Agonists of the Dioxin Receptor: Environmental Contaminants, Food Constituents, Microbial Metabolites, and Tumor Promoters

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The dioxin- or aryl hydrocarbon receptor (AhR) is a member of the Per-Arnt-Sim family of nuclear transcription factors exhibiting a basic helix-loop-helix structure. In its non-ligated state the AhR is associated with hsp 90 and the immunophilin-type XAP2. Upon ligand binding the associated proteins are released, the receptor dimerizes with the AhR nuclear translocator protein Arnt, and binds to XREs (xenobiotic-responsive elements) in the 5'-flanking region of responsive genes thus modulating their transcription.

Induction of cytochrome P450 (CYP) 1A1 and its catalytic activity measured as 7-ethoxyresorufin O-deethylase (EROD) in mammalian cells are well-established functional parameters for AhR activation. We used the analysis of CYP1A1 induction, e.g., as a tool to analyze the relative potencies of polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and biphenyls (PCBs) as dioxin-receptor agonists. In rat hepatocytes in primary culture and in the rat hepatoma cell line H4IIE, a rank order of relative inducing potencies, usually measured as EC_{50} values, was established for these compounds. In human HepG2 hepatoma cells, a lower potency and a different rank order of relative potencies was found.

Furthermore, CYP1A1 induction allows the screening for yet unknown AhR agonists, e.g., among natural compounds. This approach led to the identification of tryptanthrins, secondary metabolites formed by *Candida lipolytica* from tailor-made precursors as AhR agonists. AhR activation by tryptanthrins was confirmed by gel-retardation and supershift analysis using a labeled XRE-oligo. Another novel group of AhR agonists comprises microbial metabolites formed *in situ* such as malassezin produced by the dermal fungus *Malassezia furfur*.

Furocoumarins, a family of almost planar molecules found in a variety of plants of the *Compositae* (celery, parsnip) or *Citrus* (lemon, lime) families also act as inducers of CYP1A1 in rat liver cells without detectable activation of DNA binding of the AhR or activation of an XRE-containing reporter gene. Thus, an alternative mechanism of CYP1A1 induction seems to be operative with furocoumarins possibly related to their pronounced capacity to inhibit the catalytic activity of the enzyme.

Finally, CYP1A1 induction can serve as a parameter of AhR activation in comparison to other effects of AhR agonists. Likewise, the potent tumor-promoter of rodent liver 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is thought to suppress the rate of apoptosis usually found in preneoplastic hepatocytes. This effect thought to represent a crucial mechanism of liver tumor promotion, can also be found *in vitro* in rat hepatocytes in primary culture. Our experiments suggest that suppression of apoptosis is mediated by enhanced phosphorylation of key regulators of apoptosis such as p53. In fact, hyperphosphorylation of p53 and EROD induction upon TCDD treatment of rat hepatocytes exhibited identical concentration-response relationships suggesting involvement of the AhR in hyperphosphorylation of p53.

In conclusion, AhR agonists with various potencies can be found among environmental contaminants, food constituents, and microbial metabolites. Persistent agonists such as TCDD act as tumor promoters in rodent liver. Their tumor-promoting activity seems to be related to an AhR-mediated suppression of apoptosis of preneoplastic hepatocytes.

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Inducing Effects of Dioxin-like Polychlorinated Biphenyls on CYP1A in the Human Hepatoblastoma Cell Line HepG2, the Rat Hepatoma Cell Line H4IIE, and Rat Primary Hepatocytes: Comparison of Relative Potencies

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Polychlorinated biphenyls (PCBs) are a group of widespread environmental pollutants. Some non-*ortho*-substituted congeners with a high likelihood of coplanarity of both aromatic rings have been shown to act like 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) as agonists of the aryl hydrocarbon receptor (AhR) subsequently leading to adverse effects, such as immunosuppression and tumor promotion. Although there is a broad base of experimental data concerning the toxicity of PCBs in laboratory animals and animal-derived primary cells and cell lines, only few experimental data are available for cells of human origin. As a parameter of AhR activation, induction of CYP1A-mediated 7-ethoxyresorufin *O*-deethylase (EROD) activity was determined in the human hepatoblastoma cell line HepG2 treated with the PCBs IUPAC Nos. 77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169, and 189, and with TCDD as a positive control. Compared with results in rat primary hepatocytes and the rat hepatoma cell line H4IIE, treated HepG2 cells showed lower specific EROD activities maximally inducible by TCDD and PCBs, and EC_{50} values were shifted to higher concentrations. Furthermore, relative potency factors (REPs) for some congeners such as PCBs 81, 126, and 169 greately differed from those observed in cells derived from rats. Northern blot analyses showed that EROD activities run parallel to changes in CYP1A-specific mRNA contents. The considerable differences in EROD-derived REPs between cells of human and rat origin indicate the need for further investigations in experimental models from different species including humans in order to extend the database of biochemical and toxic responses to PCBs.

Key Words: PCB; dioxin; TCDD; Ah receptor; CYP1A; EROD; human; rat.

Polychlorinated biphenyls (PCBs) are a family of 209 compounds (congeners) differing in extent and position of chlorination of their aromatic rings. They were extensively used in industrial applications, e.g., because of their insulating and flame retardant properties, leading to widespread distribution in soil and water. Because of their lipophilic character, chemical stability, and slow rate of degradation they tend to accumulate in adipose tissue of animals and humans, and although production and use have been terminated they are still present in the food chain and environmental matrices (Danse *et al.*, 1997).

Some of the PCB congeners, especially those with non- and mono-*ortho*-chlorine substitution show patterns of toxicity in laboratory animals resembling those of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), including teratogenicity, endocrine disorders, tumor promotion and adverse effects on skin, immune system, and reproduction (Safe, 1994). There is sufficient evidence that these compounds share a common mechanism of action of toxicity, involving agonist binding to the cytosolic aryl hydrocarbon receptor (AhR; Poland and Knutson, 1982). The ligand-activated receptor forms a heterodimer with the nuclear protein ARNT ("AhR nuclear translocator"), and after binding to specific DNA elements (xenobiotic responsive elements [XRE]) increases transcription of several dioxin-activated genes (AhR gene battery) (Bock, 1993). Among these genes, increased expression of cytochrome P450 1A1 (CYP1A1) is a well-understood example frequently used as a parameter for the potency of AhR agonists (Whitlock, 1993). The 7-ethoxyresorufin *O*-deethylase (EROD) activity of CYP1A isozymes is widely accepted to be used for measuring induction by dioxin-like compounds (Leece *et al.*, 1985).

To describe the relative potency of dioxin-like compounds, the concept of Toxic Equivalency Factors (TEFs) has been developed (Eadon *et al.*, 1986; Birnbaum, 1999) with TCDD as one of the strongest AhR agonists attributed with a TEF value of 1. The TEFs published by the World Health Organization (WHO) for risk assessment in humans and wildlife are consensus values derived from available experimental *in vivo* and *in vitro* data by scientific consideration and evaluation (Van den Berg *et al.*, 1998). Following the recommendation of the WHO expert group, the values of our experiments determining

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the range of CYP1A-inducing potency of dioxin-like PCBs are referred to as relative potencies (REPs).

Usually, REPs are ratios of EC_{50} values or other hallmarks of effects of individual congeners compared to TCDD (Van den Berg *et al.*, 1998) in a single experimental model based on a series of experiments carried out under reproducible conditions. Dose-response curves running parallel under ideal conditions allow the calculation of EC_{50} values including confidence intervals (DeVito *et al.*, 1997). In *in vivo* experiments, different pharmacokinetic properties including absorption, distribution, metabolism, and elimination of congeners can affect REPs (DeVito and Birnbaum, 1995), whereas in tissue culture, different binding affinities to the Ah receptor seem to play a predominant role (Safe, 1994).

In recent years, the TEF/REP concept has been extended to dioxin-like PCBs comprising non-*ortho*- and mono-*ortho*-chlorinated congeners (Ahlborg *et al.*, 1994; Safe, 1994; Van den Berg *et al.*, 1998). A number of studies have been published presenting REPs for dioxin-like PCBs using CYP1A induction as a surrogate parameter for AhR activation. These include studies *in vivo* in rodents (DeVito *et al.*, 1993, 2000; Safe, 1990) and *in vitro* in rat hepatocytes and hepatoma cells (Schmitz *et al.*, 1995), in hepatocytes from other species (van der Burght *et al.*, 1999, 2000), and in human cell lines (Pang *et al.*, 1999).

Previously, we found considerable species differences in the potency of polychlorinated dibenzo-*p*-dioxins (PCDDs) to induce CYP1A isozymes in tissue culture (Lipp *et al.*, 1992; Schrenk *et al.*, 1991, 1995). Therefore, investigations of the CYP1A-inducing potencies of most dioxin-like PCBs were done in the human hepatoblastoma cell line HepG2 in comparison to the rat hepatoma cell line H4IIE, and in rat primary hepatocytes. For a number of dioxin-like PCBs pronounced differences were found between cells of human and rat origin.

MATERIALS AND METHODS

Chemicals. PCB 81 (3,4,4′,5-TetraCB) was kindly provided by L. Robertson (University of Kentucky, Lexington, KY). PCBs 77 (3,3′,4,4′-TetraCB), 105 (2,3,3′,4,4′-PentaCB), 114 (2,3,4,4′,5-PentaCB), 118 (2,3′,4,4′,5-PentaCB), 123 (2′,3,4,4′,5-PentaCB), 126 (3,3′,4,4′,5-PentaCB), 156 (2,3,3′,4,4′,5-HexaCB), 157 (2,3,3′,4,4′,5′-HexaCB), 167 (2,3′,4,4′,5,5′-HexaCB), 169 (3,3′,4,4′,5,5′-HexaCB), 189 (2,3,3′,4,4′,5,5′-HeptaCB), and 2,3,7,8-TCDD were purchased from Promochem (Wesel, Germany). All other chemicals were purchased from Sigma (Deissenhofen, Germany). The purity of the commercially purchased PCBs exceeded 99% according to the supplier. In addition, HPLC analyses were carried out that confirmed the supplier's information. For the chemical analysis of PCB 81 the preparation was dissolved and the solution was diluted in toluene. This solution was analyzed by high-resolution mass spectrometry. Quantification was carried out after addition of ^{13}C-labelled PCB standards. A contamination with 3.9% PCB 39 and 0.7% of another unidentified trichlorinated biphenyl was found. Other PCBs and PCDD/Fs could not be detected.

Preparation of TCDD and PCB solutions. Solutions for treatment of cell cultures were obtained by dissolving PCBs in dry dimethyl sulfoxide (DMSO) and preparation of stepwise dilutions resulting in final DMSO concentrations of 0.5 % in the culture medium.

Cell culture and treatment. Fetal bovine serum (FBS) was from Gibco (Karlsruhe, Germany), all other components of the culture medium were from Biochrom (Berlin, Germany). Type I collagen for coating was prepared from rat tail tendon (Elsdale and Bard, 1972).

The human hepatoblastoma cell line HepG2, and the rat hepatoma cell line H4IIE were a kind gift from F. Wiebel (GSF, Munich, Germany). Hepatocytes were prepared from male Wistar rats (Charles River, Kisslegg, Germany) as described earlier (Schmitz *et al.*, 1995). Preparations showing viability > 90% were seeded on collagenated Petri dishes (6 cm diameter) at a density of 70,000 cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM) containing 20% FBS, 0.1 μM dexamethasone, 100 U/ml penicillin, and 100 μg/ml streptomycin. After 3 h, medium was replaced by fresh medium, PCBs were added, and the cells were incubated for additional 48 h. DMSO alone served as a negative control. Cell lines were seeded at a density of 10,000 cells/cm^2 in the same medium. After reaching 50% confluency, medium was replaced by fresh medium and cells were treated as described above.

EROD assay. After incubation, cells were washed with ice-cold saline and scraped off with ice-cold Tris-buffered sucrose solution (10 mM Tris-HCl, 250 mM sucrose; pH 7.4). After centrifugation, cells were homogenized by disruption with a sonifier (Braun, Reutlingen, Germany) at 50 watts on ice. EROD activity in the homogenates was measured according to the method of Burke and Mayer (1974) modified by Pohl and Fouts (1980) using a fluorescence sprectrometer (Perkin-Elmer LS 50). Protein concentrations were determined according to Lowry *et al.* (1951).

Northern analysis. Total cellular RNA was isolated from rat primary hepatocytes and H4IIE cells as described previously (Schmitz *et al.*, 1997; Schrenk *et al.*, 1994), from HepG2 cells according to Chomczynski and Sacchi (1987).

After electrophoresis in a 1% agarose gel containing 2.2 M formaldehyde, RNA was blotted to nylon membranes (Magnagraph, MSI, Westborough, MA) using capillary transfer with 10× SSC. As a size standard, an RNA ladder of fragment size of 0.24–9.5 kb (Gibco) was used.

Analysis of CYP1A mRNA expression was achieved by hybridization to a 2 kb mouse CYP1A2 cDNA probe (Gonzalez *et al.*, 1984) hybridizing with rat and human CYP1A1 and 1A2 mRNAs. Loading controls were performed using a 249 bp cDNA probe for the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (Fort *et al.*, 1985). The probes were labeled with [α-^{32}P]dCTP using a random primer labeling kit (Roche, Mannheim, Germany). Membranes were prehybridized and hybridized in 50% deionized formamide, 6× SSC, 5× Denhardt's solution, and 1% sodium dodecyl sulfate (SDS). Hybridized mRNA was visualized by autoradiography at −80°C for 1 to 2 days and quantified using a scanning software (Stratagene Eagle Sight).

Statistical analysis. Treatments were carried out in duplicate in 3 independent experiments. Dose-response curves, EC_{50} values, and SDs were calculated using a computerized log-probit procedure (Origin 5.0, Microcal, Northampton, MA). Calculated no-effect concentrations (CNEC) were determined analogous to the calculation of no-effect levels (CNEL) of TCDD in a subchronic dose-response study by Van Birgelen *et al.* (1995). Briefly, a margin of 2 standard deviations was added to the EROD activity of untreated controls (EROD of control + 2 SDs). The corresponding concentration value was calculated from the output parameters of the logistic regression equation for each concentration-response curve. The 95% confidence limits were calculated from the error values of these parameters. This procedure was carried out with each cell type investigated and for any single PCB leading to EROD induction.

RESULTS

The non-*ortho* PCBs 77, 81, 126, the mono-*ortho* PCB 114, and TCDD as the reference compound induced EROD activity in human HepG2 cells as shown in Figure 1. The EC_{50}-based potencies were in the rank order TCDD > PCB 81 > PCB

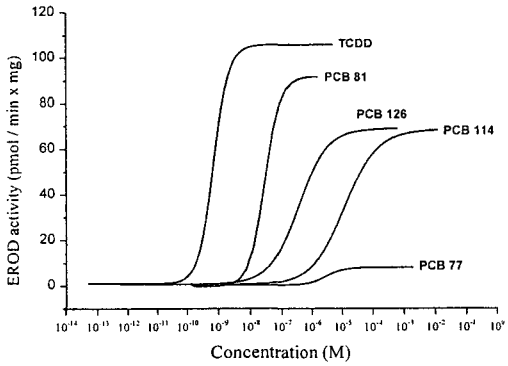

FIG. 1. EROD-inducing effects of TCDD or the PCBs 77, 81, 114, and 126 in HepG2 cells after treatment over 48 h. PCBs 105, 118, 123, 156, 157, 167, 169, and 189 did not induce EROD activities in HepG2 cells. Data were obtained from 3 independent experiments, and concentration-effect curves fitted from data sets obtained at 6–8 different concentrations of each congener were calculated using a log-probit software.

126 > PCB 77 > PCB 114. The maximal efficacies of most inducing PCBs were lower than that of TCDD while the non-*ortho* PCB 77 exhibited an extraordinary low maximal efficacy. In contrast to cells of rat origin, the other mono-*ortho* PCB congeners (105, 118, 123, 156, 157, 167, and 189) and the non-*ortho* congener PCB 169 did not induce EROD activity in HepG2 cells. The EC_{50} value for TCDD in HepG2 cells was within the same order of magnitude as the earlier published value, i.e., 0.68 nM in this vs. 0.31 nM in a previous report (Lipp *et al.*, 1992). A summary of EC_{50} values is presented in Table 1.

To better take into consideration different shapes of concentration-response curves (slope and maximum), a benchmark concept of CNEC similar to the CNEL described by Van Birgelen *et al.* (1995) for a subchronic dose-response study with TCDD was applied. In contrast to that study, our calculations are based upon a logistic regression model because, with our data, other models did not result in narrower 95% confidence intervals (data not shown).

The rank order of CNEC-based potencies was TCDD > PCB 81 ≈ PCB 126 > PCB 114 > PCB 77, revealing an even lower potency of the non-*ortho* PCB 77 compared with the mono-*ortho* PCB 114, and similar potencies for both the non-*ortho* congeners PCB 81 and PCB 126. A summary of CNEC values is presented in Table 2.

To determine whether the effects on EROD activity in HepG2 cells were due to changes in CYP1A mRNA contents or to posttranslational events, Northern blot analyses were performed. Figure 2 demonstrates that increasing EROD activities run parallel to specific mRNA contents. An increase in CYP1A mRNA could not be detected after treatment with PCB 77 and with those PCBs that failed completely to induce EROD activity (PCBs 105, 118, 123, 156, 157, 169, and 189) although GAPDH controls were positive (data not shown).

For the analysis of EROD induction in rat primary hepatocytes and the rat hepatoma cell line H4IIE, part of the data were taken from a previous publication (Schmitz *et al.*, 1995). The effects of the non-*ortho* PCB 81 and the mono-*ortho* PCBs 114, 123, 157, 167, and 189 were investigated in this study to complete the existing set of data.

Figures 3 and 4 show the fitted concentration-effect curves for EROD induction by selected non-*ortho* and mono-*ortho* PCBs in H4IIE cells. The EC_{50}-based potencies were in the rank order TCDD > PCB 126 > PCB 81 > PCB 169 > PCB 114 ≈ PCB 157 > PCB 77 ≈ PCB 156 > PCB 105 ≈ PCB 167 > PCB 118 ≈ PCB123. The maximal efficacies of TCDD and most inducing PCBs, in particular the non-*ortho* PCB 77, were considerably higher than in HepG2 cells. The mono-*ortho* PCB 189 failed to induce detectable EROD activity in H4IIE cells.

The CNEC-based potencies were in the rank order TCDD > PCB 126 > PCB 81 > PCB 77 > PCB 169 > PCB 156 > PCB 105 > PCB 114 > PCB 157 > PCB 118 > PCB 167 > PCB 123, i.e., all non-*ortho* congeners showed higher potencies than the mono-*ortho* congeners, and among mono-*ortho* congeners, the potencies of PCB 77 and PCB 169 were in the same order of magnitude.

Figures 5 and 6 show the fitted concentration-effect curves for EROD induction by selected non-*ortho* and mono-*ortho* PCBs in rat primary hepatocytes. All congeners tested including PCB 189 led to a significant induction. The maximal efficacy of most inducers was considerably higher than in

TABLE 1

EC_{50} Values ± S.D. (nM) for Induction of EROD Activity in HepG2 Cells, H4IIE Cells, and Rat Primary Hepatocytes

Inducer	Type[a]	HepG2	H4IIE	Rat primary hepatocytes
TCDD		0.68 ± 0.02	0.05 ± 0.013[b]	0.02 ± 0.005[b]
PCB 77	n	2700 ± 230	530 ± 280[b]	140 ± 55[b]
PCB 81	n	28 ± 0.7	7.4 ± 1.1	0.41 ± 0.01
PCB 126	n	340 ± 10	0.28 ± 0.08[b]	0.22 ± 0.04[b]
PCB 169	n	n.d.[c]	17 ± 8.4[b]	7.1 ± 3.5[b]
PCB 105	m	n.d.	4800 ± 2100[b]	270 ± 40[b]
PCB 114	m	12,000 ± 3700	253 ± 1	16 ± 0.5
PCB 118	m	n.d.	13,000 ± 4100[b]	660 ± 180[b]
PCB 123	m	n.d.	17,500 ± 347	1000 ± 73
PCB 156	m	n.d.	690 ± 190[b]	74 ± 19[b]
PCB 157	m	n.d.	361 ± 38	29 ± 1.4
PCB 167	m	n.d.	4740 ± 603	1670 ± 36
PCB 189	m	n.d.	n.d.	12,100 ± 6880

Note. EC_{50} values ± S.D. (nM) calculated 48 h after treatment with TCDD and dioxin-like PCBs.

[a]n, non-*ortho*; m, mono-*ortho*.

[b]Data published earlier (Schmitz *et al.*, 1995).

[c]Not detectable

TABLE 2
Calculated No Effect Concentrations (CNEC; nM) for Induction of EROD Activity in HepG2 Cells, H4IIE Cells, and Rat Primary Hepatocytes

		HepG2			H4IIE			Rat primary hepatocytes		
Inducer	Type[a]	Mean	LL[b]	UL[c]	Mean	LL	UL	Mean	LL	UL
TCDD		0.034	0.023	0.047	0.0002[d]	$8 \cdot 10^{-5}$	0.0004	$4 \cdot 10^{-5}$	$3 \cdot 10^{-5}$	$8 \cdot 10^{-5}$
PCB 77	n	336	189	523	1.24[d]	0.33	3.38	0.3	0.1	0.71
PCB 81	n	1.67	1.41	1.94	0.44	0.03	1.29	0.03	0.04	0.01
PCB 126	n	1.59	1.33	1.89	0.001[d]	0.0005	0.002	0.0004	0.0002	0.0006
PCB 169	n	n.d.[e]	n.d.	n.d.	2.27[d]	1.47	3.21	0.13	0.05	0.29
PCB 105	m	n.d.	n.d.	n.d.	6.56[d]	0.88	30.54	3.35	2.3	4.58
PCB 114	m	24.7	8.5	62.1	11	10.6	11.3	4.5	3.94	5.07
PCB 118	m	n.d.	n.d.	n.d.	307[d]	245	379	33.8	21	45
PCB 123	m	n.d.	n.d.	n.d.	4534	4299	4771	128	77	173
PCB 156	m	n.d.	n.d.	n.d.	3.07[d]	1.07	6.89	1.17	0.9	1.5
PCB 157	m	n.d.	n.d.	n.d.	29.6	14.6	48.3	6.15	4.41	7.89
PCB 167	m	n.d.	n.d.	n.d.	1786	1137	2450	324	292	357
PCB 189	m	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	209	90	399

Note. CNEC calculated 48 h after treatment with TCDD and dioxin-like PCBs.
[a]n, non-*ortho*; m, mono-*ortho*.
[b]Lower 95% confidence limit.
[c]Upper 95% confidence limit.
[d]Based on data published earlier (Schmitz *et al.*, 1995)
[e]Not detectable.

H4IIE cells. Furthermore, no inducers with extraordinary low maximal efficacy as observed in the hepatoma cells lines were found. The EC_{50}-based potencies were in the rank order TCDD > PCB 126 > PCB 81 > PCB 169 > PCB 114 > PCB 157 > PCB 156 > PCB 77 > PCB 105 > PCB 118 > PCB 123 > PCB 167 > PCB 189, thus very similar to the EC_{50}-based rank order in H4IIE cells. The maximal efficacies of TCDD and most inducing PCBs, in particular PCB 77, were considerably higher than in HepG2 cells.

The CNEC-based potencies were in the rank order TCDD >

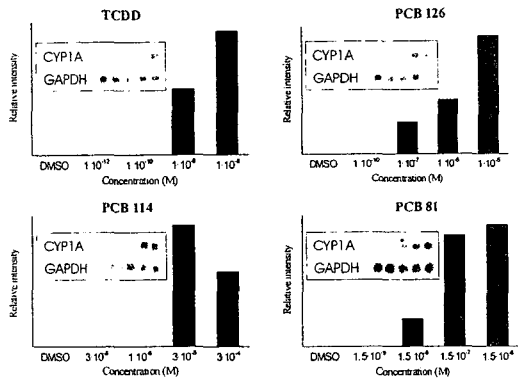

FIG. 2. Representative Northern analyses of HepG2 cells after treatment with TCDD or the PCBs 81, 114, and 126 over 48 h. CYP1A mRNA could not be detected after treatment with PCBs 77, 105, 118, 123, 156, 157, 167, 169, and 189 (GAPDH control positive; data not shown). Blots were probed with a mouse CYP1A2 cDNA, and a rat GAPDH cDNA as loading control. Autoradiographs were scanned and analyzed densitometrically. Intensities (arbitrary units) of CYP1A mRNA (verticle bars) were calculated relative to corresponding GAPDH mRNA intensities.

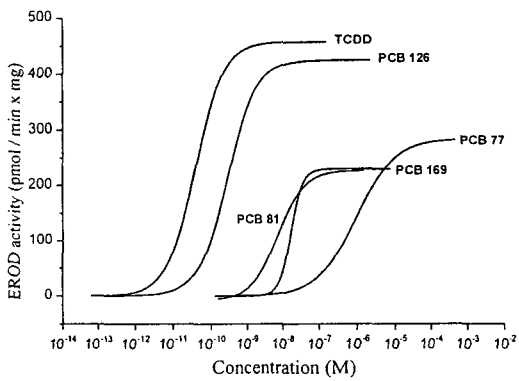

FIG. 3. EROD-inducing effects of TCDD or the non-*ortho*-substituted PCBs 77, 81, 126, and 169 in H4IIE cells after treatment over 48 h. Data for PCB 81 were obtained from 3 independent experiments, data for the other non-*ortho* PCBs were from a previous publication (Schmitz *et al.*, 1995). Concentration-effect curves fitted from data sets obtained at 6–8 different concentrations of each congener were calculated using a log-probit software.

PCB EFFECTS ON RAT AND HUMAN CYP1A

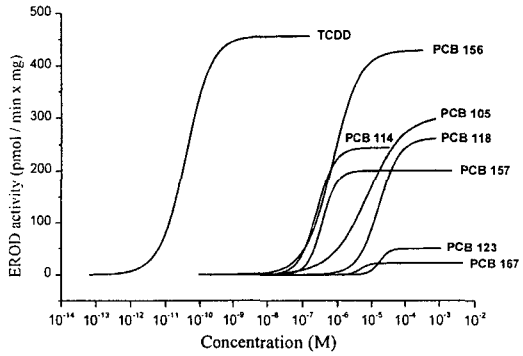

FIG. 4. EROD-inducing effects of TCDD or the mono-*ortho*-substituted PCBs 105, 114, 118, 123, 156, 157, and 167 in H4IIE cells after treatment over 48 h. PCB 189 did not induce EROD activity in H4IIE cells. Data for PCBs 114, 123, 157, 167, and 189 were obtained from 3 independent experiments, data for the other mono-*ortho*-substituted PCBs were from a previous publication (Schmitz *et al.*, 1995). Concentration-effect curves fitted from data sets obtained at 6–8 different concentrations of each congener were calculated using a log-probit software.

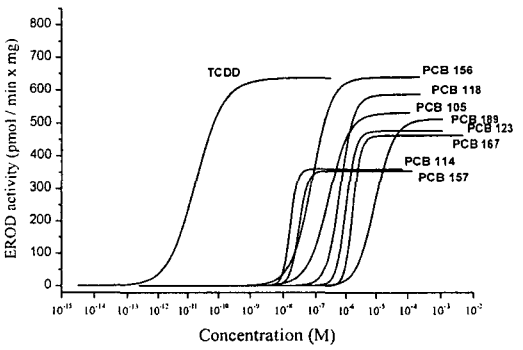

FIG. 6. EROD-inducing effects of TCDD or the mono-*ortho*-substituted PCBs 105, 114, 118, 123, 156, 157, 167, and 189 in rat primary hepatocytes after treatment over 48 h. Data for PCBs 114, 123, 15, 167, and 189 were obtained from 3 independent experiments, data for the other mono-*ortho*-substituted PCBs were from a previous publication (Schmitz *et al.*, 1995). Concentration-effect curves fitted from data sets obtained at 6–8 different concentrations of each congener were calculated using a log-probit software.

PCB 126 > PCB 81 > PCB 169 > PCB 77 > PCB 156 > PCB 105 ≈ PCB 114 > PCB 157 > PCB 118 > PCB 123 > PCB 189 > PCB 167, also similar to CNEC-based rank order in H4IIE cells. Again all non-*ortho* congeners had higher potencies than the mono-*ortho* congeners.

The fitted concentration-response curves did not strictly meet the requirements for a direct comparison of EC_{50} values. In particular, the maximal efficacies (maximum response) were very different, in some cases, for individual congeners. In addition, some indications for different slopes of the fitted curves were obtained, which led us to use a second benchmark concept better considering curve shapes, i.e., CNEC as a means for comparison of the different cell types. The analysis of both the benchmarks (Tables 1 and 2) revealed marked differences in REP values (Tables 3 and 4) for the non-*ortho* PCBs 81 and 126 between hepatoma cells of rat and human origin, and a complete lack of inducing potency in human HepG2 cells for PCB 169 and the mono-*ortho* PCBs tested with the exception of PCB 114.

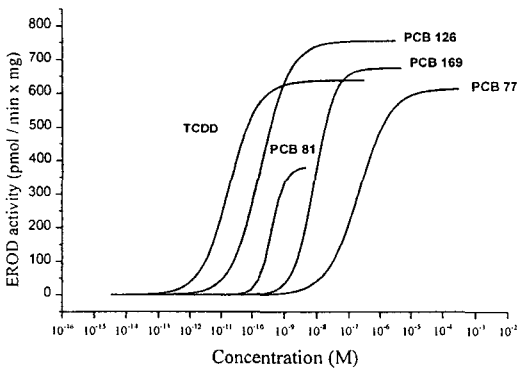

FIG. 5. EROD-inducing effects of TCDD or the non-*ortho*-substituted PCBs 77, 81, 126, and 169 in rat primary hepatocytes after treatment over 48 h. Data for PCB 81 were obtained from 3 independent experiments, data for the other non-*ortho*-substituted PCBs were from a previous publication (Schmitz *et al.*, 1995). Concentration-effect curves fitted from data sets obtained at 6–8 different concentrations of each congener were calculated using a log-probit software.

DISCUSSION

Our data show that the inducibility of CYP1A activity by TCDD in the human HepG2 line is at least 1 order of magnitude below that in the rat hepatoma cell line H4IIE. This finding is in accordance to observations by Wiebel *et al.* (1996) who found that HepG2 cells were 20 times less sensitive with respect to induction of CYP1A-related aryl hydrocarbon hydroxylase than H4IIE cells. The rank order of sensitivity of the cells towards TCDD in terms of EROD induction was rat hepatocytes > H4IIE > HepG2. Parallel to the differences in EC_{50} values run different maxima of CYP1A induction in the 3 cell types resulting in the same rank order. These findings were completed by according differences in CNEC.

The fitted concentration-response curves did not strictly meet, for all PCBs, the requirements for a direct comparison of EC_{50} values. In particular, the maximal efficacies (maximum response) were very different for individual congeners. In addition, some indications for different slopes of the fitted

TABLE 3

EROD-Specific EC_{50}-Based REP (EC_{50}-REP) Values of Dioxin-Like PCBs in HepG2 Cells, H4IIE Cells, and Rat Primary Hepatocytes, and Corresponding WHO-TEFs for Human Risk Assessment and Mammals

		EC_{50}-REP			
Inducer	Type[a]	HepG2	H4IIE	Rat primary hepatocytes	WHO-TEF[b]
TCDD		1	1	1	1
PCB 77	n	0.0003	0.00009	0.0001	0.0001
PCB 81	n	0.02	0.007	0.05	0.0005
PCB 126	n	0.002	0.2	0.09	0.1
PCB 169	n	n.d.[c]	0.003	0.003	0.01
PCB 105	m	n.d.	0.00001	0.00007	0.0001
PCB 114	m	0.0006	0.0002	0.001	0.0005
PCB 118	m	n.d.	0.000004	0.00003	0.0001
PCB 123	m	n.d.	0.000003	0.00002	0.0001
PCB 156	m	n.d.	0.00007	0.0003	0.0005
PCB 157	m	n.d.	0.0001	0.0007	0.0005
PCB 167	m	n.d.	0.00001	0.00001	0.00001
PCB 189	m	n.d.	n.d.	0.000002	0.0001

[a]n, non-*ortho*; m, mono-*ortho*.
[b]Van den Berg *et al.* (1998)
[c]Not determined for lack of EROD induction.

curves were obtained. A comprehensive review of studies with dose-response curves for different biological endpoints of PCBs revealed that different shapes of the curves usually do not compromise the TEF concept, i.e., measured TCDD equivalencies are according to a dose additive model (van den Berg *et al.*, 1998). This statement corresponds to our own experimental findings with relative potencies of PCB mixtures (Schmitz *et al.*, 1995). Nevertheless, a second benchmark concept better considering curve shapes was used in this study to compare cell types and congeners, i.e., the calculation of CNEC from untreated control EROD activities, acoording to a method described by Van Birgelen *et al.* (1995).

A striking result of this analysis were the different EC_{50}: CNEC ratios for different congeners and cell types, e.g., the CNEC of TCDD was 1 order of magnitude below the EC_{50} in HepG2 cells but 2 in H4IIE cells and even 3 orders of magnitude in rat primary hepatocytes. The reason for these differences are the slopes of the left branch of the sigmoidal induction curves. A steeper slope of the left branch means a greater effect at low concentrations leading to a lower CNEC value and a higher CNEC-based potency compared to the corresponding EC_{50}-based pontency. This effect is independent of the maximum response of the cell type to an inducing agent and the median slope of the corresponding induction curve and may be of great importance for the evaluation of inducers in a low concentration range. On the other hand, measurable effects of TCDD at extremely low concentrations (fmolar range in cells of rat origin) and thus an corresponding low CNEC of TCDD may lead to CNEC-based REP values significantly different from the corresponding EC_{50}-based ones and even more different from TEFs. This was the case, in particular, for the mono-*ortho* PCBs with weak inducing potencies, no matter which cell type was used. The CNEC-based potency of PCB 123 e.g., was 2 orders of magnitude lower than the EC_{50}-based potency and even 3 orders of magnitude lower than the proposed TEF value. Likewise, PCBs showing a slope of the induction curve at low concentrations similar to TCDD did not reveal a great discrepancy between EC_{50}-based and CNEC-based REP values.

The PCBs investigated showed much higher EC_{50} values and CNEC values in human than in rat cells, i.e., they were less potent. Among the non-*ortho* congeners, PCBs 126 and 169 elicited the most extensive species differences. The EC_{50} value of PCB 126 was found to be 2, the CNEC values even 3 to 4 orders of magnitude higher in human than in rat cells, whereas PCB 169 failed completely to induce EROD activity. Furthermore, PCB 81 (non-*ortho*) was the most potent inducer in HepG2 cells among the PCB congeners tested resulting in an EC_{50}-based REP of 0.02. It also showed a high potency at low concentrations leading to the same CNEC-based REP value. This finding is in accordance to a previous report by Pang *et al.* (1999) who found PCB 81 to be the most potent inducer among the non-*ortho* PCBs tested of CYP1A1-dependent estrogen metabolism in HepG2 cells. In other experimental systems REPs for EROD induction by PCB 81 of 0.0069 in wild-type H4IIE cells (Sanderson *et al.*, 1996) and 0.2 in chicken hepa-

TABLE 4

EROD-Specific CNEC (Calculated No-Effect Concentration)-Based REP (CNEC REP) Values of Dioxin-Like PCBs in HepG2 Cells, H4IIE Cells, and Rat Primary Hepatocytes, and Corresponding WHO-TEFs for Human Risk Assessment and Mammals

		CNEC-REP			
Inducer	Type[a]	HepG2	H4IIE	Rat primary hepatocytes	WHO-TEF[b]
TCDD		1	1	1	1
PCB 77	n	0.0001	0.0002	0.0001	0.0001
PCB 81	n	0.02	0.0005	0.001	0.0005
PCB 126	n	0.02	0.2	0.1	0.1
PCB 169	n	n.d.[c]	0.00009	0.0003	0.01
PCB 105	m	n.d.	0.00003	0.00001	0.0001
PCB 114	m	0.001	0.00002	0.000009	0.0005
PCB 118	m	n.d.	0.0000007	0.000001	0.0001
PCB 123	m	n.d.	0.00000004	0.0000003	0.0001
PCB 156	m	n.d.	0.00007	0.00003	0.0005
PCB 157	m	n.d.	0.000007	0.000007	0.0005
PCB 167	m	n.d.	0.0000001	0.0000001	0.00001
PCB 189	m	n.d.	n.d.	0.0000002	0.0001

[a]n, non-*ortho*; m, mono-*ortho*.
[b]Van den Berg *et al.* (1998).
[c]Not determined for lack of EROD induction.

tocytes (Kennedy *et al.*, 1996) were reported suggesting a high potency of this congener in birds. On the other hand, it should be taken into consideration that PCB 81 can be metabolized relatively easily due to the presence of 2 pairs of adjacent nonsubstituted carbon atoms. One possible explanation for its high potency in HepG2 cells may therefore be a reduced specific metabolic activity in this cell line, provided there is sufficient time for metabolization during 48 h of incubation. PCB 81 is found in mother's milk (Hong *et al.*, 1994), and in a variety of environmental samples including fish (Echols *et al.*, 1997). Further work is required to analyze if the TEF of 0.0005 currently used for PCB 81 may understimate the potency of this congener in humans or if the elevated potency in HepG2 is a special feature of this cell line.

The third non-*ortho* PCB acting as EROD inducer in HepG2, PCB 77, showed an extremely low maximal efficacy and was inactive as an inducer of CYP1A mRNA expression. This corresponds to the even higher CNEC value compared to the mono-*ortho* PCB 114 and to results of other investigators who found no EROD induction in HepG2 cells at 50 μM PCB 77 (Dubois *et al.*, 1996).

As a possible explanation for a reduced maximal efficacy (maximal induction) of EROD inducers reported in various cell culture systems (Kennedy *et al.*, 1996; Lipp *et al.*, 1992; Schrenk *et al.*, 1991; van der Burght *et al.*, 1999) reduced receptor activation/transactivation has been suggested (van der Burght *et al.*, 1999). However, Abnet *et al.* (1999) have shown that the human AhR transfected into COS-7 cells showed a higher transactivating efficacy on a CYP1A reporter plasmid than those derived from rainbow trout or zebrafish. Furthermore, transactivation capacities with PCB 77 and TCDD via the human receptor were almost equivalent.

Our results with PCB 77 in HepG2 cells rule out that the extremely low maximal efficacy concerning EROD induction is due to CYP1A inhibition. Since no CYP1A mRNA was found, a suppression of the nuclear transactivation/transcription of AhR/CYP1A in HepG2 cells is a likely explanation. Possibly, these effects are not mimicked in an extranuclear reporter gene model for the human AhR.

In contrast, the marked decrease in EROD activity frequently found at higher concentrations of CYP1A inducers may be related to different mechanisms. It has been proposed that cytotoxicity, or inhibition of the catalytic activity of CYP1A may explain this effect (Hahn *et al.*, 1993; Pang *et al.*, 1999; van der Burght *et al.*, 1999). Furthermore, a possible role of porphyrin accumulation has been suggested (Tysklind *et al.*, 1995).

The fourth congener showing EROD-inducing potency in HepG2 cells was PCB 114 (mono-*ortho*). The EC_{50}-based REP calculated as 0.0006 was in the same range as the REPs found for EROD induction in hepatocytes from pigs (van der Burght *et al.*, 2000) and monkeys (van der Burght *et al.*, 1999). The relatively low CNEC of PCB 114 (25 nM), i.e., the relatively high inducing potency at low concentrations compared to the non-*ortho* PCB 77, supports the observation in Northern RNA analysis. However, other mono-*ortho* PCBs exhibiting a comparable (PCBs 105, 118, 123, 156, 157, and 189) or even higher (PCB 167) potency in pig hepatocytes were compeletely inactive as inducers in HepG2. Furthermore, those PCBs being inactive in HepG2 cells (PCB 105, 118, and 167) showed REPs > 0.0001 in hepatocytes from Cynomolgus monkeys. These data suggest that PCB 114 may have distinct properties that make it an AhR agonist in the HepG2 system in contrast to a variety of other mono-*ortho* congeners.

The EC_{50} values for PCBs 81, 114, and 126 in HepG2 were confirmed by Northern blotting. In addition, these data revealed that treatment with congeners not inducing EROD activity did not lead to an increase in CYP1A mRNA.

The reason for the highly reduced potencies of most dioxin-like PCBs in HepG2 cells may either be related to differences in uptake, metabolism, etc. or be based on a reduced ability to activate the AhR complex. The uptake of PCBs through the membrane is mainly governed by their lipophilic character and molecular size. The fact that only congeners with a limited range of chlorine atoms (4 to 7) were investigated leads to the conclusion that differnces in uptake are unlikely to be of importance in this study. Additionally, the experimental time period of 48 h is probably too short to cause any significant metabolization of PCBs. Therefore differences in activation of the Ah receptor complex and/or transcription may be dominating factors explaining the variation in the *in-vitro* systems investigated. The structure of the XREs of human CYP1A genes alone cannot hold fully responsible for reduced induction since the difference in the case of TCDD is only 10-fold. Furthermore, it should be taken into account that HepG2 as a tumor cell line may have developed an aberrant AhR-XRE activation and/or transcription pathway. Future *in vitro* studies with human primary hepatocytes may elucidate this possibility. Lower levels of AhR that were found in human hepatocytes (Safe, 1986) or species differences in the hepatic expression patterns of the CYP isozymes 1A1 and 1A2 (Xu *et al.*, 2000) may also contribute to the reduced sensitivity of human cells.

In summary, our results demonstrate striking differences between the rat and human hepatoma cell lines H4IIE and HepG2 with respect to both types of REPs of EROD induction for most dioxin-like PCBs. In particular, the non-*ortho*-substituted PCB 169 was inactive, and PCB 126 was less potent by 2 orders of magnitude in HepG2 cells. These findings and findings in hepatocytes of other primate species (van der Burght *et al.*, 1999) show the necessity of widening the base of experimental data for human risk assessment of PCBs by screening their toxicity in cells of human origin.

The benchmark concept of CNEC used in this study is not completely independent of the shape of induction curves. Since a logistic regression model proved best to fit our data the slope of the left branch of the curve (i.e., at low concentrations) has a dominating influence on potencies based upon CNEC values. Whereas EC_{50}-based REPs showed sufficient additivity in

many studies despite of different curve shapes, the great differences between EC_{50}-based and CNEC-based REPs, particularly for dioxin-like PCB congeners present at higher concentrations in technical and environmental samples, make the latter values less applicable for prescreening and assessing the TEQ of mixtures of inducers. On the other hand, the CNEC concept may provide an appropriate means to support the assessment of low-concentration effects of inducing agents.

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Inhibition of apoptosis in rat hepatocytes treated with 'non-dioxin-like' polychlorinated biphenyls

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Polychlorinated biphenyls (PCBs) are among the most prominent persistent environmental pollutants exhibiting neurotoxic, teratogenic and tumour-promoting effects in experimental animals. 'Dioxin-like' properties have been assigned to a number of PCBs whereas other PCBs have been classified as 'non-dioxin-like'. Many of the latter congeners are inducers of cytochrome P450 (CYP) 2B1 and 2B2 similar to the liver tumour promoter phenobarbital. In contrast, 'dioxin-like' PCBs induce CYP1A isozymes, and other congeners have been classified as 'mixed-type' inducers. Inhibition of apoptosis of pre-neoplastic hepatocytes is thought to play a central role in tumour promotion in rat liver. We have used the inhibition of UV-induced apoptosis in rat hepatocytes in primary culture as an *in vitro* model for mechanistic studies on the inhibition of apoptosis. It could be shown that phenobarbital, and the 'non-dioxin-like' PCBs 28, 101 and 187 completely inhibit UV-induced apoptosis. The concentration–response curves and EC_{50} values for this effect, however, were different from those of induction of CYP2B1/2B2-catalysed 7-pentoxyresorufine *O*-dealkylase or CYP1A-catalysed 7-ethoxyresorufine *O*-deethylase activities. The PCBs and phenobarbital did not affect the spontaneous incidence of apoptotic nuclei. In conclusion, 'non-dioxin-like' PCBs are likely to promote liver carcinogenesis *via* the suppression of apoptosis. The signaling events in rat hepatocytes leading to induction of 2B1/2B2 activity by the compounds investigated are assumed to differ from those leading to inhibition of apoptosis.

Introduction

Polychlorinated biphenyls (PCBs) are among the most extensively investigated persistent environmental pollutants. Major sources of PCB emissions have been the use of technical PCB mixtures as hydraulic oils, flame retardants and lubricants in a number of technical processes (1). Furthermore, PCBs are formed during the incineration of organic materials in the presence of chlorine-containing compounds (2). Acute PCB intoxications as a result of the accidental ingestion of contaminated rice oil resulted in severe bone pain, chlorakne and malformations in newborn after exposure of pregnant women (3). Chronic exposure to increased levels of PCBs, e.g. in fish were suggested to result in delayed learning in children of women exposed during pregnancy (4).

In experimental models, PCBs lead to neurotoxicity, endocrine disturbances and tumour promotion in rodent liver (1,5,6). In a study of workers exposed to PCBs a significant increase in liver cancer was observed (7).

For practical classification, PCBs were subdivided into 'dioxin-like' and 'non-dioxin-like' congeners (6). This principle is based on the fact that a number of PCBs exert biological effects similar to those of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most toxic dioxin congener. In particular, 'dioxin-like' PCBs bind to the dioxin or aryl hydrocarbon receptor (AhR) and lead to characteristic effects on the expression of AhR-regulated genes including cytochrome P450 (CYP)1A1 (6). In contrast, a number of 'non-dioxin-like' PCBs are inactive or almost inactive as AhR-agonists but induce a battery of drug-metabolizing enzymes including CYP2B1/2B2 known as phenobarbital-inducible genes (6,8). Therefore, these PCBs are sometimes categorized as 'phenobarbital-like' inducers. It has to be kept in mind, however, that these compounds do not share many other major pharmacological or toxicological properties with the hypnotic drug phenobarbital. In addition, inducing properties concerning drug-metabolizing enzymes have not been investigated for a number of other PCBs. A variety of PCBs induce both CYP 2B1/2B2 and 1A isozymes in rat liver, and, therefore, have been categorized as 'mixed-type' inducers (8).

A common feature of TCDD, phenobarbital and a number of PCBs is their tumour-promoting potency in rat liver, when the animals have been treated previously with a genotoxic (initiating) carcinogen (5,9–11). It is assumed that the subsequent treatment with a tumour-promoting agent facilitates the clonal expansion of cells bearing a critical damage in their genome. This clonal expansion raises the risk of malignant transformation of genetically altered cells eventually resulting in the development of malignant tumours. A number of mechanisms have been proposed to explain the supportive effect of tumour promoters on the growth of preneoplastic cells (12). According to a current hypothesis, the inhibition of apoptosis, intrinsically enhanced in pre-neoplastic clones, may play a central role in the mechanism of tumour promotion (13). In fact, inhibition of apoptosis in pre-neoplastic enzyme-altered foci has been demonstrated for the liver tumour promoters phenobarbital, TCDD, and others (13–15). In the case of tumour-promoting PCBs, no data on the effect on apoptosis in pre-neoplastic rat liver are available. In rat hepatocytes in primary culture and in rat hepatoma cell lines, anti-apoptotic effects were described for phenobarbital, tumour-promoting peroxisome proliferators and TCDD (16–19).

This study was designed to investigate a possible relationship between 'phenobarbital-type' induction of CYP isozymes/activities, and the effects on UV-initiated apoptosis in rat hepatocytes after treatment with phenobarbital and certain 'non-dioxin-like' PCBs.

Abbreviations: AhR, aryl hydrocarbon receptor; CYP, cytochrome P450; EROD, 7-ethoxyresorufin *O*-deethylase; DMEM, Dulbecco's modified Eagle's medium; PCBs, polychlorinated biphenyls; PROD, 7-pentoxyresorufin *O*-dealkylase; TCDD, 2,3,7,8-tetrachlordibenzo-*p*-dioxin; TEFs, toxicity equivalency factors.

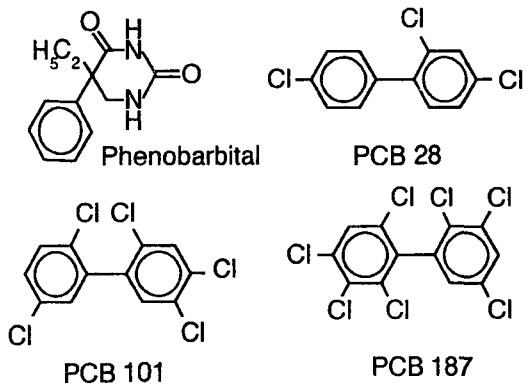

Fig. 1. Chemical structures of phenobarbital and selected 'non-dioxin-like' PCBs.

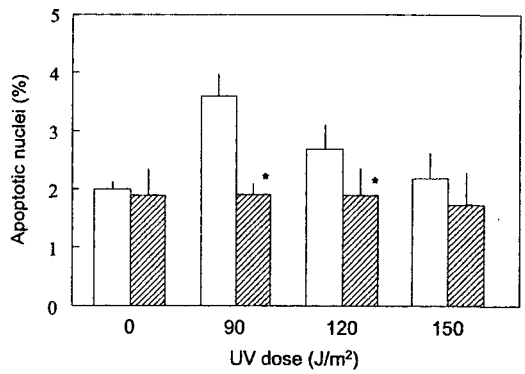

Fig. 2. Inhibition of apoptosis in rat hepatocytes in primary culture. Cultures were treated with saline (open bars) or 2 mM phenobarbital (hatched bars) 30 min after UV irradiation. Twelve hours after irradiation the number of apoptotic nuclei was determined. The bars and error bars show means and standard deviations form three independent experiments. *Significantly different from the corresponding saline-treated cultures ($P \leq 0.05$).

Materials and methods

Chemicals

Bovine serum albumin, collagenase type IV and phenobarbital were obtained from Sigma (Taufkirchen, Germany), Dulbecco's modified Eagles's medium (DMEM) from Seromed (Berlin, Germany), and Waymouth's medium MD 705/1 and fetal calf serum from Gibco-BRL (Heidelberg, Germany), ITS and ITS$^+$ from Becton Dickinson (Heidelberg, Germany), and the PCBs (Figure 1) IUPAC number 28 (2,4,4'-trichlorobiphenyl), 101 (2,2',4,5,5'-pentachlorobiphenyl) and 187 (2,2',3,4',5,5',6-heptachlorobiphenyl) from Promochem (Wesel, Germany). All other chemicals were purchased at the highest purity commercially available.

Hepatocytes and cell culture

Male Wistar rats were obtained from Charles River (Kisslegg, Germany) and were kept under standard conditions. Adult animals at a body weight of 150–180 g were anesthetized, and hepatocytes were isolated as described (20) using a modification of the sequential perfusion technique originally described by Seglen (21). The cells were cultured using the collagen sandwich procedure (22). For the preparation of collagen, collagen-rich fibres were isolated from eight rat tails, and were dissolved in 800 ml 3% acetic acid at 4°C. Unsoluble material was removed by centrifugation at 2300 *g* and 4°C over 90 min. Then, 1/5 volume 30% NaCl solution was added to the supernatant, and the precipitated collagen was collected by centrifugation at 2300 *g* and 4°C over 30 min. The pellet was resuspended in 175 ml 5% NaCl solution, and was centrifuged at 2300 *g* and 4°C over 30 min. The pellet was dissolved in 25 ml 0.6% acetic acid, and was filled up to a total volume of 400 ml by adding 0.6% acetic acid. After dialysis against 1 mM HCl over 48 h, the collagen solution was lyophilized. Prior to use, 1.5 mg collagen were dissolved in 1 ml 1 mM HCl to obtain a stock solution. This solution was diluted with nine volumes of 10× DMEM, the culture dishes were coated with 700 µl of the dilution, and were then kept in a tissue culture incubator for 1 h at 37°C. After hardening of the gel the cells were seeded and incubated as described (17). The cells were covered with 500 µl collagen solution in 10× DMEM 1 h before treatment.

Induction of cytochromes P450

Hepatocytes were seeded at a density of 100 000/cm^2 on collagen-coated 60 mm Petri dishes and were incubated as described (17). After 3 h the cells were covered with 700 µl of collagen dilution in 10× DMEM. Twelve hours after seeding, PCBs were added dissolved in DMSO, phenobarbital dissolved in sterile saline (0.9% NaCl). The added volume of DMSO did not exceed 0.5% of the total volume per dish. Controls were treated with DMSO or saline only. The cultures were washed, harvested and homogenized 48 h after addition of the inducers, and 7-ethoxyresorufine *O*-deethylase (EROD) and 7-pentoxyresorufine *O*-dealkylase (PROD) activities were analysed using the method of Burke and Mayer (23).

Inhibition of apoptosis

Hepatocytes were seeded at a density of 60 000/cm^2 on Quadriperm dishes (Heraeus, Frankfurt, Germany) of an area of 20 mm^2. After 12 h medium was replaced by fresh medium, and after 15 h the cells were treated with UV light as described (17). Treatment with phenobarbital or PCBs was performed 30 min after irradiation.For the counting of apoptotic nuclei, the cells were fixed, washed and air-dried 12 h after tretament with the tumour promoters as described (20), and stained with an aqueous 8 µM solution of 4',6-diamidino-2-phenylindole (DAPI) and 10 µM sulphorhodamine 101. The microscopic analysis of the encoded slides was carried out using a Zeiss (Jena, Germany) fluorescence microscope (Axioskop) equipped with a BP 450–490 excitation filter and a LP 520 emission filter. The slides were stored at 4°C protected from light. All experiments were carried out in double, and 3×1000 nuclei were examined on each slide. Condensed, half moon-shaped, and scattered nuclei were summarized as apoptotic nuclei as described earlier (17).

Statistical analysis

Means and standard deviations were calculated from independent experiments. For (multiple) comparisons of means of treated cultures with untreated controls Dunnett's test for independent samples was used.

Results

In rat hepatocytes cultured between two layers of collagen ('sandwich culture') UV pulse-irradiation at a specific intensity of 90 J/m^2 almost doubled the number of apoptotic nuclei after 12 h (Figure 2). After 6 or 18 h the numbers of apoptotic nuclei were significantly lower (data not shown). Therefore, the number of apoptoic nuclei was determined 12 h after UV treatment in all subsequent experiments. With a dose of 120 J/m^2 less apoptotic nuclei were detected, while no significant increase in apoptosis was observed 12 h after treatment with 150 J/m^2. The latter dose resulted in massive acute cell death (not shown) which probably prevented the onset of the apoptotic pathways. The liver tumour promoter phenobarbital which was reported previously to suppress UV-induced apoptosis in rat hepatocytes (17) was used as a reference compound. Addition of 2 mM phenobarbital 30 min after UV irradiation completely suppressed the increase in apoptosis (Figure 2). For further experiments with the PCBs 28, 101 and 187 standard conditions were used (single UV irradiation with 90 J/m^2, determination of apoptotic nuclei after 12 h). It was found that phenobarbital and the three PCBs tested inhibited the increase in apoptosis in a concentration-dependent manner. This effect reached a level of at least 90% (at least 90 % inhibition of additional, UV-induced apoptosis) with phenobarbital at 10^{-7} M, PCB 28 at 10^{-9} M, PCB 101 at 10^{-7} M and PCB 187 at 10^{-6} M (Figure 3A–D), i.e. the range

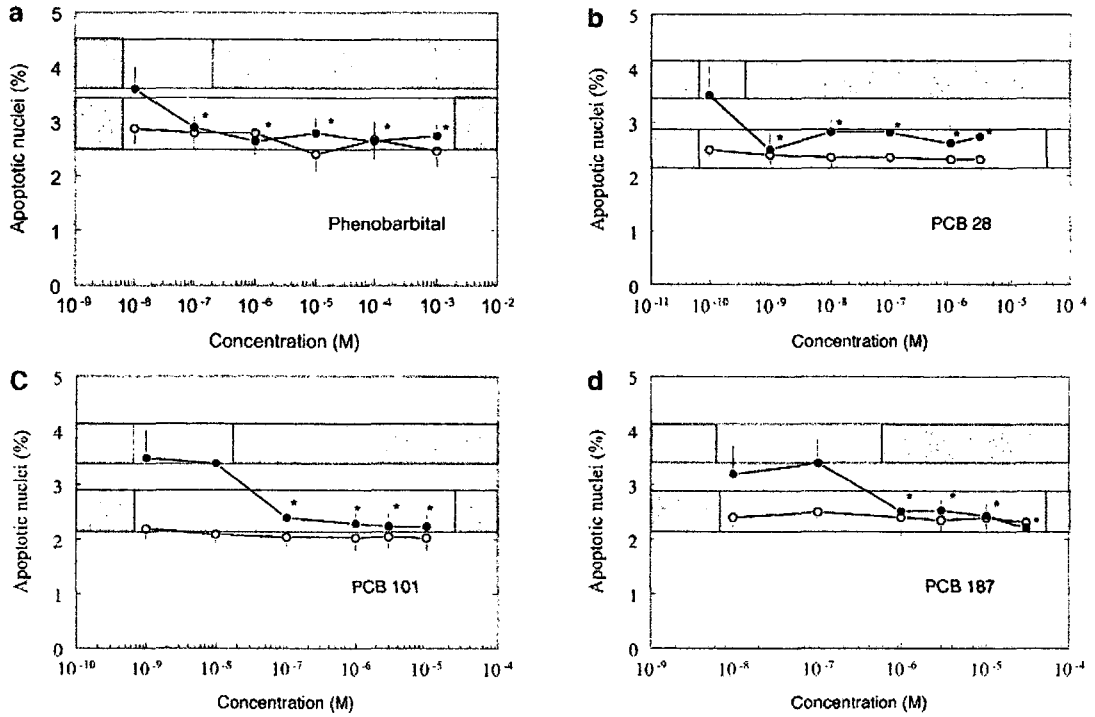

Fig. 3. Effects of phenobarbital, PCB 28, 101 or 187 on apoptosis in rat hepatocytes in primary culture 12 h after irradiation with UV-light (90 J/m^2). The compounds were added 30 min after irradiation. Symbols and error bars represent means and standard deviations from three independent experiments. *Significantly different from results obtained with corresponding DMSO-treated cultures, represented by two bands ($P \leq 0.05$). The upper bands show the standard deviation range of apoptosis in UV-treated, the lower band in DMSO-treated cultures.

of 'spontaneous' apoptosis found without UV irradiation was achieved.

All PCBs tested as well as phenobarbital also led to a concentration-dependent induction of CYP 2B1/2B2-catalysed PROD activity in cell homogenates (Figure 4A–D). Phenobarbital and the PCBs 101 and 187 exhibited a maximum efficacy in the range of 19–30 pmol/min×mg protein whereas treatment with PCB 28 resulted in a maximum level between 10 and 15 pmol/min×mg protein. With phenobarbital a complete induction curve was obtained for the CYP1A-catalysed EROD activity, whereas the PCBs were inactive as EROD inducers. Fitting of a sigmoidal dose–response function (not shown) to the experimental data using a log-probit procedure allowed the calculation of EC_{50} values and 95% confidence intervals (Table I). With respect to PROD induction, phenobarbital was ~10-fold less potent than PCB 28. The inducing potencies of the PCBs as inducers of PROD followed the rank order PCB 187 > PCB 28 > PCB 101.

Discussion

The tumour-promoting potency of PCBs and related environmental pollutants represents an important parameter in risk assessment of this group of chemicals. A variety of PCBs are members of the large and diverse group of tumour promoters which support the clonal expansion of pre-neoplastic cell clones in rat liver thus enhancing the risk of malignant transformation (12). The molecular mechanism(s) underlying tumour promotion are poorly understood. A number of hypotheses exist, however, including the notion that inhibition of apoptosis intrinsically enhanced in pre-neoplastic hepatocyte clones may play an important role (13).

The situation is complicated by the fact that PCBs can be divided into compounds with a 'dioxin-like' pattern of biochemical and toxic effects, and those with a 'non-dioxin-like' mode of action. In many cases, however, no clear borderline can be drawn between both groups. The classification of non-*ortho*-substituted PCB congeners as 'dioxin-like' is mainly based on their agonistic potency as ligands of the Ah or dioxin receptor (AhR) leading to characteristic molecular and cellular events (1,6) including the induction of CYP1A isozymes. Those 'dioxin-like' PCBs investigated so far act as tumour promoters in rat liver (5,8,9) thus resembling the most potent agonist within the family of 'dioxins', 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 10,24).

Among the 'non-dioxinlike' PCBs there are also a number of congeners acting as tumour promoters in rat liver (1,5,8,9). With another promoting agent, phenobarbital, they have in common the induction of a battery of certain drug-metabolizing enzymes including CYP 2B1/2B2 (6,8). The signalling pathway leading to 'phenobarbital-type' induction of CYP2B genes involves the constitutively active receptor (CAR) which acts as a transactivator of a distal enhancer in responsive genes called the phenobarbital-responsive enhancer module (25).

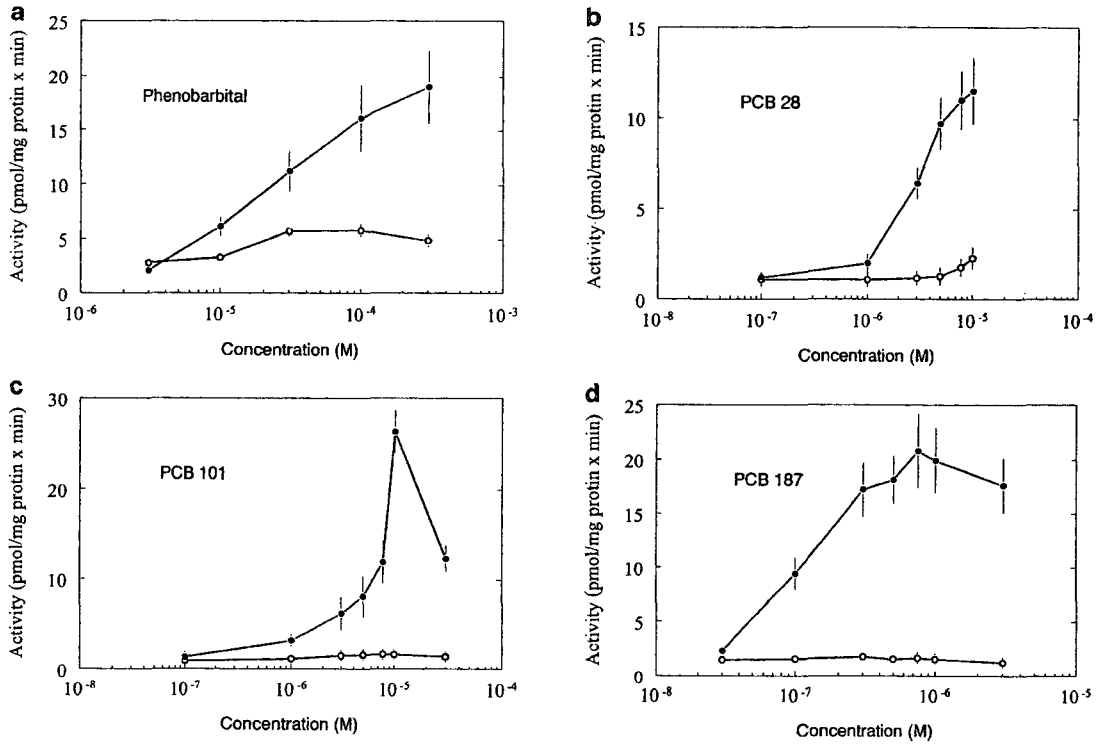

Fig. 4. Effects of phenobarbital, PCB 28, 101 or 187 on EROD activity (open circles) and PROD activity (closed circles) in rat hepatocytes after treatment with various concentrations. Forty-eight hours after addition of inducers, cells were harvested, homogenized and enzyme avctivities were determined as described under Materials and methods. Data represent means ± SD from three independent series of experiments (three different animals).

Table I. EC_{50} values for induction of PROD- and EROD-activities in homogenates from rat heptocytes in primary culture after treatment with phenobarbital, PCB 28, 101, 138 or 187

Inducer	PROD induction EC_{50} ± SD (M)	EROD induction EC_{50} ± SD (M)	Inhibition (90%) of UV-induced apoptosis (M)
Phenobarbital	$2.4\times10^{-5} \pm 0.17\times10^{-5}$	$1.8\times10^{-5} \pm 1.2\times10^{-5}$	10^{-7}
PCB 28	$3.3\times10^{-6} \pm 0.34\times10^{-6}$	–	10^{-9}
PCB 101	$5.7\times10^{-6} \pm 0.97\times10^{-6}$	–	10^{-7}
PCB 187	$1.2\times10^{-7} \pm 0.2\times10^{-7}$	–	10^{-6}

It was the major aim of the present study to investigate a possible relationship between induction of CYP1A or 2B1/2B2 activities and the inhibition of apoptosis as a possible *in vitro* surrogate for the tumour-promoting action of that class of compounds.

It was found, that UV light-induced apoptosis in rat hepatocytes was completely suppressed with phenobarbital and the three 'non-dioxinlike' PCBs 28, 101 and 187. A similar result was obtained in a previous study in rat hepatocytes using the liver tumour promoter TCDD (17). With TCDD and the PCBs used in this study, 'spontaneous' apoptosis could not be inhibited, however, arguing for two distinct types of apoptosis. One type is elicited by UV-irradiation probably initiated by DNA damage or another type of UV-induced cellular stress. Previous studies showed, that irradiation of hepatocytes resulted in induction of p53 as a characteristic response to this type of damage (26). Interestingly, both TCDD and phenobarbital could almost completely suppress the UV-induced rise in p53. The other type of apoptosis, occuring 'spontaneously' in culture is probably more abundant *in vitro* than in the liver. This may be due to various types of cellular stress during the procedures of isolation, plating, and culture of the cells.

Analysis of PROD activity as a functional parameter for CYP2B1/2B2 indicates that all PCBs tested are relatively potent inducers of this enzyme(s). In contrast, AhR-regulated

CYP1A activity (EROD) was almost unchanged with the exception of phenobarbital.

From *in vivo* experiments in rats (8) the following rank order of potency for PROD induction in the liver, probably modified by toxicokinetic influences, was reported: PCB 101 ≈ PCB187 > PCB28 which is slightly different from our *in vitro* findings where PCB 187 is found to be about 2-fold more potent than PCB 101. Comparison of EC_{50} values of PROD induction with 90% values of inhibition of apoptosis revealed no quantitative relationship. This result is of interest in several respects. First, it suggests that measurement of CYP 2B1/2B2 induction as a parameter for an anti-apoptotic potency of PCBs may be misleading for quantitative comparisons. Second, it can be speculated that the signalling events leading to induction of CYP2B1/2B2 are not directly related to those operative in inhibition of apoptosis.

Systematic investigations on possible links between the induction of CYP isozymes and liver tumour promotion are rare. With the AhR-agonist TCDD tumour promotion was observed in the livers of female but not of male Sprague–Dawley rats in spite of the fact that efficient induction of CYP1A isozymes/activities was evident in both sexes (27). On the other hand, there are indications of a relationship between the relative tumour promoting and CYP1A-inducing potencies for the class of 2,3,7,8-substituted PCDDs in the liver of female rats (2,10). Thus, it appears probable that AhR agonists may act as liver tumour promoters in a certain experimental model such as the female rat according to their affinity to the receptor. For the more diverse familiy of PCBs and other tumour promoters in rodent liver, there are some correlations between the induction of drug-metabolizing enzymes and their promoting potency (5,28). These correlations are incomplete, however, and may reflect the existence of several mechanisms in tumour promotion by phenobarbital-like inducers (29). Alternatively, a common pathway of induction and tumour promotion may diverge at a certain level, and both branches may be subject to strong modulating effects, e.g., by adaptation. An example is the enhanced formation of peroxides after phenobarbital treatment, leading to an adaptive increase in catalase activity (18).

In summary, our results show for the first time that 'non-dioxin-like' PCBs suppress apoptosis in rat hepatocytes. Furthermore, it is shown that UV-initiated but not constitutive apoptosis is inhibited and that both inhibition of apoptosis and 'phenobarbital-type' induction of cytochrome P450 activity can exhibit distinct concentration-response relationships. Further experiments are required to identify the mechanisms of action of 'non-dioxin-like' PCBs critical for tumour promotion and their links to the inhibition of apoptosis.

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