DIOXIN-LIKE POTENCIES OF HYDROXYLATED POLYCHLORINATED BIPHENYLS IN DR-CALUX ASSAY

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Introduction

Hydroxylated polychorinated biphenyls (OH-PCBs) are metabolites of PCBs formed by the cytochrome P450 (CYP) monooxygenase enzyme system. Due to their persistence and bioaccumulative potential, PCBs and OH-PCBs are still found in the tissues of humans[1, 2] as well as animals—from pets[3,4] to various wildlife species[3–6]—several decades after the ban on PCBs in many countries. The abundance of OH-PCBs relative to their parent compounds in animal tissues varies widely with the metabolic capacity of the species and can be up to an order of magnitude higher in some carnivores[3]. To assess potential adverse effects for animals accumulating high levels of OH-PCBs, toxicological characterization of these compounds is necessary.

The most well-known toxic endpoints of OH-PCBs are associated with thyroid hormone (TH) homeostasis, where they have been reported to cause disturbance at multiple levels, including TH transport protein[7] and TH receptor[8]. Results from recent *in vitro* studies indicate that these compounds can also affect endpoints mediated by the aryl hydrocarbon receptor (AhR)[9,10] as well as several steroid hormone receptors[9,11]. Although some OH-PCBs have been expected to also bind to the AhR and exhibit dioxin-like (DL) effects to various extents due to their molecular structure being very similar to that of their parent compounds PCBs, reports on their potencies have been somewhat conflicting. An early investigation on DL potency of a few OH-PCB congeners using a recombinant rat hepatoma cell line with 24-h exposure time reported very low relative potency factors (REPs) compared with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), in the range of 10^{-6} or lower[8]. A more recent study screened 84 congeners using a yeast assay with 6-h exposure time and reported that 52 compounds exhibited AhR agonism, many of them were more potent than their analogous PCBs and some were as potent as the reference compound β-napthoflavone[10]. However, with the yeast assay procedure, metabolism may not be taken into account and thus it is difficult to conclude whether the reported activities indicated persistent, dioxin-like effects.

This study used the Dioxin-Responsive Chemical-Activated Luciferase gene eXpression (DR-CALUX) to determine the DL potencies of 34 OH-PCBs, aiming to understand the potential contribution of OH-PCBs to the adverse effects caused by dioxin-like compounds (DLCs) accumulated in animals and human.

Materials and methods

Chemicals

Thirty-four mono-OH tri–heptachlorobiphenyl congeners were tested (Table 1). Among them, seventeen were synthesized by thermal diazo-coupling between a chlorophenol and a chloroaniline diazonium salt, and then characterized using ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy and high resolution mass spectrometry as described elsewhere[10]. The other compounds and were obtained from AccuStandard, Inc. and Wellington Laboratories, Inc. For each compound, a series of standard solutions was prepared by serial dilution from a 1-mM stock solution in dimethyl sulfoxide (DMSO). TCDD was used as the reference compound, and the calibration standard solutions were prepared from a 50-μg/ml TCDD solution in DMSO (Cambridge Isotope Laboratories, Inc.).

DR-CALUX assay

Dioxin-like activity was measured using DR-CALUX with a rat hepatoma cell line stably transfected with a rat AhR-regulated luciferase gene construct (H4IIE-luc, BioDetection Systems b.v.). The conditions for cell culture and the assay procedure have been described in detail elsewhere[12]. Briefly, the cells were sub-cultured at 37 °C under 5% CO_2 and high humidity in α-minimal essential medium (αMEM) supplemented with 10% fetal

calf serum (FCS). The cells were seeded in a 96-well microplate with αMEM supplemented with 10% FCS, incubated for 24 h, and then exposed to TCDD or individual OH-PCBs at DMSO concentration of 0.8%. After 24 h of exposure, the medium was removed, and the cells were lysed with Triton-based buffer. Upon addition of luciferin solution, the luciferase activity was measured with a luminometer. The measurement was conducted in three wells on the same microplate and repeated three times for each compound. Levels of luciferase induction by OH-PCBs were expressed in terms of the percentage of the maximum level of induction by TCDD after correction for background activity of the DMSO control. Compounds with more than 5% induction were considered as having significant AhR agonistic activity.

Table 1. OH-PCB congeners tested in this study and their DR-CALUX activity at maximum tested concentration

BZ#	Chemical name	Provider	Activity[†]
4'-OH-CB25	2',3,4'-trichlorobiphenyl-4-ol	synthetic	-
4-OH-CB26	2,3',5-trichlorobiphenyl-4-ol	synthetic	-
3'-OH-CB28	2',4,4'-trichlorobiphenyl-3-ol	synthetic	-
4-OH-CB31	2,4',5-trichlorobiphenyl-4-ol	synthetic	-
6-OH-CB31	3,4',6-trichlorobiphenyl-2-ol	synthetic	++
5'-OH-CB33	3',4',6-trichlorobiphenyl-3-ol	synthetic	-
4'-OH-CB35	3,3',4'-trichlorobiphenyl-4-ol	synthetic	+++
6'-OH-CB35	3',4',5-trichlorobiphenyl-2-ol	synthetic	-
2'-OH-CB35	3,3',4'-trichlorobiphenyl-2-ol	synthetic	++
6'-OH-CB36	3',5,5'-trichlorobiphenyl-2-ol	synthetic	-
3'-OH-CB37	3',4,4'-trichlorobiphenyl-3-ol	synthetic	++
2'-OH-CB39	3',4,5'-trichlorobiphenyl-2-ol	synthetic	+
3'-OH-CB53	2,2',5',6-tetrachlorobiphenyl-3-ol	synthetic	-
3'-OH-CB61	2',3',4',5'-tetrachlorobiphenyl-3-ol	AccuStandard	+
4'-OH-CB61	2',3',4',5'-tetrachlorobiphenyl-4-ol	AccuStandard	+
4'-OH-CB65	2',3',5',6'-tetrachlorobiphenyl-4-ol	AccuStandard	+
3-OH-CB66	2,3',4,4'-tetrachlorobiphenyl-3-ol	synthetic	+++
4'-OH-CB72	2',3,5,5'-tetrachlorobiphenyl-4-ol	AccuStandard	-
3'-OH-CB74	2',4,4',5'-tetrachlorobiphenyl-3-ol	synthetic	++
2'-OH-CB79	3,3',4',5-tetrachlorobiphenyl-2-ol	synthetic	+++
4'-OH-CB86	2,2',3',4',5'-pentachlorobiphenyl-4-ol	AccuStandard	+
4'-OH-CB93	2,2',3',5',6'-pentachlorobiphenyl-4-ol	AccuStandard	-
3'-OH-CB101	2,2',4',5,5'-pentachlorobiphenyl-3-ol	AccuStandard	-
6'-OH-CB101	2',3,4',5',6-pentachlorobiphenyl-2-ol	AccuStandard	+
4'-OH-CB101	2,2',4',5,5'-pentachlorobiphenyl-4-ol	Wellington	-
6'-OH-CB106	2',3',4',5,5'-pentachlorobiphenyl-2-ol	AccuStandard	++
4'-OH-CB106	2',3,3',4',5'-pentachlorobiphenyl-4-ol	AccuStandard	-
4-OH-CB107	2,3,3'4',5-pentachlorobiphenyl-2-ol	Wellington	+
2'-OH-CB121	2',3,4',5,6'-pentachlorobiphenyl-2-ol	synthetic	++
4'-OH-CB121	2',3,4',5,6'-pentachlorobiphenyl-4-ol	AccuStandard	-
4-OH-CB146	2,2',3,4',5,5'-hexachlorobiphenyl-4-ol	Wellington	+
4'-OH-CB159	2',3,3',4',5,5'-hexachlorobiphenyl-4-ol	Wellington	++
4'-OH-CB165	2',3,3',5,5',6'-hexachlorobiphenyl-4-ol	AccuStandard	-
4-OH-CB187	2,2',3,4',5,5',6-heptaachlorobiphenyl-4-ol	Wellington	-

[†]induction (at 8 μM, or approximately 1 μM for 4-OH-CB107/-CB146/-CB187) relative to 300 pM TCDD: -, +, ++ and +++ indicate <5%, 5%–<20%, 20%–<50% and ≥50%, respectively

Data analysis

The dose–response curves for each compound was fitted to a three-parameter sigmoid $y = a_0 / [1 + (x / a_1)^{a2}]$, where y is the measured luciferase induction, x the concentration, a_0 the maximum luciferase induction (approximately 100% for TCDD), a_1 the 50% effective concentration (EC_{50}), and a_2 the slope. In the case where maximum induction could not be reached at the maximum available concentration (8 μM), the dose–response

curve was fitted to a sigmoid with a fixed value of a_0 (between 50% and 100%) until best fitting characteristics was obtained. REP at *i*% induction was defined as $REP_i = EC_{i,TCDD} / REC_{i,OH\text{-}PCB}$, with $EC_{i,TCDD}$ and $REC_{i,OH\text{-}PCB}$ being the effective concentration of TCDD and the TCDD-relative effective concentration of OH-PCB, respectively. In this report, only REP_{20} values were calculated.

Results and discussion

Dioxin-like potency

Among the 34 tested OH-PCBs, 19 congeners significantly induced luciferase activity in the DR-CALUX assay (Table 1) in a dose-dependent manner (see example in Fig. 1) at concentrations of 0.02 µM or higher. However, REP_{20} values could be derived for only nine congeners, for which 20% induction (relative to 300 pM TCDD) was achieved. Fig. 1 shows the dose–response curves and REP_{20} values for several tested OH-PCB congeners with the highest activity. Among the nine congeners with calculable REPs, 3-OH-CB66 had the highest potency (3.7×10^{-5}), and 2'OH-CB35 had the lowest value (1.4×10^{-6}). Regarding their molecular structure, the most potent compounds tended to have from three to four chlorines. Two congeners (4'-OH-CB35 and 3'-OH-CB37) have no *ortho* subtitution (non-*ortho*), five have one *ortho* position substituted with either a chlorine (3-OH-CB66 and 4'-OH-CB159) or an hydroxy group (6-OH-CB31, 2'-OH-CB35 and 2'-OH-CB79) (mono-*ortho*), one has two substituted *ortho* positions (6'-OH-CB106) and one has three substituted *ortho*s (2'-OH-CB121). Activity of the latter two congeners is surprising because non- and mono-*ortho* configurations are considered as criteria for dioxin-like activity of PCBs.

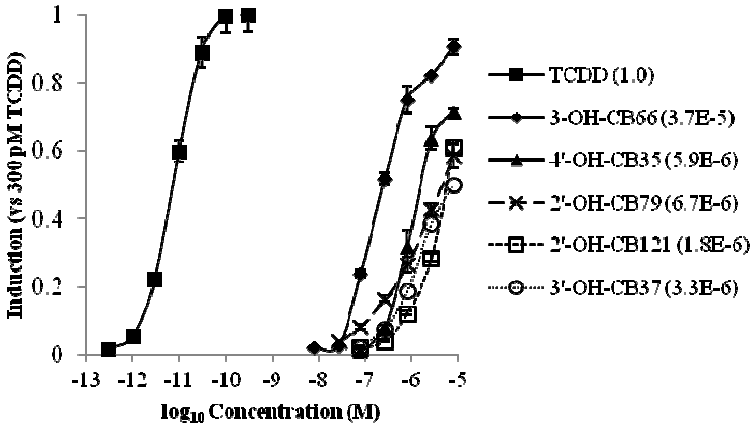

Fig. 1. DR-CALUX dose–response curves of TCDD and the most potent OH-PCBs tested. Each data point represents the average of at least three measurements. REP_{20} values are given in parentheses.

There are some discrepancies between the results obtained with DR-CALUX cells (rat hepatoma, this study) and those obtained with yeast cells containing human AhR[10]. The most potent compound in the yeast assay (2'-OH-CB39) showed very low activity compared with other congeners in DR-CALUX (Table 1). On the other hand, the most potent compound in DR-CALUX (3-OH-CB66) had comparatively lower potency in the yeast assay. Such conflicting results may be caused by the difference in ligand interactions with rat/human AhRs, cell uptake efficiency and/or by metabolism in the rat hepatoma cells. Further investigation is necessary before the results with these bioassays can be generalized as dioxin-like potency for OH-PCBs.

Relevance for total dioxin-like toxic equivalents (TEQs)

The DR-CALUX REP_{20} values calculated for some OH-PCB congeners in this study were in the same order as those reported for mono-*ortho* PCBs[13]. Considering that the total OH-PCB concentrations in blood of some terrestrial carnivorous animals are known to be similar to or higher than those of total PCBs, up to the nanogram per gram range[3,4], it is necessary to evaluate the contribution of OH-PCBs to the PCB-TEQs in these animals. However, two of the most potent OH-PCB congeners in this study (3-OH-CB66 and 4'-OH-CB35) so far have not been detected in biological tissues[4,6]. Moreover, three congeners abundantly found in biota (4-OH-CB-107, 4-OH-CB146 and 4-OH-CB187) showed weak activity in DR-CALUX (Table 1). It can then be inferred that the TEQ contribution of OH-PCBs targeted so far in chemical analysis of biological samples is low. Nevertheless, it is highly likely that biological tissues contain many unidentified OH-PCB congeners[3,4,6]. The occurrence of *ortho*-hydroxylated congeners in particular has not been investigated in details because of the current focus on

para- and *meta*-hydroxylated compounds due to their relevance to thyroid hormone-related effects. In this study, some *ortho*-OH-PCBs were found to exhibit significant DL activity. Considering the possible occurrence of *ortho*-OH-PCBs in animal tissues and their potential DL activity, these compounds need to be considered in future chemical analysis as well as toxicological studies.

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