TOX IV

STRUCTURE DEPENDENT INDUCTION OF CYP1A BY POLYCHLORINATED BIPHENYLS IN CYNOMOLGUS MONKEY HEPATOCYTES (*Macaca fascicularis*)

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INTRODUCTION

Polychlorinated biphenyls (PCBs) are industrial contaminants which have been detected in the environment in large quantities[1]. Their chemical stability, physical properties, inflammability and dielectric properties caused the commercial interest in these compounds in the past. However, due to their lipophilic nature and resistence to biotransformation, PCBs tend to accumulate in the food chain[2]. Different classes of PCB congeners, depending on the position and amount of chlorine substitutions, elicit a complex spectrum of biological and toxic responses both *in vivo* as *in vitro*[3,4]. Some toxic effects of the *non-ortho* or *mono-ortho* chlorine substituted PCBs, include reproductive toxicity, neurotoxicity, dermal toxicity and hepatoxicity. These effects are similar to 2,3,7,8 TCDD and most if not all are mediated by the *Ah*-receptor. Some biochemical responses include significant induction of phase I and II metabolizing enzymes in many vertebrate species eg. the induction of CYP1A1 and CYP1A2[4].

The biochemical responses of another group of PCB congeners, those with two or more *ortho* chlorine substitutions, resembles phenobarbital to some extent, e.g by inducing CYP2B1/2 and CYP3A1/2 in rat[5,6,7]. However, CYP1A2 protein levels could also be induced in C57BL/6J mouse after treatment with *di-ortho* PCBs[8]. Little is known about the biochemical and toxic effects of these multiple *ortho* chlorine substituted PCBs, although their occurence in the biotic and abiotic environment is abundant[3]. Some responses are: promotor effects in the presence of some genotoxic compounds[9], effects on the thyroid hormones and vitamin A metabolism[10], interaction on vitamin K metabolism[11], and neurotoxic effects[12].

Some PCB congeners, which have either a *mono-ortho* or *di-ortho* chlorine substitution pattern can elicit both an *Ah*-receptor mediated as well as a phenobarbital type of respons and are referred to as 'mixed inducers'[4].

In addition to pronounced differences in mechanism of action between these PCBs, there are marked species differences in activity and regulation of cytochrome P450 isoenzymes. Consequently, it is difficult to extrapolate effects found in animal studies to the human situation. It has been suggested that non human primates could be a suitable model for humans to study induction of cytochrome P450 enzymes[13]. In our study, hepatocytes from Cynomolgus monkeys (*Macaca Fascicularis*) were used to study the effect of different PCBs on the CYP1A family. It was shown earlier that hepatocytes of Cynomolgus monkeys express a CYP1A enzyme which is inducable by *non* as well as multiple *ortho* substituted PCBs[14].

MATERIALS AND METHODS

Chemicals

The selection of PCBs was based on a multivariate physico-chemical characterization of all tetra- to hepta-chlorinated congeners[15]. The PCBs used in this study were selected as described earlier by Tysklind *et al*[16].

PCBs were purchased from Ultra Scientific (North Kingstown, RI, USA) (ie. 2,3,3',5 TeCB (PCB#58), 3,3',4,5 TeCB (PCB#78), 2,2',3,3',4,5,6 HpCB (PCB#173), 2,2',3,4',5,6,6' HpCB (PCB#188) and 2,3,3',4,4',5,6 HpCB (PCB#190)) and from AccuStandard (New Haven, CT, USA) (ie. 2,2',3,4 TCB (PCB#41), 2,2',4,6' TCB (PCB#51), 2,3,4,4' TCB (PCB#60), 2,3',4,5' TCB (PCB#68), 2,2',3,4',6 PCB (PCB#91), 2,2',4,4',5 PCB (PCB#99), 2,2',4,6,6', PCB (PCB#104), 2,3,3',5,6 PCB (PCB#112), 2,3,4,4',6 PCB (PCB#115), 3,3',4,4',5 PCB (PCB#126), 2,2',3,4,5,6' HxCB (PCB#143), 2,2',4,4',5,5', HxCB (PCB#153), 3,3',4,4',5,5' HxCB (PCB#169), 2,2',3,4,4',6,6' HpCB (PCB#184), 2,3,3',4',5,5',6 HpCB (PBC#193)). All PCBs were analyzed by HRGC/LRMS (MD800, Fisons, UK) and had more than 99% purity. Bovine serum albumine (BSA), gentamicin, insulin, hydrocortison, L-glutamine, Hanks' balanced salt solution (H.B.S.S), ethylene glycol-bis-(ß-amino ethyl ether) N,N,N',N' tetra acetic acid (EGTA) and resorufin were all purchased from Sigma (St.Louis, MO, USA). Fetalclone serum was from Greiner (Alphen a.d. Rijn, The Netherlands). Collagenase and ethoxyresorufin were obtained from Boehringer Mannheim (Mannheim, Germany). Methoxy resorufin was obtained from Brunschwig Chemie BV (Amsterdam, The Netherlands).

Animals, cell isolation and cell culture

Cynomolgus monkeys (*Macaca fascicularis*) were bred at the National Institute of Public Health and Environmental Protection (RIVM, Bilthoven, The Netherlands) and served as donors for kidney cells which are used for the production of the poliomyelitis vaccine.

Hepatocytes, from male or female animals, were isolated as described by Mennes *et al*[17]. The cells were plated in 12 wells plates (Greiner, Alphen a/d Rijn, The Netherlands) at a density of $0.75*10^6$ cells/well in 1.5 ml Williams'E medium, supplemented with 5% (v/v) fetal clone serum and 1 µM insulin, 10 µM hydrocortisone and 50 mg/L gentamicin.

Cells were incubated in a humified atmosphere of air (95%) and CO_2 (5%) at 37°C. During the first 4 hours, 4 mM $CaCl_2$ and 4mM $MgCl_2$ were added to the medium. After 4 hours, the medium was replaced and the cells were preincubated for 16 hours. Next, the medium was replaced by medium with different inducers (PCBs or DMSO 0.1%, v/v) for 24 hours, in the absence of serum.

Cytochrome P450 enzyme activity

The methoxyresorufin (MR) and ethoxyresorufin (ER) analysis were performed as described by Burke *et al*[18]. Hepatocyte monolayers were washed with Hanks' balanced salt solution. The incubation was performed by adding 10 µM MR or 5 µM ER and 10 µM dicumarol to the medium. The formation of resorufin van determined fluorimetrically.

Protein content

Protein content in each well was determined according to Bradford *et al*[19]. using bovine serum albumin (BSA) as standard.

RESULTS and DISCUSSION

The results, shown in table 1 and 2, summarize the dose-dependent induction of CYP1A activity in monkey hepatocytes by different PCBs. These PCB congeners were divided in two groups, a standard and validation set, which can be of use for principle component analysis. This technique will be used to determine the physico-chemical properties and structural requirements which determine the CYP1A induction by PCBs in Cynomolgus monkeys[15]

Both methoxyresorufin and ethoxyresorufin are substrates for CYP1A enzymes in different species[20,21,22]. The different affinities of these substrates for CYP1A are not known in Cynomolgus monkey hepatocytes.
The maximum induction of the CYP1A activity was not identical for the different PCBs. Therefore, besides EC-50 values of the different PCBs in both sets also the maximum induction of both EROD and MROD activity relative to PCB#169 is presented in table 1 and 2.

Table 1. EC-50 (nM) of ten different PCBs in Cynomolgus monkey hepatocytes (♂) based on MROD or EROD activity (standard set of PCBs), using a sigmoidal curvefit.

Congener (IUPAC No.)	EC-50 (nM) EROD activity	EC-50 (nM) MROD activity	% of maximum EROD activity induced by PCB#169	% of maximum MROD activity induced by PCB#169
PCB#41	n.d.	n.d.	-	-
PCB#58	n.d.	n.d.	-	-
PCB#60	617	477	69	63
PCB#99	n.d.	n.d.	-	-
PCB#104	n.d.	n.d.	-	-
PCB#115	7150	10850	20	25
PCB#169	14.1	3.6	100	100
PCB#173	8470	7130	25	56
PCB#184	n.d.	n.d.	-	-
PCB#190	8310	9475	14	30

n.d. not detectable

In table 1, it is shown that five different substituted PCBs were capable of inducing CYP1A activity, measured as MROD and EROD activity, in hepatocytes of Cynomolgus monkeys. These are PCB#60, PCB115, PCB#169, PCB#173 and PCB#190.
PCB#60 and PCB#169 are *mono-* respectively *non-ortho* substituted congeners and are known CYP1A inducers in different species[4]. PCB#190 (two *meta* and both *para* positions substituted) has little *Ah*-receptor agonist activity in rodents[4], and is able to induce CYP1A slightly. But PCB#115 and PCB#173 are *di-*, and *tri-ortho* substituted PCBs respectively and were not known as CYP1A inducers so far. These congeners also belong to the group of phenobarbital-like inducers[4].
The effect on CYP1A activity of the validation set of PCBs is presented in table 2. PCB#78 (*non-ortho*) and PCB#126 (*non-ortho*) are potent inducers of the CYP1A activity in monkey hepatocytes. Both PCBs are efficient *Ah* receptor agonist[4], comparable with PCB#60 and PCB#169 in the standard set. However, PCB#153 (*di-ortho*), PCB#188 (*tetra-ortho*) and PCB#193 (*di-ortho*) are clear CYP1A inducing PCBs in the Cynomolgus monkeys, besides being phenobarbital-type of inducers[4,23].

The EC-50 values of PCB#169 for EROD- and MROD-activity in hepatocytes isolated from both the female or a male Cynomolgus monkey used in our study was comparable, approximately 15 nM. Therefore we consider the data from both tables comparable, which allows us to perform principle component analysis. PCB#126 is about a 30 times more potent CYP1A inducer in these monkey hepatocytes than PCB#169.

Table 2. EC-50 (nM) of ten different PCBs (and PCB#169 as a standard) in Cynomolgus monkey hepatocytes (♀) based on MROD or EROD activity (validation set of PCBs), using a sigmoidal curvefit.

Congener (IUPAC No.)	EC-50 (nM) EROD activity	EC-50 (nM) MROD activity	% of maximum EROD activity induced by PCB#169	% maximum MROD activity induced by PCB#169
PCB#51	n.d.	n.d.	-	-
PCB#68	n.d.	n.d.	-	-
PCB#78	299	317	83	89
PCB#91	n.d.	n.d.	-	-
PCB#112	n.d.	n.d.	-	-
PCB#126	0.350	0.522	63	90
PCB#143	n.d.	n.d.	-	-
PCB#153	17200	21900	32	46
PCB#169	15.7	16	100	100
PCB#188	8900	8400	43	50
PCB#193	21800	28400	28	46

n.d. not detectable

In conclusion, in hepatocytes of Cynomolgus monkeys not only typical CYP1A inducers[4] (*Ah* receptor agonists) induce a CYP1A enzyme. Congeners, which were earlier characterized as phenobarbital-type of inducers, can also induce CYP1A activity in Cynomolgus monkey hepatocytes. Principle component analysis will be used to determine the QSAR of these PCBs for CYP1A induction in Cynomolgus monkeys in more detail.

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