PHASE I OXIDATION OF α- AND γ-HEXABROMOCYCLODODECANE BY CYTOCHROME P450 ENZYMES: SIMULATION OF THE STEROISOMERISM OF HYDROXYLATED METABOLITES

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Introduction

The flame retardant hexabromocyclododecane (HBCD) is persistent, bioaccumulative and undergoes long-range environmental transport. HBCD is a potential endocrine disrupter[1,2] and may induce developmental neurotoxicity[3]. Recently, hydroxylated derivatives of HBCD were found in biota[4-6] and after incubation of HBCD with liver microsomes[4,6]. It may be assumed that these derivatives contribute to the potentially adverse effects of HBCD in biota. Hydroxy-HBCDs are typical products of phase I metabolism mediated by cytochromes (CYPs) as shown in Fig. 1. Indeed, a significant induction of CYP2B and CYP3A were induced in rats on subacute exposure to HBCD[7] in freshwater fish[8] as well as in hepatocyte cultures[9]. Chicken hepatocytes[10] and chicken eggs[11] reacted with significant upregulation of CYP3A and CYP2H.

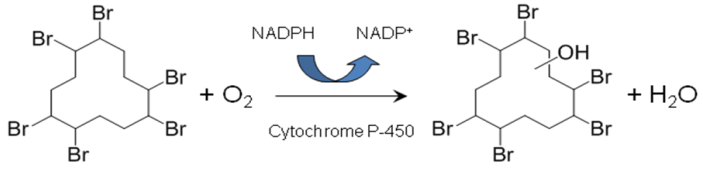

Fig. 1: Phase I hydroxylation of HBCD

The objectives of this work were firstly to provide experimental evidence for the involvement of specific CYPs in the metabolism of α- and γ-HBCD. Secondly, the interactions of α- and γ-HBCD stereoisomers with the relevant CYPs were explored by means of docking experiments and molecular dynamics simulations. The two main questions were: Does in silico docking allow predicting the activity of a given CYP regarding the phase I hydroxylation of HBCD? Is it possible to derive a relative susceptibility of the different hydrogen atoms in the HBCD molecule towards oxidative attack in the active site and thus predict the likely stereoisomerism of hydroxylated HBCD?

Materials and methods

Hydroxy-HBCD isomers: InVitroSomes™, human recombinant cytochrome (hrCYP) P450 enzymes 1A1, 1A2 and 3A4 were from InVitro Technologies (Leipzig, Germany). Incubations of the hrCYPs with individual HBCD isomers were conducted in micro-centrifuge tubes at 37 °C. For metabolite identification solution of the respective HBCD stereoisomer was used as solution in DMSO (33.6 - 54.8 µM in case of single enantiomers[12] and 76.0 - 78.7 µM in case of diastereomers (±)-α and (±)-γ). A mixture consisting of 2 µL of this DMSO solution, 170 µL potassium phosphate buffer (pH 7.4, including 0.1 M $MgCl_2$), and 20 µL of the respective hrCYP was pre-incubated for 3 min at 37 °C. The reaction was then initiated by addition of 10 µL of a 0.67 mM nicotinamide adenine dinucleotide phosphate (NADPH) solution. The reaction was run for 50 min and stopped by adding ethyl acetate (about 4 °C) to the incubation mixture, which was then extracted three times with ethyl acetate using a Vortex shaker (Heidolph, Schwabach, Germany). The combined organic phases were evaporated using a gentle stream of nitrogen and re-dissolved in methanol for HPLC-MS/MS analysis as described in detail elsewhere[13].

Semi-flexible docking of HBCD stereoisomers to cytochrome active sites: The structures of cytochromes CYP1A1 (PDB entry 2HI4), CYP2B4 (3IBD), CYP2B6 (1PO5), and CYP3A4 (1TQN, 1WOE, 2JOD) and α-, and γ-HBCD stereoisomers were parameterized using the Merck Molecular force field[14]. 20 dockings for each

HBCD enantiomer and each cytochrome were carried out using the Flexible Alignment and DOcking **(**FADO)[15] procedure.

Solvent accessible areas of HBCD hydrogens: The solvent accessible areas (SAS) of the hydrogens of both α-HBCD and γ-HBCD enantiomers were calculated using the Contour-Buildup algorithm[36]. First, for each enantiomer, we retrieved the minimal energy geometry by a Hybrid-Monte-Carlo (HMC) sampling[17] with 500,000 steps. The convergence was detected by the Gelman and Rubin Criterion[18]. Then, the trajectories for each enantiomer were minimized by the conjugate gradient method[19] and the global minimum of each trajectory was determined. The conformations belonging to the global minima were used for calculation of SAS of the hydrogens under consideration.

Dipole moments of monohydroxy-HBCD: A HMC sampling with 120,000 steps at T=1,500 K was performed for each of the six monohydroxy-HBCD structures theoretically possible as metabolite from each of the enantiomers of α- and γ-HBCD. The trajectories were minimized and the geometries corresponding to the global minima of the trajectories were used as inputs for the calculation with Gaussian 09[20]. The quantum mechanical calculations were performed on the B3LYP hybrid density functional level with the 6-31+G* basis sets. The geometries were optimized and the dipole moments were calculated.

Accessibility of HBCD hydrogen to oxidative attack in the cytochrome active site: CYP3A4 (PDB entry 1WOE) and the enantiomers of α- and γ- were parametrized by using the AMBER force field[21]. The heme group of CYP3A4 with the dioxygen attached was modeled using the parameters derived by Park et al.[22]. For each α- and γ-HBCD enantiomer, the 20 geometries found by FADO were used as input structures for the molecular dynamics (MD) simulation with the GROMACS package[23]. Water molecules using the TIP3P[24] model were selected as solvent. All-atom models for the liganded CYP3A4 were immersed in rectangular boxes containing about 20000 TIP3P water molecules. Out of 20 trajectories for each α- and γ-HBCD enantiomer, the energetically and geometrically most favorable was chosen for further analysis. Then we started at the end point of the chosen trajectory and rotated the corresponding HBCD enantiomer 60 times according to the vertices of an icosahedron. For each rotation, we performed energy minimization, 20 ps restrained equilibration and 20 ps unrestrained equilibration. Only short times for the equilibration were meaningful in this case, because ligands should keep their orientation relative to the heme group. Afterwards, a short MD simulation of 400 ps was carried out.

Dynamics Analysis: We analyzed the trajectories with regard to the distances between the hydrogens of HBCDs and the attacking oxygen and with regard to the interacting energies between HBCDs and the CYP3A4 structure. The analyses were performed with the g_dist and g_energy modules of GROMACS. For each hydrogen under consideration those steps of produced MD trajectories were chosen, which displayed a distance between hydrogen and the attacking oxygen < 2.3 Å. We approximate the potential energy by the sum of the Lennard Jones and Coulomb energies between the respective HBCD ligand and the CYP3A4.

Results and discussion

The docking of the four α- and γ-HBCD stereoisomers as ligands to the CYP structure was done semi-flexibly with flexible ligand and rigid CYP structure. The results are comprised in Table 1 in terms potential energy as sum of the Lennard Jones and Coulomb energies of the most favorable alignment of the respective HBCD ligand in the active site of the cytochrome. Obviously, the HBCD stereoisomers fit into the active site of CYP3A4 (2JOD) comparably well as the co-cyrstallized ligand.

Table 1: Semi-flexible docking of HBCD stereoisomers to cytochrome active sites: potential energy in kJ mol^{-1}

	CYP 1A2 (2HI4)	CYP 2B6 (3IBD)	CYP 2B4 (1PO5)	CYP 3A4 (1TQN)	CYP 3A4 (1WOE)	CYP 3A4 (2JOD)
Ligand						
PDB ligand[a]	-145[b]	-44[c]	-	-	-	-74[d]
(-)-α-HBCD	237	294	-41	-65	-96	-33
(+)-α-HBCD	322	685	-74	-28	-80	-65
(-)-γ-HBCD	215	217	-73	-42	-112	-96
(+)-γ-HBCD	277	233	-99	-14	-124	-73

[a] Co-crystallized ligand in the PDB structure; [b] alpha-naphthoflavone; [c] 4-(4-chlorophenyl)imidazole; [d] Erythromycin

The other CYP3A4 structures investigated in silico also display negative potential energies while the data for CYP1A2 suggests no ready alignment with HBCD ligands and confirms the experiment which showed no enzymatic activity. CYP2B6 yields energies similar to CYP1A2 while the closely related CYP2B4 displays negative potential energies suggesting a favorable fit of HBCD in its active site. This is in accordance with the induction of unspecified CYP2B induced by HBCD in vivo[7]. This encourages in our view the docking of substrates to the active site of the PDB x-ray structures in order to predict potential metabolic activity of a given cytochrome.

The incubation of hrCYP3A4 with the HBCD stereoisomers resulted in the following numbers of hydroxy-HBCD. (-)-α: 3; (+)-α: 4; (-)-γ: 2; (+)-γ: 3. Table 2 summarizes the solvent accessible areas of the H-atoms in the positions which would give rise to different hydroxy-HBCD diastereomers along with the mean interaction energies P of the respective HBCD isomer in the active site of hrCYP3A4. The molecules were positioned in the active site such that the distance of the hydrogen under consideration to the hem oxygen was below 2.3 Å (Fig. 2). The table also displays the dipole moments DP of the respective hydroxy-HBCD. It should be noted that the other H-atoms in the HBCD molecules are equivalent to the listed ones for symmetry reasons (Fig. 3). H-atoms on carbons bound to bromine were not considered.

Table 2: Solvent accessible surface (SAS in Å^2) of the H-atom in the parent HBCD, interaction energy P of the HBCD with the same H aligned to the oxygen in the active site of CYP3A4, and dipole moments (DP in Debye) of the respective hydroxy-HBCD derivatives

Position of H or OH	(-)-α-HBCD			(+)-α-HBCD			(-)-γ-HBCD			(+)-γ-HBCD		
	SAS	P	DP	SAS	P	DP	SAS	P	DP	SAS	P	DP
3R	9.9	- 154	1.6	8.0	-153	2.0	5.9	- 145	3.8	7.6	- 153	1.8
3S	8.0	- 152	2.0	9.9	- 155	1.6	7.6	- 149	1.8	5.9	- 161	3.8
4R	11.1	- 135	1.1	3.6	- 148	3.3	9.5	- 146	1.8	0.37	- 128	2.2
4S	3.6	- 143	3.3	11.1	- 174	1.1	0.33	- 142	2.5	9.5	- 154	1.8
7R	2.8	- 136	3.1	12.6	- 146	1.6	2.8	- 143	2.7	14.7	- 157	2.3
7S	12.6	- 158	1.6	2.8	- 147	3.1	14.7	- 157	2.3	2.9	- 147	2.7

The probability for an oxidative attack of the different hydrogens in the HBCD molecules by CYP3A4 depends on two conditions. First, the solvent accessible surface SAS of the respective hydrogen has to be sufficiently large for the attack. Second, the respective HBCD molecule must be able to adopt an energetically feasible position inside the active site of CYP3A4 in order to allow for the attack (column "P" in Table 2).

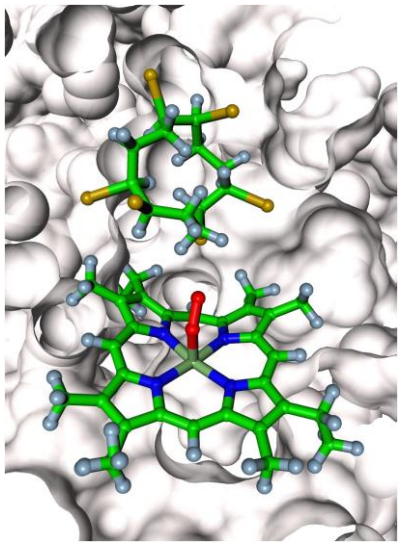

Fig. 2: HBCD in the active site of CYP3A4

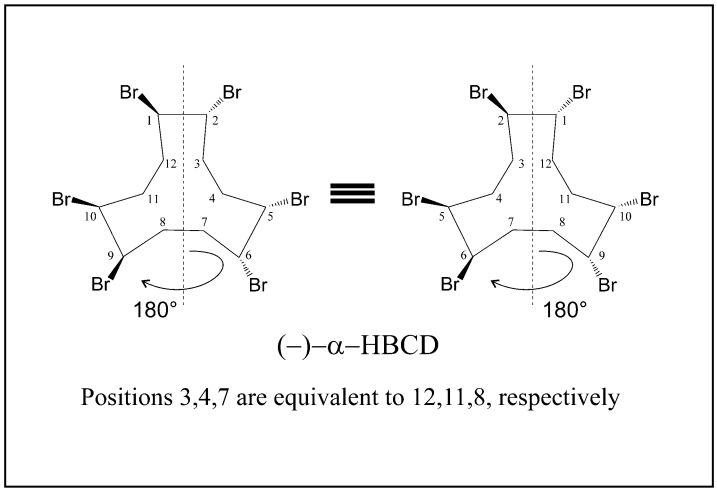

Fig. 3: Symmetry of α- and γ-HBCD exanplified for (-)-α

This means for (-)-α that positions *4S* and *7R* can be excluded from further considerations (SAS too small). of the remaining hydrogens display an energetically feasible binding position each (*3R*, *3S*, and *7S*) which correspond to the three hydroxy-HBCD found in the experiment. In case of (+)-α *4R* and *7S* can be excluded due to small SAS and from the four remaining ones *4S* is the preferred one regarding the energy of the binding positions. In case of (-)-γ *7R, 4S* and probably *3R* can be excluded (SAS). *7S* has a large SAS as well as a preferred binding position and is the most probable of the two experimentally observed γ-HBCD-OH. For (+)-γ *4R,7S* and probably *3S* can be ruled out. The three remaining positions display similar interaction energies and three hydroxy-HBCD were observed from this steroisomer. The order of elution of the respective diastereomic hydroxy-HBCD during HPLC analysis depends on their interaction with the cyclodextrin moiety which would have to be simulated separately. A tentative assignment of the elution order might be attempted using the dipole moments.

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