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In Vitro Toxicity Profiling of Ultrapure Non–Dioxin-like Polychlorinated Biphenyl Congeners and Their Relative Toxic Contribution to PCB Mixtures in Humans

Timo Hamers,*[,1] Jorke H. Kamstra,* Peter H. Cenijn,* Katerina Pencikova,† Lenka Palkova,† Pavlina Simeckova,† Jan Vondracek,†[,‡] Patrik L. Andersson,§ Mia Stenberg,§ and Miroslav Machala†

*Institute for Environmental Studies (IVM), VU University Amsterdam, 1081 HV Amsterdam, The Netherlands; †Department of Toxicology, Pharmacology and Immunotherapy, Veterinary Research Institute, 621 00 Brno, Czech Republic; ‡Department of Cytokinetics, Institute of Biophysics AS CR, 612 65 Brno, Czech Republic; and §Department of Chemistry, Umeå University, SE-901 87 Umeå, Sweden

[1]To whom correspondence should be addressed at Institute for Environmental Studies (IVM), VU University Amsterdam, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands. Fax: +31 20 598 9553. E-mail: timo.hamers@ivm.vu.nl.

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The toxic equivalency concept used for the risk assessment of polychlorinated biphenyls (PCBs) is based on the aryl hydrocarbon receptor (AhR)–mediated toxicity of coplanar dioxin-like (DL) PCBs. Most PCBs in the environment, however, are non-dioxin-like (NDL) PCBs that cannot adopt a coplanar structure required for AhR activation. For NDL-PCBs, no generally accepted risk concept is available because their toxicity is insufficiently characterized. Here, we systematically determined *in vitro* toxicity profiles for 24 PCBs regarding 10 different mechanisms of action. Prior to testing, NDL-PCB standards were purified to remove traces of DL compounds. All NDL-PCBs antagonized androgen receptor activation and inhibited gap junctional intercellular communication (GJIC). Lower chlorinated NDL-PCBs were weak estrogen receptor (ER) agonists, whereas higher chlorinated NDL-PCBs were weak ER antagonists. Several NDL-PCBs inhibited estradiol-sulfotransferase activity and bound to transthyretin (TTR) but with much weaker potencies than reported for hydroxylated PCB metabolites. AhR-mediated expression of uridine-glucuronyl transferase isozyme UGT1A6 was induced by DL-PCBs only. Hierarchical cluster analysis of the toxicity profiles yielded three separate clusters of NDL-PCBs and a fourth cluster of reference DL-PCBs. Due to small differences in relative potency among congeners, the highly abundant indicator PCBs 28, 52, 101, 118, 138, 153, and 180 also contributed most to the antiandrogenic, (anti)estrogenic, antithyroidal, tumor-promoting, and neurotoxic potencies calculated for PCB mixtures reported in human samples, whereas the most potent AhR-activating DL-PCB-126 contributed at maximum 0.2% to any of these calculated potencies. PCB-168 is recommended as an additional indicator congener, given its relatively high abundance and antiandrogenic, TTR-binding, and GJIC-inhibiting potencies.

Key Words: **polychlorinated biphenyls; endocrine disruption; thyroid hormone; steroid hormone; cell communication; neurotoxicity.**

Polychlorinated biphenyls (PCBs) are synthetic organic compounds, with high resistance against electrical, thermal, chemical, or biological breakdown. Despite the ban on their production, PCBs remain to be an environmental problem due to their high persistency and ongoing "leaking" to the environment from existing applications and waste.

Based on their toxicological mechanism of action, PCB congeners can be divided into two groups. The first group consists of PCB congeners, which can adopt a coplanar structure, because they have zero or one chlorine substitution in the *ortho* position. Similar to polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs), these coplanar PCBs can bind to the aryl hydrocarbon receptor (AhR) transcription factor and exert dioxin-like (DL) toxicity via AhR activation. For PCDD/Fs and DL-PCBs, the World Health Organization derived toxicity equivalency factors (TEF values), expressing their relative AhR-mediated toxicity compared with 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (Van den Berg *et al*., 2006). A toxic equivalency (TEQ) concentration of a DL compound is determined by multiplying its concentration with its compound-specific TEF value. TEQ concentrations of individual DL compounds can be summarized according to the principle of concentration addition, and risk assessment of a mixture of DL compounds in humans is performed by comparing the total TEQ intake to a tolerable weekly intake of 14 pg TEQ/kg body weight (SCF, 2000, 2001).

In contrast to DL-PCBs, the toxicity profiles of non–dioxin-like (NDL-PCBs) are insufficiently characterized. Consequently, no risk assessment model similar to the TEQ concept is available for NDL-PCBs, although various adverse effects on thyroid, liver, brain, immune system, estrous cycling, reproduction, and neurodevelopment have been reported for animal studies with individual NDL-PCB congeners (EFSA, 2005). The risk characterization of NDL-PCBs is hampered by the fact that only a minor contamination of the NDL-PCBs with DL compounds such as DL-PCBs and PCDD/Fs is sufficient to explain the observed effects, given the much higher toxicity of DL compounds to affect certain endpoints. Because most studies

used non-purified NDL-PCB standards, contaminations with DL-PCBs and PCDD/Fs are realistic and it cannot be concluded whether the observed effects are true NDL-PCB effects or caused by DL contaminations.

In order to improve the hazard characterization of NDL-PCBs, the aims of the present systematic *in vitro* screening study were (1) to screen the toxic potency of structurally representative and highly purified NDL-PCBs to identify relevant mechanisms of action for NDL-PCBs in man and wildlife, (2) to classify NDL-PCBs into clusters with similar toxicity profiles, and (3) to determine the relative importance of individual NDL-PCB congeners to the calculated toxic potency of a complex mixture of PCBs present in human samples (blood and adipose tissue).

Most PCB research has focused on a set of seven indicator PCB congeners representing the most abundant and well-studied PCBs in the environment, i.e., PCBs 28, 52, 101, 118, 138, 153, and 180. PCB-118 is the only DL-PCB in this set for which a TEF value has been derived, whereas the other 6 NDL-PCB congeners are considered to represent about 50% of the total PCB level in food (EFSA, 2005). The test set of PCBs in the present study consisted of the seven indicator PCBs extended with another set of 13 mono- to tetra-*ortho*-chlorinated PCBs 19, 47, 51, 53, 74, 95, 100, 104, 122, 128, 136, 170, and 190 previously selected by Stenberg and Andersson (2008) to span the structural and chemical variation domain of NDL-PCBs. This set was further extended with PCB-80 (a positive control for the transthyretin [TTR]-binding assay; Chauhan *et al*., 2000) and PCBs 125 and 168, representing PCB congeners with two chlorine substitutions in *ortho* position on one phenyl ring and none on the other. Finally, the non–*ortho*-substituted PCB-126 was added to the set as a reference structure for DL-PCBs. The complete test set of PCBs including their substitution pattern, molecular weight, and structural formula is given in Supplementary figure 1.

All 24 PCBs were tested for their potencies to interact with the estrogen receptor (ER) and the androgen receptor (AR), to inhibit sulfonation of estradiol (E2) by estradiol-sulfotransferase (E2SULT), to compete with thyroid hormone thyroxine (T4) for binding to its plasma transport protein TTR, to alter expression of uridine-glucuronyl transferase isozymes (UGT1A1 and UGT1A6) and deiodinase-1 (DI-1) involved in thyroid hormone metabolism, and to inhibit gap junctional intercellular communication (GJIC), a parameter related closely to tumor promotion. Altogether, all compounds were tested in eight different bioassays for 10 different mechanisms of action (Table 1). For each mechanism of action, relative potency (REP) factors were calculated for each individual PCB congener as the ratio between the EC_{50} values of a positive reference compound (Table 1) and the PCB congener of interest. Classes of PCBs with similar toxicity profiles were determined using hierarchical cluster analysis (HCA) and evaluated for possible common structural characteristics. Finally, the relative contribution was estimated of different PCB congeners, or their classes, to the overall toxic potency of complex PCB mixtures determined in human samples. To this end, PCB concentrations determined in blood and adipose tissue from normal human populations were converted into equivalent concentrations of specific reference compounds for each specific mechanism of action studied using the REP factors determined for the individual PCB congeners.

MATERIALS AND METHODS

Chemicals. All 24 PCB standards (Supplementary fig. 1) were purchased from Neosyn Inc. Each PCB was dissolved in n-hexane and purified on a charcoal carbon column mixed with celite, as described in detail by Danielsson *et al*. (2008). Purification efficiency was assessed by applying the cleaned standard on a second carbon/celite column eluting the PCB using n-hexane followed by backflushing the column with toluene for analysis of DL-PCBs and PCDD/Fs. These tests indicated only trace levels (< pg/g) of DL compounds in the purified NDL-PCB standards. Ultimately, 25mM stock solutions and further dilution series of the PCB standards were prepared in dimethylsulfoxide (DMSO). Using a similar approach as described for the PCB standards, only trace levels (< pg/g) of DL impurities were identified in the DMSO batch used in all experiments.

TABLE 1
Overview of the Eight *In Vitro* Bioassays Used to Determine the Toxic Potency of NDL-PCBs for 10 Different Mechanisms of Action Ordered per Type of Activity

Activity	Bioassay	Reference material	Mechanism of action	Maximum PCB test concentration (μM)
Estrogenic	ER-CALUX	17β-estradiol (E2)	ER activation	25
	E2SULT	Pentachlorophenol (PCP)	Inhibited sulfonation (metabolism) of E2	
Antiestrogenic	ER-CALUX	ICI-182,780	Competing with E2 for ER	25
Androgenic	AR-CALUX	DHT	AR activation	10
Antiandrogenic	AR-CALUX	Flutamide (Flu)	Competing with DHT for AR	10
Antithyroidal	TTR binding	T4	Displacing T4 from TTR	10
	UGT1A1 expression	Hexabromocyclododecane	Induced glucuronidation of T4	10
	UGT1A6 expression	Aroclor 1254	Induced glucuronidation of T4	10
	deiodinase-1 expression	3,3′,5-triiodothyronine (T3)	Induced deiodinization of T4	10
Tumor promoting	GJIC inhibition	PCB-136	Decreased communication between neighboring cells	50

Bioassay data analysis. A complete list of bioassays together with its mechanism of action, reference compound, and maximum test concentration is provided in Table 1. For a detailed description of experimental procedures, see Supplementary data. Dose-response curves were calculated, and concentrations corresponding with 50% effect (EC_{50}) or inhibition (IC_{50}) were determined at half (50%) of the maximum response level induced by the reference compounds (Table 1). EC_{50} or IC_{50} values exceeding the highest test concentration were set at a minimum value (Table 4).

If possible, *in vitro* toxic potencies of the PCBs were classified based on EC_{50} or IC_{50} values according to the criteria previously used by Hamers *et al.* (2006):

(1) No potency: Response < 20% at 10μM
(2) Weak potency: (EC_{50} or IC_{50}) > 10μM and response > 20% at 10μM
(3) Moderate potency: 1μM < (EC_{50} or IC_{50}) ≤ 10μM
(4) High potency: 0.1μM < (EC_{50} or IC_{50}) ≤ 1μM
(5) Very high potency: 0.01μM < (EC_{50} or IC_{50}) ≤ 0.1μM

Although no exact IC_{50} values could be calculated for the E2SULT inhibition assay (see Results section), its results could still be classified according to these criteria because the order of magnitude of IC_{50} values could be assessed from the observed dose-response relationships. Finally, gene expression results were only determined at 10μM exposure concentrations. By setting the highest UGT1A6 induction by PCB-126 to 100%, it could be deduced that the 50% induction level was reached by the other PCB congeners in the range 1–10μM (class 3).

Toxicity profiles based on classified bioassay responses were analyzed with HCA in SPSS (version 16.0 for Windows) using the between-group linkage method based on squared Euclidean distances between class values, similar as described for polybrominated diphenyl ethers (PBDEs) by Hamers *et al.* (2006). In HCA, PCBs were clustered together according to similarity in toxicity profile, and the results are presented as a dendrogram, i.e., a diagram illustrating which PCBs cluster together at successive stages of HCA and indicating the relative distance in toxicity profile between the individual PCB congeners and between the distinguished clusters of PCBs.

Toxic potency of complex PCB mixtures in human samples. To determine total toxic potencies of PCBs in human samples for each endpoint, 3 studies were selected, in which "average" human levels were reported for as many of the tested 24 congeners as possible, including the most potent AhR-activating PCB-126 (reference DL-PCB). Wingfors *et al.* (2000) reported PCB levels in blood and adipose tissue for two general populations in Spain and in Sweden. Hirai *et al.* (2005) analyzed PCBs in whole blood samples from healthy adult Japanese volunteers, and Wang *et al.* (2010b) in adipose tissue from a rural and an urban population in Southeast China. Out of the 24 congeners for which REP factors are reported in the present study, levels of 13 (54%; Spain and Sweden) to 15 (63%; Japan and China) PCB congeners were available. Mean PCB concentrations and number of samples analyzed reported in the three selected studies are presented in Supplementary table 1. For each mechanism of action, the total toxic potencies were calculated as a sum of the mean levels of each congener in humans multiplied by its respective REP factor for that mechanism of action determined in the present study (see Supplementary data for more details). Similarly, neurotoxic potencies of the PCB levels in human samples were calculated using neurotoxic REP values (Supplementary table 2) determined by Simon *et al.* (2007).

RESULTS

Bioassay Results

All tested PCBs showed antiandrogenic effects in the AR-CALUX bioassay (Table 2), and none of them had androgenic effects. The highest AR-antagonistic potencies (IC_{50} between 0.1 and 1μM) were found for PCB congeners 168, 125, 19, 122, 104, 128, 51, 126, 47, 136, and 28. Most PCBs had higher AR-antagonistic potency than the reference compound flutamide, an antiandrogenic drug used in prostate cancer treatment, whereas their potencies were in the same order of magnitude as those reported for PBDEs PBDE-100 and PBDE-47 (IC_{50} = 0.1 and 1.0μM, respectively), which are the strongest AR antagonists with environmental relevance reported to date (Hamers *et al.*, 2006). For most PCB congeners, the AR-antagonistic potencies could be counteracted by addition of an excess concentration of 200nM dihydrotestosterone (DHT), but not for PCBs 118, 122, 180, and 190. Nevertheless, visual observations and the resazurin viability assay did not indicate any cytotoxic effects of these PCBs in the U2OS cells of the AR-CALUX bioassay at 10μM.

Out of the tri- or tetrachlorinated congeners, PCBs 19, 28, 47, 51, and 53 exhibited significant but weak estrogenic activity in the ER-CALUX bioassay with EC_{50} values ranging from 5 to 15μM, but PCB-52 and PCB-74 did not (Table 3). Out of the pentachlorinated congeners, PCB-104 was the most potent estrogenic congener of the whole test set (EC_{50} = 3μM), whereas PCB-100, and to a lesser extent PCB-95 and PCB-125, also

TABLE 2
AR-Antagonistic Responses of the 24 PCBs and the Positive Reference Compound Flutamide in the AR-CALUX Assay

	AR antagonistic		
Compound	IC_{50} (μM) ± SD	Flutamide-REP ± SD	Luciferase activity at 10μM PCB (% control)
PCB-19	0.38 ± 0.05	7.8 ± 0.1	19
PCB-28	0.76 ± 0.36	4.6 ± 1.9	1
PCB-47	0.60 ± 0.03	5.6 ± 0.2	1
PCB-51	0.50 ± 0.03	5.8 ± 0.3	8
PCB-52	3.2 ± 1.5	1.1 ± 0.5	29
PCB-53	1.3 ± 0.2	2.4 ± 0.4	19
PCB-74	2.1 ± 0.2	1.6 ± 0.0	11
PCB-80	1.6 ± 0.3	2.0 ± 0.3	29
PCB-95	1.4 ± 0.2	2.1 ± 0.0	8
PCB-100	1.0 ± 0.2	3.3 ± 0.6	7
PCB-101	2.7 ± 0.4	1.1 ± 0.0	21
PCB-104	1.9 ± 0.6	1.7 ± 0.5	9
PCB-118	0.47 ± 0.01	7.0 ± 0.4	1
PCB-122	0.39 ± 0.00	8.6 ± 0.7	0
PCB-125	0.25 ± 0.03	13 ± 2	2
PCB-126	0.53 ± 0.14	5.7 ± 0.8	5
PCB-128	0.48 ± 0.02	6.9 ± 0.2	0
PCB-136	0.71 ± 0.16	4.2 ± 0.5	0
PCB-138	1.0 ± 0.3	3.2 ± 0.9	0
PCB-153	8.5 ± 4.1	0.43 ± 0.23	22
PCB-168	0.16 ± 0.01	20 ± 1	2
PCB-170	1.1 ± 0.1	3.1 ± 0.0	3
PCB-180	2.1 ± 0.3	1.4 ± 0.33	8
PCB-190	1.3 ± 0.2	2.2 ± 0.0	2
Flutamide	3.1 ± 0.3	1.0 ± 0.1	16

Note. AR-antagonistic potencies were tested in the presence of 200pM DHT; $n = 2$.

TABLE 3
ER-Agonistic and Antagonistic Responses of the 24 PCBs and Positive Reference Compounds E2 and ICI-182,780, Respectively, in the ER-CALUX Assay

	ER agonistic			ER antagonistic		
Compound	EC_{50} (μM) ± SD	E2-REP ± SD	Response at 10μM (% E2 max)	IC_{50} (μM) ± SD	ICI-182,780 REP ± SD	Luciferase activity at 10μM PCB (% control)
PCB-19	12 ± 4	13×10^{-8}	55	—	—	120
PCB-28	26 ± 2	6.0×10^{-8}	15	—	—	100
PCB-47	21 ± 2	7.4×10^{-8}	31	—	—	100
PCB-51	12 ± 2	13×10^{-8}	61	—	—	101
PCB-52	—	—	8	—	—	100
PCB-53	25 ± 12	6.1×10^{-8}	18	—	—	110
PCB-74	—	—	2	—	—	100
PCB-80	—	—	3	—	—	114
PCB-95	—	—	10	—	—	105
PCB-100	13 ± 1	12×10^{-8}	58	—	—	85
PCB-101	—	—	0	—	—	83
PCB-104	3 ± 1	46×10^{-8}	122	—	—	171
PCB-118	—	—	0	—	—	100
PCB-122	—	—	0	—	—	90
PCB-125	—	—	7	—	—	100
PCB-126	—	—	0	—	—	100
PCB-128	—	—	10	—	—	93
PCB-136	6 ± 1	25×10^{-8}	80	—	—	124
PCB-138	—	—	0	16 ± 1	1.8×10^{-6}	65
PCB-153	—	—	0	24 ± 3	1.3×10^{-6}	69
PCB-168	—	—	2	—	—	125
PCB-170	—	—	0	24 ± 2	1.3×10^{-6}	80
PCB-180	—	—	0	14 ± 5	2.2×10^{-6}	60
PCB-190	—	—	0	18 ± 3	1.7×10^{-6}	68
E2	$(16 \pm 6.1) \times 10^{-7}$	1.0	100	ND	ND	ND
ICI-182,780	ND	ND	ND	$(30 \pm 4.9) \times 10^{-6}$	1.0	0

Note. ER-antagonistic potencies were tested in the presence of 6pM E2; $n = 3$; —, no response; ND; not determined.

exhibited ER-mediated activity. PCB-136 was the only hexachlorobiphenyl in the test set eliciting ER-mediated activity ($EC_{50} = 6\mu M$). Other pentachlorobiphenyls (including mono-*ortho*-chlorinated PCB-118 and coplanar PCB-126) and hexachlorobiphenyl PCB-128 elicited no effect in the ER-CALUX bioassay. All other hexa- and heptachlorinated PCBs studied (i.e., PCBs 138, 153, 168, 170, 180, and 190) exhibited weak antiestrogenic activities at micromolar concentrations (IC_{50} values ranging from 14 to > 25μM).

E2SULT-inhibiting potency was found for many tested PCBs at concentrations ranging between 1 and 10μM (Table 4). Highest E2SULT-inhibiting activity was found for PCBs 100, 101, and 138, although no clear dose-response relationship could be observed for the latter congener. For PCBs 47, 100, 101, and 104, dose-response curve fits were too unreliable to report IC_{50} values in Table 4, as shown in Figure 1.

TTR-binding capacity was observed for only a few PCBs (Table 4): full dose-response curves could only be determined for PCBs 80, 122, 125, and 168. Full inhibition (i.e., 100% replacement of T4 from TTR) was observed for PCB-125 and PCB-168, which have high structural similarity to T4, whereas maximum inhibition by PCB-80 and PCB-122 leveled off to inhibition levels of 40–55%, respectively. Increasing test concentrations of these 2 congeners did not lead to further inhibition. As a result, no IC_{50} value could be determined for PCB-80 (Fig. 1). Based on its relatively high TTR-binding potency at sub-micromolar levels (Fig. 1), however, PCB-80 was arbitrarily classified in class 3 (moderately high) and not in class 1 for this bioassay. Very weak TTR-binding activities were found for PCB congeners 138, 153, 170, 180, and 190, showing > 20% inhibition at the highest test concentration (10μM).

In the rat hepatoma H4IIE cell line, exposure to 10μM of DL-PCBs 74, 118, or 126 caused an upregulation of UGT1A6 messenger RNA (mRNA) expression up to a factor 2.8. PCB-138 was the only NDL-PCB in the test set causing an upregulation of UGT1A6 up to a factor 1.5. None of the PCB congeners tested affected mRNA expression of UGT1A1 or DI-1.

NDL-PCB congeners with 2,2′6 substitution (PCBs 19, 51, 53, 95, 104, and 136) had moderate GJIC-inhibiting potencies with IC_{50} values ranging from 5 to 8μM (Table 5), except for 2,2′4,4′6-substituted PCB-100 ($IC_{50} = 16\mu M$). Out of the six indicator PCBs, PCB-52 also had a moderate

TABLE 4
E2SULT-Inhibiting and T4-TTR–Competing Potencies of the 24 PCBs and the Positive Reference Compounds T4 and PCP, Respectively, in the TTR-Binding and E2SULT Assays

	E2SULT inhibition[a]		TTR binding		
Compound	IC_{50} (μM) ± SD	E2SULT activity at 10μM compared with control (%)	IC_{50} (μM) ± SD	T4-REP ± SD	T4 binding at 10μM compared withcontrol (%)
PCB-19	+	71	—	—	126
PCB-28	+	42	—	—	88
PCB-47	++[b]	47	—	—	103
PCB-51	++	38	—	—	122
PCB-52	++	53	—	—	98
PCB-53	+	45	—	—	114
PCB-74	—	98	—	—	99
PCB-80	—	94	—[c]	—	60
PCB-95	++	59	—	—	107
PCB-100	+++[b]	44	—	—	104
PCB-101	+++[b]	63	—	—	101
PCB-104	++[b]	53	—	—	112
PCB-118	+	78	—	—	82
PCB-122	—	113	0.79 ± 0.23[d]	0.083 ± 0.035	45
PCB-125	+	82	3.7 ± 0.4	0.014 ± 0.003	6
PCB-126	—	97	> 15	< 0.0038	78
PCB-128	+	85	—	—	83
PCB-136	—	91	—	—	84
PCB-138	+[e]	74	> 15	< 0.0038	66
PCB-153	—	97	> 15	< 0.0038	73
PCB-168	—	90	1.3 ± 0.2	0.041 ± 0.011	10
PCB-170	—	97	> 15	< 0.0038	74
PCB-180	—	93	> 15	< 0.0038	73
PCB-190	—	106	> 15	< 0.0038	77
T4	ND	ND	0.057 ± 0.010	1.0 ± 0.0	11
PCP	0.29 ± 0.06	3	ND	ND	ND

Note. $n = 2$; —, no response; ND, not determined.

[a]No IC_{50} values could be determined for the E2SULT assay. +, > 20% inhibition at 10μM; ++, > 20% inhibition at 3μM; +++, > 20% inhibition at 1μM.

[b]E2SULT-inhibiting compounds with maximum inhibition levels (plateau) of 38–58% of the maximum inhibition by PCP.

[c]No IC_{50} value could be determined because at PCB-80 concentrations > 1μM, the dose-dependent decrease in T4-TTR binding leveled off to a plateau level of 60% of the maximum T4-TTR binding at 10μM (see Fig. 1).

[d]Although 50% inhibition was observed at $IC_{50} = 0.8$μM, T4-TTR binding leveled off from this concentration to a plateau level of 45% of the maximum T4-TTR binding at 10μM.

[e]> 20% inhibition was found at 0.3–10μM, but not in a dose-dependent way.

GJIC-inhibiting potency ($IC_{50} = 9$μM), whereas the most abundantly occurring PCBs in the environment, PCB-138 and PCB-153, along with other di-*ortho*- and mono-*ortho*-substituted PCBs, had weak potencies ($IC_{50} > 10$μM). Out of these weak GJIC-inhibiting congeners, heptachlorinated PCBs 170, 180, and 190 had again weaker GJIC inhibitory potencies ($IC_{50} \geq 23$μM) than tri- to hexachlorinated PCBs ($IC_{50} < 23$μM). Non–*ortho*-substituted PCBs 80 and 126 had no effect on GJIC.

Toxicity Profiling

Based on their toxicity profiles, the test set of PCBs could be divided in four different clusters by multivariate HCA (Fig. 2). Cluster I consisted of NDL-PCBs with moderate to high AR-antagonistic potencies and weak TTR-binding, GJIC-inhibiting, and antiestrogenic potencies. This cluster contained the most abundant environmental PCB congeners: 138, 153, 170, and 180. Cluster II had moderate to high AR-antagonistic and TTR-binding potencies and weak GJIC-inhibiting potencies. Cluster III consisted of DL-PCBs with moderate UGT1A6-inducing potencies via AhR activation and moderate to high AR-antagonistic potencies. Cluster IV consisted of NDL-PCBs with moderate to high AR-antagonistic potencies, weak to moderate GJIC-inhibiting and E2SULT-inhibiting potencies, and no TTR-binding potencies. Two subclusters could be further distinguished, based on the absence (IV.1) or presence (IV.2) of weak ER-agonistic potencies. The tetra-*ortho*-substituted PCBs 104

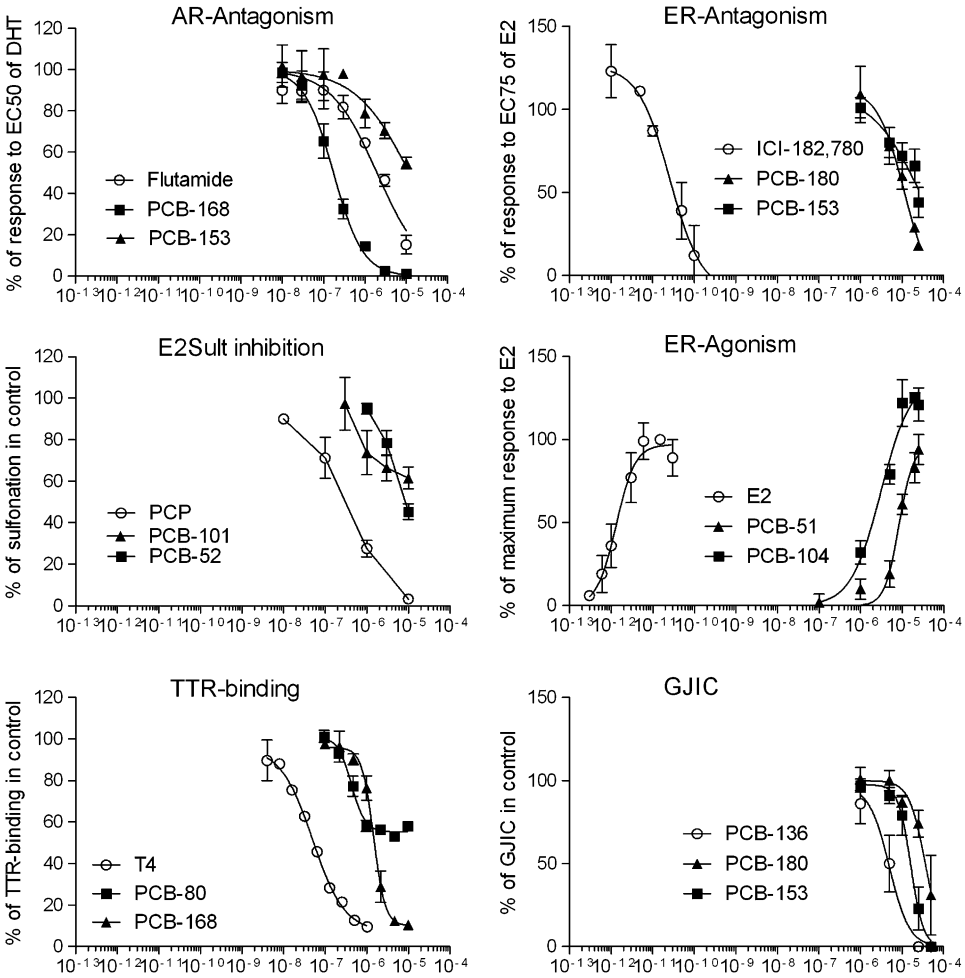

FIG. 1. Typical examples of dose-response curves for NDL-PCBs in the in vitro bioassays: antagonistic responses toward the AR (A) and ER (B), inhibition of E2SULT (C), agonistic responses toward the ER (D), competition with T4 for TTR binding (E), and inhibition of GJIC (F). For each mechanism of action, results are presented for one or more exemplary NDL-PCBs and the positive reference compound used to calculate REP values. Values on the x-axis represent molar concentrations.

and 136 could not be classified in any of the four clusters and stood apart from the other congeners, given their moderate ER-agonistic potencies.

Contribution of Individual NDL-PCB Congeners to the Toxic Potency in Complex Mixtures

For the congeners tested in the present study, PCB concentrations in human samples declined in the order Spain ≈ Sweden > Japan > China (Table 6). In all five data sets, PCB congener composition in human samples was dominated (> 50%) by hexachlorinated PCBs, followed by heptachlorinated PCBs in Europe and Japan and by pentachlorinated PCBs in China (Fig. 3; Supplementary table 1). Wang *et al.* (2010b) attributed the relatively low PCB concentrations and the different congener pattern in Chinese samples, as compared with European studies, to a shorter time span of production and usage of PCBs and lower influence of dietary intake in China.

Due to the relatively small variation in REP values (Tables 2–5), the contribution of different PCB congeners to the overall GJIC-inhibiting, AR-antagonistic, and neurotoxic potencies generally reflected the molar composition pattern of the samples, with most abundant congeners contributing most to the calculated potency (Fig. 3). This observation was most obvious for the GJIC-inhibiting potency. Relatively high REP values caused a higher contribution to the neurotoxic potencies by PCB-180 in the European and Japanese studies and by PCB-118 in the Chinese study. Similarly, a relatively high AR-antagonistic REP value resulted in a higher antiandrogenic contribution of PCB-118 than expected based on its analyzed levels, whereas a relative low REP value resulted in a lower contribution of PCB-153. In addition, the Chinese study made clear that PCB-168, which had the highest AR-antagonistic REP value but was not analyzed in the other studies, is an important contributor to overall AR-antagonistic potency (Fig. 3). Also for the TTR-binding potency of PCBs in human samples, the Chinese case study data identified PCB-168

TABLE 5
Inhibition of GJIC by the 24 PCBs and REP toward the Most Potent Congener PCB-136 in the Scrape-Loading Dye Transfer Assay with WB-F344 Cells

Compound	IC_{50} (μM) ± SD	PCB-136 REP	% Control GJIC at 10μM
PCB-19	6 ± 0.8	0.7	11
PCB-28	15 ± 2.4	0.3	73
PCB-47	13 ± 1.0	0.4	55
PCB-51	7 ± 0.7	0.6	21
PCB-52	9 ± 2.2	0.5	44
PCB-53	6 ± 0.6	0.8	7
PCB-74	18 ± 1.3	0.3	82
PCB-80	—	—	100
PCB-95	8 ± 2.0	0.6	24
PCB-100	16 ± 1.8	0.3	77
PCB-101	13 ± 3.4	0.4	60
PCB-104	8 ± 1.2	0.6	26
PCB-118	19 ± 0.5	0.2	72
PCB-122	22 ± 4.2	0.2	82
PCB-125	13 ± 1.0	0.4	66
PCB-126	—	—	96
PCB-128	13 ± 0.7	0.3	60
PCB-136	5 ± 0.9	1.0	0
PCB-138	14 ± 1.0	0.3	68
PCB-153	16 ± 2.3	0.3	73
PCB-168	23 ± 6.7	0.2	83
PCB-170	30 ± 2.9	0.2	92
PCB-180	43 ± 10.3	0.1	87
PCB-190	23 ± 4.7	0.2	85

Note. $n = 3$; —, no response.

as an important congener, contributing to > 80% of the calculated T4-competing potency. Estrogenic potencies could be attributed by 76–100% to PCB-28, whereas antiestrogenic potencies mostly reflected the composition of predominant higher chlorinated PCBs 138, 153, 170, 180, and 190.

DISCUSSION

Epidemiological studies have found associations between exposure to PCBs and effects on reproduction and (neuro)-development, thyroid system, nervous system, immune system, cardiovascular system, growth, lipid metabolism, diabetes, and obesity (e.g., reviews by ATSDR, 2000; EFSA, 2005; Hatch *et al*., 2010; Korrick and Sagiv, 2009; Meeker and Hauser, 2010; Wang *et al*., 2010a). Many of the observed associations are still under scientific study and debate because they lack mechanistic support, are not expected at the measured exposure levels, or could not be confirmed in other studies.

In general, observed associations between PCB exposure and human effects are difficult to be attributed to either DL- or NDL-PCBs because humans are simultaneously exposed to both classes of PCBs. By comparing results from animal studies with single DL- or NDL-PCB congeners, however, Rice (2004) concluded that, except for PCB-126, "there is not much difference in potency between the DL and NDL congeners" on reproductive/developmental, thyroid, immune, and neuropsychological functions, which are "presumably the most sensitive functional endpoints" to PCB exposure. This comparison, however, is hampered by the limited number of congeners that have been studied to date and the possible DL contamination of the NDL-PCB standards used in these animal studies.

Results from the present study with ultrapure PCB congeners, however, indicate that individual NDL-PCB congeners may exert specific mechanisms of action *in vitro* that may be well associated with some of the effects observed in epidemiological and animal studies. Interference with sex steroid signaling (AR- and ER-CALUX and E2SULT inhibition bioassays) may be linked to the observed association in humans between PCB exposure and effects on reproductive health in males (semen quality and sperm DNA integrity) and females (menstrual irregularities, miscarriage, fetal death, and decreased gestational age) and on sex ratio of newborns (e.g., ATSDR, 2000; Brouwer *et al*., 1999; EFSA, 2005; Meeker and Hauser, 2010). Thyroid hormone disruption (TTR-binding assay and UGT gene expression) may be linked to hypothyroidism, which in turn affects neurodevelopmental outcomes such as psychomotor development, mental development, IQ, memory, and hearing (Brouwer *et al*., 1999; Trnovec *et al*., 2008). Decreased GJIC may be closely linked to the carcinogenic potency of PCBs, which has unequivocally been demonstrated in animal studies (ATSDR, 2000) but is still an issue of debate for the human situation. A recent weight of evidence evaluation (Golden and Kimbrough, 2009) did not support a causal relationship between PCB exposure and human cancer, although the U.S. Environmental Protection Agency and the International Agency for Research on Cancer have classified PCBs as "probably carcinogenic to humans."

In Vitro Toxicity of NDL-PCBs in Relation to Their Structure

All PCBs in the test set had AR-antagonistic potencies similar to or higher than flutamide, an antiandrogenic drug used in prostate cancer treatment. Structural requirements for AR-antagonistic potency of PCBs were not obvious, but highest potencies were found for PCB-168 and PCB-125, with di-*ortho* chlorine substitution in one phenyl ring and non-*ortho* substitution in the other ring. PCB-190 also meets this description, but its AR-antagonistic potency was not higher than observed for other congeners. Relative AR-antagonistic potencies of PCB-138 > PCB-153 > PCB-180 observed in DHT-exposed AR-CALUX cells confirmed results in R1881-exposed CHO cells transiently transfected with an AR-responsive luciferase reporter gene (Bonefeld-Jørgensen *et al*., 2001). In the present study, DL-PCBs and NDL-PCBs had similar AR-antagonistic potencies, suggesting that *ortho* substitution in PCBs is a less critical requirement for antiandrogenic potencies than in PBDEs (Harju *et al*., 2007). Similar to PBDEs, none of the tested PCB congeners exhibited AR-activating potencies, possibly because

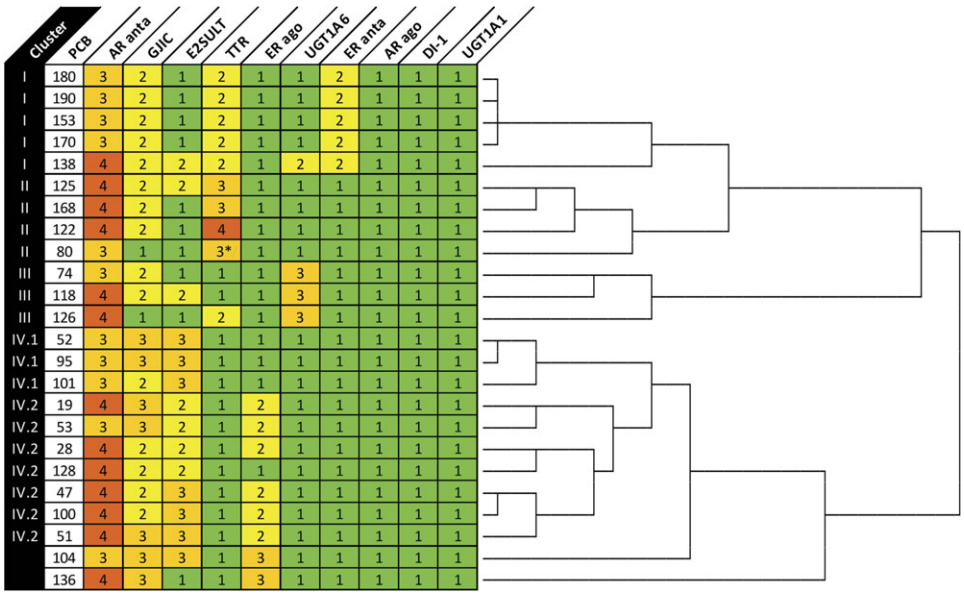

FIG. 2. HCA of the classified in vitro toxicity profiles of 24 PCBs. Classes (1–5) indicate no toxic potency (1) to very high toxic potency (5) for the different mechanisms of action according to the criteria given in the Materials and Mehtods section, with an exception for the TTR-binding potency of PCB-80 (see Results section).

PCBs have no hydrogen-bonding ability, unlike the natural ligands testosterone and DHT (Harju *et al.*, 2007).

Weak ER-agonistic potency was observed for lower chlorinated PCBs with 2,2′,6- or 2,4,4′ substitution and combinations thereof. Further substitution in *meta* positions 3 or 5 clearly diminished or abolished ER-agonistic potencies, except for tetra-*ortho*-chlorinated PCB-136, which was, together with the other tetra-*ortho*-chlorinated PCB-104, the most potent ER agonist among the tested PCBs. Potent ER binding by tetra-*ortho*-chlorinated PCBs has been described before (Matthews and Zacharewski, 2000). Possibly, the rotational rigidity of this molecule reflects the planar rigidity of E2 and favors fit of the molecule into the ER pocket (DeCastro *et al.*, 2006). In general, the observed structure-activity relationships for ER-agonism confirm earlier studies with PCBs and structurally related PBDEs in the ER-CALUX (Hamers *et al.*, 2006; Meerts *et al.*, 2001; Pliskova *et al.*, 2005) and with PCBs in an MCF-7 proliferation test (Andersson *et al.*, 1999), with highest estrogenic potency for lower halogenated compounds preferably with a substitution pattern in two to four *ortho* positions and one *para* position. The observed ER-antagonistic potency for higher chlorinated PCBs also confirmed earlier results in the ER-CALUX bioassay, the MCF-7 proliferation assay, and in transiently transfected reporter gene assays with MCF-7 and MDA-MB-231 cells (Bonefeld-Jørgensen *et al.*, 2001; Pliskova *et al.*, 2005).

Except for mono-*ortho*-chlorinated PCB-28, all other mono-*ortho* (PCBs 74, 118, and 122) and non-*ortho* (PCB-126) congeners did not show any estrogenic or antiestrogenic activity in the ER-CALUX bioassay. In contrast, Krishnan and

TABLE 6
PCB Concentrations (nmol/g Lipid) of the 19 PCB Congeners that Were Tested in the Present Study and Analyzed in Human Samples, together with Their Overall Calculated Toxic Potencies Expressed in Equivalent Concentrations (nmol/g lipid) of the Reference Compounds

Reference	Tarragona, Spain Wingfors *et al.* (2000)	Örebro, Sweden Wingfors *et al.* (2000)	Japan Hirai *et al.* (2005)	Anhui Province, China Wang *et al.* (2010b)	Jiangsu Province, China Wang *et al.* (2010b)
ΣPCB	2.70	2.51	0.250	0.0492	0.0332
ΣAR-antagonistic potency (Flu-Eq)	5.75	5.44	0.520	0.301	0.205
ΣER-agonistic potency (E2-EQ)	0.126	0.105	0.0392	0.0177	0.0186
ΣER-antagonistic potency (ICI-182,780-EQ)	4.04	3.67	0.308	0.0360	0.0243
ΣTTR-binding potency (T4-EQ)	0.00905	0.00846	0.000726	0.000459	0.000309
ΣGJIC-inhibiting potency (PCB-136-EQ)	0.631	0.615	0.063	0.0127	0.0081
ΣNeurotoxic potency (PCB-53-EQ)	0.494	0.416	0.046	0.00707	0.00457

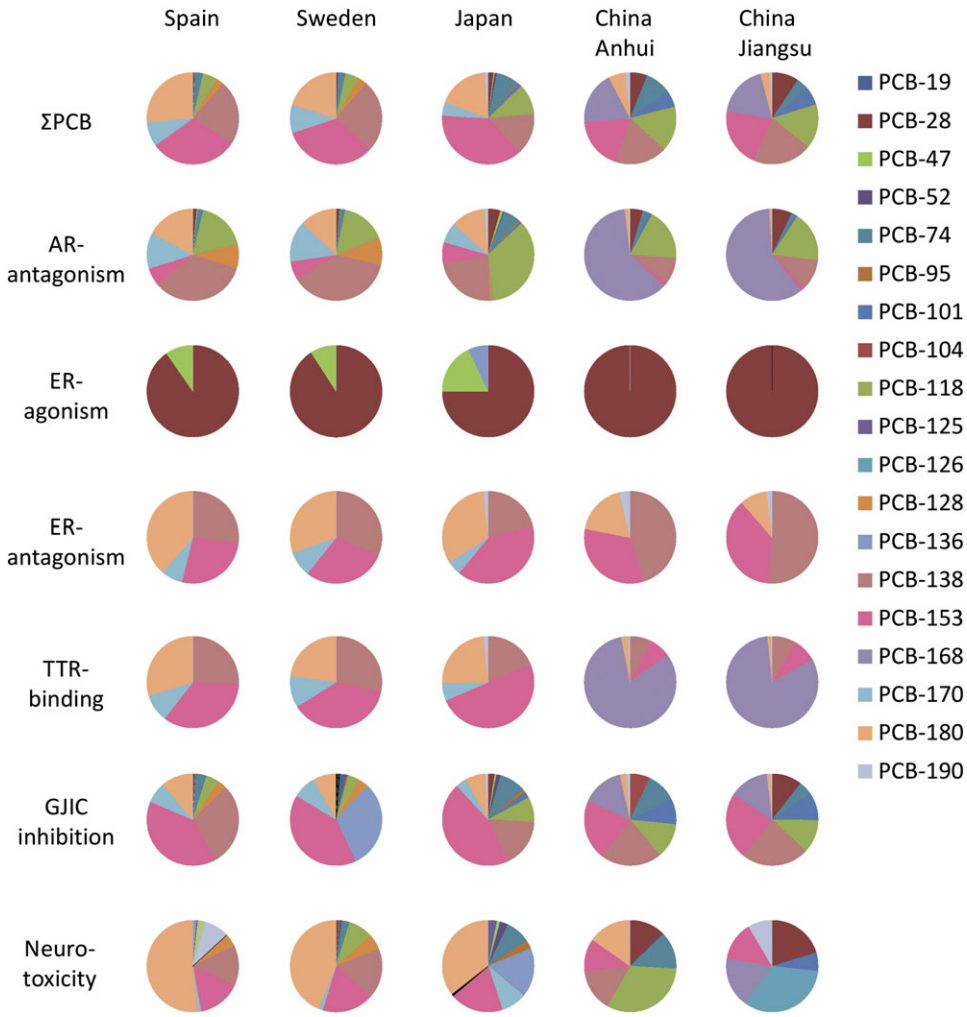

FIG. 3. Contribution of the individual congeners to the molar composition of the PCB mixture and its calculated toxic potencies in human samples. ΣPCB and total toxic potencies are given for each study in Table 6. Not all congeners were analyzed in each study (see Supplementary table 1).

Safe (1993) reported that antiestrogenic potencies of coplanar PCBs in MCF-7 cells correlated with their AhR-activating potencies. A possible explanation for AhR-mediated inhibition of E2-induced transactivation is that the activated AhR binds to inhibitory dioxin-responsive elements (iDREs) present in promoter regions of E2-responsive target genes (Safe *et al.*, 1998). The lack of iDREs in the reporter construct of the ER-CALUX bioassay may explain why antiestrogenic potency of DL-PCBs was not observed in the ER-CALUX bioassay (Table 3; Pliskova *et al.*, 2005).

Inhibition of E2SULT activity is an alternative mechanism by which compounds can exert estrogenic properties. By inhibiting the formation of inactive E2 sulfonates, indirect estrogenic effects may appear due to the enhanced bioavailability of E2 in target tissues. Weak E2SULT-inhibiting potencies were observed for many PCBs at the highest test concentration of 10μM. Dose-dependent E2SULT inhibition could only be determined for four NDL-PCBs, but curve fits were considered unreliable and not all PCBs inhibited E2SULT activity in a dose-dependent way. Tetra- or pentachlorinated PCBs, with a 2,2′4- or 2,2′5-substitution pattern, had the highest E2SULT-inhibiting potencies.

Not only parent NDL-PCB compounds but also their metabolites may exert (anti)estrogenic activity. *In vitro* ER-(ant)agonistic potencies of hydroxylated or methyl-sulfonyl PCB metabolites have been reported at similar micromolar effect concentrations as found for their parent compounds (e.g., Andersson *et al.*, 1999; Letcher *et al.*, 2002). Moreover, E2SULT-inhibiting potencies of hydroxylated PCB metabolites were up to four orders of magnitude higher than observed for their parent compounds, i.e., IC_{50} concentrations in the below nanomolar range (Kester *et al.*, 2000). In conclusion, (anti)-estrogenic activities of PCB metabolites should be taken into account in NDL-PCB hazard characterization.

TTR-binding potencies of PCBs were very low or absent. Up to 10μM test concentrations, full dose-response curves

were only obtained for PCBs 80, 122, 125, and 168. Full inhibition of T4-TTR binding was only observed for PCBs 125 and 168. The TTR-binding congeners contained a non–*ortho*-substituted phenyl ring with chlorine substitution in 3,5 or 3,4,5 positions. In addition, TTR-binding potencies were further increased when the other phenyl ring was substituted in one (PCB-122) or two (PCB-125 and PCB-168) *ortho* positions, giving the congener a structure resembling reversed triiodothyronine or T4, respectively.

Chauhan *et al.* (2000) also tested the TTR-binding potency of 47 different PCB congeners, but surprisingly poor correspondence was observed for the 16 congeners that were also tested in the present study. Similar to the present study, Chauhan *et al.* (2000) found highest TTR-binding potency for PCB-80 ($IC_{50} = 7$nM), but no IC_{50} value could be estimated in the present study because TTR binding leveled off to a maximum of 40% T4-TTR–binding inhibition (Fig. 1). For the remaining 15 PCB congeners, even bigger differences were found between both studies. At 10μM test concentrations, we found very weak TTR binding for PCBs 138, 153, and 180 and no TTR binding at all for PCBs 95, 100, and 101, whereas Chauhan *et al.* (2000) reported IC_{50} values for these congeners ranging from 28 to 690nM. No explanations are available for the discrepancies between both studies, especially when taking into account that sensitivities toward the natural ligand T4 were highly comparable, i.e., IC_{50} values of 57 (Table 4) versus 49nM (Chauhan *et al.*, 2000).

Similar to E2SULT inhibition, hydroxylated PCB metabolites have TTR-binding potencies in the same order of magnitude as the natural ligand T4, whereas their parent compounds have very low or no binding potency at all (Lans *et al.*, 1993). To determine the effect of PCB exposure on T4-TTR binding, information is required about biotransformation rates of PCBs, as well as the identity, fate, and TTR-binding potency of their hydroxylated metabolites.

Alternative to TTR binding, PCBs can act as thyroid hormone disruptors by inducing T4 glucuronidation causing increased hepatic clearance of T4 (Hood and Klaassen, 2000). Therefore, the effect of PCBs was tested on mRNA expression of T4-glucuronidating isozymes UGT1A1 and UGT1A6 (Vansell and Klaassen, 2002). Upregulation of UGT1A6 expression by DL-PCBs, as observed in the rat hepatoma H4IIE cell line, is most likely caused by AhR activation. Upregulation of UGT1A6 by NDL-PCBs is *in vivo* most likely caused by activation of the constitutive androstane receptor (CAR) (Shelby and Klaassen, 2006). It is not clear, however, if the observed *in vitro* upregulation of UGT1A6 by PCB-138 in H4IIE cells is also CAR mediated because the H4IIE cell line hardly expresses CAR (Fery *et al.*, 2010).

The GJIC-inhibiting potencies of highly purified PCB congeners corresponded with the previous data obtained with 37 PCB standards that were not purity checked (Machala *et al.*, 2003). In both studies, noncoplanar congeners had highest GJIC-inhibiting potencies, but the observation that 2,2′, 6-substituted congeners had higher potencies than 2,2′- or 2,6-substituted congeners was more pronounced in the present study. In addition, PCBs 52, 95, and 136 were > two times more potent than previously reported. Also Hemming *et al.* (1991) have suggested that GJIC inhibition increases with the number of chlorinated *ortho* positions. These results suggest that the PCB-induced acute closure of GJIC is a result of a specific mechanism and is not due to unspecific membrane perturbations. PCBs can induce long-term disruption of GJIC via increased internalization and degradation of connexin43 and via inhibition of connexin43 transport, as was recently demonstrated for PCB-153 in rat liver epithelial cells (Simeckova *et al.*, 2009).

Toxicity Profiling

Multivariate statistical methods pointed out that the PCB test set could be divided into at least four different clusters. Such classification can be used to identify common structural features that are typical for a given toxicity profile. As expected, DL-PCB congeners 74, 118, and 126 were classified together in a single cluster (cluster III) based on their ability to induce AhR-mediated UGT1A6 expression. Two additional mono-*ortho*-chlorinated congeners in the test set, i.e., PCBs 28 and 80, displayed no UGT1A6 induction and were classified in different clusters. PCB-80 was classified in cluster II together with congeners sharing a non-*ortho* 3,4,5-chlorination pattern on one ring and 1 or 2 chlorinated *ortho* positions on the other ring. Classification in cluster II was clearly driven by the TTR-binding activity, which is not surprising given the overlap in substitution pattern between these PCB congeners and T4. Other hexa- to heptachlorinated PCBs with di-*ortho* substitution in 2,6 or 2,2′ positions were classified in a separate cluster I, mainly based on their weak ER-antagonistic potency. PCB-28 was classified in cluster IV together with congeners characterized by a 2,5-substitution pattern (subcluster IV.1) or a 2,6- or 2,(3,)4-substitution pattern (subcluster IV.2) in one of the phenyl rings. The tetra-*ortho*-chlorinated PCBs 104 and 136 both had deviating toxicity profiles due to their moderate ER-agonistic potencies, which were not observed for any of the other PCBs. The toxicity profile of PCB-136 did not fit into any of the four clusters distinguished in the test set, whereas the profile of PCB-104 was in-between cluster IV (2,6 substitution in one ring) and PCB-136.

Toxic Potency of PCBs in Human Samples and Contribution of the Individual Congeners

Based on the IC_{50} levels, AR antagonism was apparently the most sensitive endpoint affected by the tested NDL-PCB. The calculated total potency in human samples ranged from 0.2 to 6 nmol Flu-Eq/g lipid (Table 6). In flutamide treatment of prostate cancer, the main metabolite 2-hydroxy-flutamide has an average therapeutic steady-state concentration of 940 ng/ml in plasma (Schulz *et al.*, 1988), which can be calculated into

6 μmol Flu-Eq/g lipid, based on a sevenfold higher affinity of the metabolite for the human AR than flutamide (Simard *et al.*, 1986), a molecular weight of flutamide of 276.2 g/mol, and a plasma lipid content of 4 g/l. These data suggest that the calculated AR-antagonistic potency of complex PCB mixtures in humans is 1000–30,000 times lower than the therapeutic potency, which can effectively compete with natural androgenic hormones for binding to prostate AR in humans. It should be realized, however, that this margin of safety was calculated for "average" and not for highly exposed human populations and that 2-hydroxy-flutamide is cleared from the body much faster (half-life < 1 day) than the persistent PCBs. This holds especially for the most abundant NDL-PCBs 138, 153, 170, 180, and 168, which also contributed most to the AR-antagonistic potency in human samples (Fig. 3).

The observation that most abundant NDL-PCBs contribute most to the calculated total toxic potency in the human samples does not hold only for the individual congeners but of course also for different classes of congeners, based on *ortho*-substitution level, chlorination level, status as indicator PCB (yes or no), or toxicity profiles (Supplementary figs. 2–5). For instance, the dominance of di-*ortho*-substituted PCBs (> 69%) and hexa- and heptachlorinated biphenyls (> 63%) in the molar composition of the human samples was reflected by a > 68 and > 51% dominance, respectively, of these groups in the calculated toxic potencies. Two exceptions were observed, i.e., (1) the ER-agonistic potency is 100% dominated by tri- to pentachlorinated PCBs, of which 76–100% by PCB-28, and (2) the neurotoxic potency in the Chinese study is dominated for > 58% by mono-*ortho*-substituted tri- to pentachlorinated PCBs 28, 74, and 118. The most potent AhR-activating DL-PCB-126 contributed at maximum 0.2% to any of the calculated toxic potencies, i.e., the AR-antagonistic potency in the Japanese study.

The complex PCB mixtures in human samples consisted for 71–86% of indicator PCBs including PCB-118 and for 55–81% excluding PCB-118. Except for the AR-antagonistic and TTR-binding potencies in the Chinese case study, the set of indicator PCBs including PCB-118 could explain 74–100% of the calculated toxic potencies in the human samples and 48–100% excluding PCB-118. From the Chinese case study, it became clear that PCB-168, which was not analyzed in the European and Japanese studies, is the most important "non-indicator" congener. On average, a 0.96 ratio was found between molar concentrations of PCB-168 and the most abundant congener PCB-153. Making up 18–19% of the molar composition of the PCB mixture, PCB-168 explained 60–61% of the AR-antagonistic, 81–82% of the TTR-binding, and 14% of the GJIC-inhibiting potencies calculated for the Chinese study. Assuming a similar PCB-168:PCB-153 ratio for the European and Japanese case study, it was estimated that PCB concentrations would increase on average by 30% (Europe) and 7% (Japan) if PCB-168 was taken into account. Consequently, estimated AR-antagonistic potencies would increase by a factor 3.8 for the European and a factor 1.7 for the Japanese case study, TTR-binding potencies by a factor 4.7 and 2.0, and GJIC-inhibiting potencies by a factor 1.2 and 1.1, respectively.

Representative PCBs from all four distinguished toxicity profiles were only analyzed in the Chinese study. In general, almost half of the ΣPCB levels consisted of cluster I congener PCBs 138, 153, 170, and 180, whereas the other half was equally divided by clusters II, III, and IV. As could be expected from the toxicity profiles, all four clusters contributed to GJIC and AR-antagonistic potencies, albeit that the latter was dominated by cluster II (PCB-168) rather than by cluster I. ER-agonistic potency was completely attributed to cluster IV (PCB-28) and ER-antagonistic potency to cluster I. Cluster II (PCB-168) dominated the TTR-binding potency, whereas it did not contribute to the neurotoxic potency.

CONCLUSIONS

The present study demonstrates that ultrapure NDL-PCBs have *in vitro* toxic potencies, which are not mediated through AhR activation but may well be associated with reprotoxic, neurotoxic, and carcinogenic effects observed in animal studies and epidemiological studies. Four different *in vitro* toxicity profiles could be distinguished for the set of 24 tested PCBs, of which one could be attributed to AhR activation by DL-PCBs and three others to combinations of non–AhR-mediated mechanisms of action. Due to the small variation in REP values, most abundant congeners also contributed most to the calculated toxic potencies of complex PCB mixtures analyzed in human samples. PCB-168 was recognized as a congener that is not regularly monitored but may contribute considerably to the AR-antagonistic and TTR-binding potency of the complex mixture of PCBs in human samples. Therefore, it is recommended to extend the set of seven indicator PCBs with PCB-168 for future human monitoring studies. Based on the current knowledge of PCB levels in human tissues and toxic potencies of individual congeners, the seven indicator PCBs plus PCB-168 may explain on average > 74% of the calculated toxic potency of PCB mixtures in humans. The studied bioassays reflect critical mechanisms of action that may well underlie toxicities observed in *in vivo* and in epidemiological studies. Current risk assessment procedures for PCBs only consider the AhR-activating DL-PCBs. According to the results of the present study, however, DL-PCBs only have a minor contribution to the non–AhR-mediated toxic potencies calculated for complex PCB mixtures found in the general human population. Therefore, NDL-PCBs should be included in future risk assessment concepts.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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