136 to hydroxylated products, 145 which suggests that the binding site of rat CYP2B1 can only accommodate up to 146 hexachlorinated PCBs (Warner et al., 2009). Yet, in a contradictory report, 3'-OH-CB183 147 and 5-OH-CB183 were detected in the serum of rats intraperitoneally dosed with 148 phenobarbital as a CYP2B inducer, followed by CB183 (Ohta et al., 2007). This 149 contradiction can be attributed to the different experimental conditions as one study was 150 conducted in vitro and the other in vivo. CYP2B1 is a frequently used isoform among rat 151 CYP2B subfamilies for in vitro experiments. However, there are other CYP2B 152 subfamilies in rats, such as CYP2B2, CYP2B3, CYP2B12, CYP2B15, and CYP2B21.

Therefore, it is possible that CB183 is metabolized by other rat CYP2B subfamilies.

153

154 As the retention time of methylated M1 was consistent with that of the authentic 155 standard S1 (3'-MeO-CB183), M1 was identified as 3'-OH-CB183 (Table S2). Similarly, 156 M2 was identified as 5-OH-CB183. Their peaks also matched the isotope ratios $([M+2]^+:[M+4]^+)$ of MeO-heptachlorobiphenyl, indicating that the peaks represent 157 158 hydroxylated heptachloro compounds (Figure S1, Table S3). According to the 159 fragmentation patterns of the CB183 metabolites, M1 and M2 had larger peaks in [M+2-160 $COCH_3$ ⁺ than in [M+2-CH₃Cl]⁺ (Figure S2). The peak of [M+2-COCH₃]⁺ indicated that 161 the substituted hydroxyl group was located at the meta- or para-position (Kunisue and 162 Tanabe, 2009). This result was consistent with the identification of M1 as 3'-OH-CB183 163 and M2 as 5-OH-CB183. Further, CYP2B subfamilies preferentially hydroxylate at meta-164 position of PCBs whereas CYP1A1 preferentially hydroxylates at para-position (Mise et 165 al., 2016). 3'-OH-CB183 and 5-OH-CB183 were also detected in the serum of rats 166 injected with CB183 and the concentration of these metabolites increased due to the 167 presence of phenobarbital (Ohta et al., 2007). These reports support our observation that 168 CB183 can be metabolized to 3'-OH-CB183 and 5-OH-CB183 by the CYP2B subfamily.

169 *3.2. Production rates of hydroxylated CB183 by human CYP2B6*

170 Higher concentrations of metabolites were obtained in case of (-)-CB183 compared to 171 (+)-CB183, with the concentration of 3'-OH-CB183 being 5.5 times higher and that of 5-172 OH-CB183 being 4.3 times higher (Figure 3). The production rates of 3'-OH-CB183 from 173 (+)-CB183 and (-)-CB183 were 2.7 and 3.5 times higher than those of 5-OH-CB183, 174 respectively. The proposed metabolic pathways of CB183 are shown in Figure 4. It has 175 been reported that (+)-CB183 is more abundant than (-)-CB183 in human breast milk, 176 while a racemic CB183 is present in fish, which is a major source of PCB intake (Konishi 177 et al., 2016). These results suggest that CYP2B6 is related to the metabolism of (-)-178 CB183 in the human body. In contrast, it was reported that human CYP2B6 did not show 179 enantioselective metabolism of racemic CB183 (Nagayoshi et al., 2018; Warner et al., 180 2009). This suggests that atropisomers influence each other's metabolism. This 181 hypothesis is supported by the study in which (-)-CB132 inhibited the biotransformation of (+)-CB132 in the racemic mixture owing to the difference in the binding affinity of 182 183 each atropisomer toward rat CYP2B1 (Lu and Wong, 2011).

184 *3.3. Docking models of CB183 with human CYP2B6*

185 To understand the molecular basis of enantioselective metabolism and different 186 production rates of the two metabolites, both the atropisomers of CB183 were docked in 187 the substrate-binding cavity of human CYP2B6 (PDB ID: 3IBD) (Figure S3A). The 13 crystal structures of human CYP2B6 obtained from PDB were superimposed to identify 188 189 the substrate-binding cavity of human CYP2B6 (Figure S3B). Binding modes of eight 190 conformations showed that either the 3'- or 5-position of CB183 approaches the heme 191 iron in the substrate-binding cavity (Figure S3A). From these results, a plausible 192 conformation of CB183 (displayed in red) in the cavity was selected (Figure S3A). Our

193 docking models indicate that the (-)-CB183 conformation experiences no steric hindrance 194 in the substrate-binding cavity (Figure 5A). In contrast, steric hindrance between Cl at 3-195 position of (+)-CB183 and the cavity was observed (Figure 5B). Bulky side chains of 196 amino acids 1101, 1209, and V474 were positioned 2.8, 2.9, and 3.3 Å away from the Cl 197 residue at 3-postion, respectively (Figure 5C). This adversely affects the conformational 198 stability of (+)-CB183, resulting in its lower metabolizing activity. In the future studies, 199 a binding energy calculated in the docking simulation is compared between human 200 CYP2B6 or rat CYP2B1 and atropisomers of CB183 to support these models.

201 The selective production of two metabolites could be associated with two factors. 202 Firstly, CB183 has seven chlorines and is composed of a trichlorophenyl and a 203 tetrachlorophenyl ring (Figure 1). The electrostatic potential map of CB183 is shown in 204 Figure S4. The tetrachorophenyl ring is electron-deficient due to the presence of four 205 electron-withdrawing chlorine groups, whereas the trichlorophenyl ring has three 206 chlorines, making it relatively electron-rich, which facilitates the reaction at 3'-position. 207 The dipole moment of the tetrachloro-substituted phenyl ring was larger than that of the 208 trichloro-substituted ring. Therefore, it is possible that dipole-induced dipole interactions 209 occur in the lipophilic area (Figure 6A). Conversely, when a trichlorophenyl ring occupies 210 the right side of the cavity, the stability is decreased, causing low hydroxylation activity 211 at the 5-position (Figure 6B). Thus, the preferential metabolism of (-)-CB183 212 atropisomer to the 3'-OH-CB183 metabolite by human CYP2B6 can be explained by 213 these docking models.

214

215 4. Conclusions

216 All chiral PCBs are considered to have no dioxin-like toxicity because the expression 217 of dioxin-like toxicity requires a co-planar structure that does not contain chlorine or has 218 only one chlorine at the ortho-position. However, chiral PCBs alter calcium regulation 219 associated with neuronal signaling through RyR (Wong and Pessah, 1996). (-)-CB95 and 220 (-)-CB136 are known to cause enantioselective neurodevelopmental toxicity via RyR 221 (Feng et al., 2017; Yang et al., 2014). Since CB183 also has RyR-binding activity, 222 enantioselective binding activities of CB183 atropisomers have been observed (Pessah et 223 al., 2006). Furthermore, it has been reported that chiral PCBs with a hydroxyl group, 224 especially at the meta-position, decrease the binding activity toward RyR. These results 225 suggest that hydroxylation of chiral PCBs by P450 species plays an important role in 226 decreasing toxicity. Atropisomer-specific binding activity of CB183 toward RyR should 227 be examined to understand how human CYP2B6 alters toxicity patterns.

228 In this study, we have shown that the atropisomers of chiral CB183 are metabolized 229 to 3'- and 5-hydroxylated heptachloro metabolites by human CYP2B6. Furthermore, (-)-230 CB183 was metabolized to a greater extent than (+)-CB183. These observations were 231 explained with the help of docking models of CB183 atropisomers with human CYP2B6. 232 To our knowledge, this is the first study to explore the structural basis of chiral PCB 233 metabolism by P450 species. Our results will accelerate research on the clarification of 234 the structural basis of chiral PCB metabolism and precise toxicity evaluation of chiral 235 PCBs. In addition to the biotransformation of chiral PCBs, factors like differences in the 236 binding activity of transporter proteins toward chiral PCBs and cell membrane 237 permeability should be taken into consideration for enantiomeric enrichment of chiral 238 PCBs (Nagayoshi et al., 2018).

239

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244

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Figure 2 Chromatograms of hydroxylated heptachloro metabolites of CB183 by human
CYP2B6 and rat CYP2B1 analyzed by high-resolution gas chromatography/highresolution mass spectrometry.

(A): S1 and S2 are the authentic standards 3'-MeO-CB183 and 5-MeO-CB183,
respectively. (B) and (C): Metabolism of (+)-CB183 by human CYP2B6, with and
without NADPH, respectively. (D) and (E): Metabolism of (-)-CB183 by human
CYP2B6, with and without NADPH, respectively. (F) and (G): Metabolism of (+)-CB183

- 402 by rat CYP2B1, with and without NADPH, respectively. (H) and (I): Metabolism of (-)-
- 403 CB183 by rat CYP2B1, with and without NADPH, respectively. Metabolites are
- 404 represented as M1 and M2, respectively.







Figure 5 Preferential metabolism of (–)-CB183 by human CYP2B6.

464 Docking models of (-)-CB183 (A) and (+)-CB183 (B) in the binding cavity of human
465 CYP2B6. Blue and green spheres in CB183 indicate the hydrogen and chlorine atoms,
466 respectively. Steric hindrance between the Cl atom at 3-position of (+)-CB183 and bulky
467 side chains of amino acids I101, I209, and V474 that are present in the human CYP2B6
468 cavity is observed (C).

