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Atropisomers of 2,2',3,3',6,6'-hexachlorobiphenyl (PCB 136) exhibit stereoselective effects on activation of nuclear receptors *in vitro*

Kateřina P n ěková¹, Petra Brenerová¹, Lucie Svržková¹, Eva Hrubá¹, Lenka Pálková¹, Jan Vondrá ěk², Hans-Joachim Lehmler³, and Miroslav Machala^{1,*}

¹Department of Chemistry and Toxicology, Veterinary Research Institute, 62100 Brno, Czech Republic

²Department of Cytokinetics, Institute of Biophysics of the Czech Academy of Sciences, 62165 Brno, Czech Republic

³Department of Occupational and Environmental Health, College of Public Health, University of Iowa, Iowa City, 52242, IA, USA

Abstract

PCB 136 is an environmentally relevant chiral PCB congener, which has been found *in vivo* to be present in form of rotational isomers (atropisomers). Its atropselective biotransformation or neurotoxic effects linked with sensitization of ryanodine receptor suggest that it might interact also with other intracellular receptors in a stereospecific manner. However, possible atropselective effects PCB 136 on nuclear receptor transactivation remain unknown. Therefore, in this study, atropselective effects of PCB 136 on nuclear receptors controlling endocrine signaling and/or expression of xenobiotic and steroid hormone catabolism were investigated. PCB136 atropisomers were found to exert differential effects on estrogen receptor (ER) activation; (+)-PCB 136 was estrogenic, while (–)-PCB 136 was antiestrogenic. In contrast, inhibition of androgen receptor (AR) activity was not stereospecific. Both PCB136 stereoisomers induced the constitutive androgen receptor (CAR)-dependent gene expression; however, no significant stereospecificity of PCB 136 atropisomers was observed. PCB136 was a partial inducer of the pregnane X receptor (PXR)-dependent gene expression. Here, (–)-PCB 136 was a significantly more potent inducer of PXR activity than (+)-PCB 136. Taken together, the present results indicate that at least two nuclear receptors participating in endocrine regulation or metabolism, ER and PXR, could be regulated in an atropselective manner by chiral PCB 136. The enantioselective enrichment of PCB atropisomers in animal and human tissues may thus have significant consequences for endocrine-disrupting effects of chiral *ortho*-substituted PCB congeners.

Keywords

androgen receptor; atropisomer; constitutive androstane receptor; chiral; estrogen receptors; polychlorinated biphenyl; pregnane X receptor

*corresponding author: Miroslav Machala: machala@vri.cz.

Introduction

Polychlorinated biphenyls (PCBs) are a group of widely distributed organic pollutants, which have been produced as complex mixtures, and used as electrical insulating fluids, in building construction materials or added as plasticizers or flame retardants to a variety of commercial products. Despite the ban on their production, and their generally declining levels, both in the environment and in exposed human populations, PCB contamination remains a significant environmental problem in many parts of the world, because they continue to be released into the environment through the use or disposal of various PCB-containing products (Lehmann et al., 2015). As PCBs and products of their metabolism have been shown to induce a range of toxicities in humans and wildlife, in a substitution pattern-dependent manner, these important persistent organic pollutants remain a serious concern for human health (Grimm et al., 2015).

Seventy eight PCB congeners with unsymmetrical chlorine substitutions in both phenyl rings display axial chirality because they can exist as rotational isomers, or atropisomers, that are non-superimposable mirror images of each other (Kania-Korwel and Lehmler 2016a, 2016b; Lehmler et al. 2010). However, only nineteen congeners, such as 2,2',3,3',6,6'-hexachlorobiphenyl (PCB 136), form rotational isomers that are stable under physiological conditions and at temperatures routinely used for enantioselective gas chromatographic analyses (Harju and Haglund 1999). These chiral PCB congeners are present in commercial PCB mixtures as racemate (i.e., a 1:1 mixture) (Kania-Korwel and Lehmler 2016a) and both atropisomers have been detected in environmental samples, including wildlife and humans (Kania-Korwel and Lehmler 2016b; Lehmler et al. 2010). Enantioselective enrichment of PCB atropisomers has been reported both for environmental and biological samples, including a limited number of humans samples (Kania-Korwel et al. 2015; Zheng et al. 2016). A growing number of studies demonstrate that atropselective biotransformation by cytochrome P450 enzymes may contribute to the atropisomeric enrichment observed in mammals and in humans (Lu et al. 2013; Lu and Wong 2011; Uwimana et al. 2016; Warner et al. 2009).

Although the existence of chiral polychlorinated biphenyl (PCB) congeners has been proposed more than forty years ago (Kaiser 1974), little is currently known about their atropselective effects on the expression of drug metabolizing enzymes and other important targets of PCB-mediated toxicity. Exposure of zebrafish (*Danio rerio*) to pure PCB atropisomers results in atropselective effects on amino acid metabolism pathways and causes changes in the expression of drug metabolizing enzymes *in vivo* (Chai et al. 2016a; Chai et al. 2016b). Similarly, the atropisomers of several PCB congeners have been shown to differentially alter the expression of drug metabolizing enzymes in chick embryo hepatocyte cultures and in the liver of PCB-exposed rats (Püttmann et al. 1990; Püttmann et al. 1989; Rodman et al. 1991). *In vitro* neurotoxicity studies have further demonstrated that the atropisomers of PCB 84 (2,2',3,3',6-pentachlorobiphenyl) selectively alter [³H] phorbol ester binding in rat cerebellar granule cells and ⁴⁵Ca²⁺-uptake in rat cerebellum (Lehmler et al. 2005). (–)-PCB 136, but not (+)-PCB 136 displays enantiomeric specificity toward ryanodine receptors (RyR) and affects morphometric and functional parameters of neuronal connectivity in cultured rat hippocampal neurons by mechanisms involving RyRs (Pessah et

al. 2009; Yang et al. 2014). The above results of Pessah *et al.* have demonstrated for the first time atropispecific binding and functional modification of intracellular receptors by an environmentally-relevant chiral PCB congener.

Modulation of estrogen- and androgen-dependent gene expression appears to be one of major modes of action contributing to the endocrine-disrupting effects of PCBs. Estrogen receptors (ER) and androgen receptor (AR) belong to the superfamily of nuclear receptors and both have been reported to be either transactivated or antagonized by PCBs in a congener-specific manner. Lower chlorinated PCBs, but also racemic ($\pm$)-PCB136, have been found to exert estrogenic responses, whereas higher chlorinated congeners typically elicit antiestrogenic activity, inhibiting the 17 β -estradiol (E2)-induced gene expression (Plíšková et al. 2005; Hamers et al. 2011; Zhang et al. 2014). A number of PCB congeners are potent antagonists of the AR-dependent gene expression, including ($\pm$)-PCB 136 (Bonenfeld-Jorgensen et al. 2001; Hamers et al. 2011). Constitutive androstane receptor (CAR) and pregnane X receptor (PXR) are further members of the nuclear receptor superfamily that are transactivated by various xenobiotics, including PCBs (Al-Salman and Plant 2012; Gahrs et al. 2013; Wahlang et al., 2014), and this leads to upregulation of major detoxification genes, such as the phase I cytochrome P450 (CYP) enzymes, e.g. CYP2B6 and CYP3A4. Effects of PCBs on CAR/PXR-mediated gene expression have been also linked to enhanced CYP-dependent steroid hormone metabolism and disturbances in metabolism of other endogenous compounds (Konno et al. 2008; Moreau et al. 2008; Tolson and Wang 2010). Interactions of PCBs with PXR have been proposed to potentially inhibit detoxification of steroids, bioactive dietary compounds, or xenobiotics (Tabb et al., 2004).

Although PCBs have been shown to affect the activities of multiple members of the nuclear receptor family, their atropselective effects on these important cellular targets of PCB toxicity remain unknown. Therefore, we analyzed the impact of purified atropisomers of PCB 136 on the activation of ER-, AR-, CAR- and PXR-dependent gene expression using a variety of *in vitro* models derived from human breast, prostate and liver carcinoma cells. Our present data suggest that PCB 136 exhibits atropselective effects on ER and PXR activation, while modulation of both AR and CAR activities seems to be similar for both of its atropisomers. The enantioselective enrichment of PCB atropisomers in animal and human tissues may thus have significant consequences for endocrine-disrupting effects of chiral *ortho*-substituted PCB congeners.

Materials and Methods

Chemicals and reagents

($\pm$)-PCB 136 was synthesized using the Ullmann coupling reaction and characterized as reported previously (Kania-Korwel et al. 2007). Subsequently, milligram quantities of PCB 136 atropisomers were separated by reversed phase-high pressure liquid chromatography on two serially connected Nucleodex β -PM columns (4.6 mm diameter; 250 mm length; Macherey-Nagel, Düren, Germany) (Haglund 1996, Kania-Korwel et al. 2008). The enantiomeric purity of the PCB 136 atropisomers was determined using a HP6890 gas chromatograph equipped with a ^{63}Ni -micro electron capture (μ -ECD) detector and a Chirasil-Dex column (25 m length; 0.25 mm inner diameter; 0.25 μm film thickness; Varian,

Palo Alto, CA) (Kania-Korwel et al. 2008). The enantiomeric fractions (EFs) of (–)-PCB 136 and (+)-PCB 136 were 0.01 and 1.00, respectively, with $EF = \text{peak area (+)-PCB 136} / [\text{peak area (+)-PCB 136} + \text{peak area (–)-PCB 136}]$ (Harner et al. 2000; Kania-Korwel et al. 2008). The racemic (±)-PCB 136 for *in vitro* experiments was prepared by mixing the pure atropisomers in a 1:1 ratio. Dihydrotestosterone (DHT), 17β-estradiol (E2), phenobarbital (PB) and rifampicin (RIF) were supplied by Sigma-Aldrich (Prague, Czech Republic).

Cultivation of cells for qRT-PCR analyses

The human breast carcinoma MCF-7 cells (passage numbers 15 – 18), kindly provided by A. M. Soto (Tufts University, Boston, MA) were grown in DMEM/Ham's F-12 nutrient mixture (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 7.5% FBS (GE Healthcare Life Sciences, Little Chalfont, UK). For exposure, cells were maintained in phenol-red free DMEM/F12 (Gibco) with 5% charcoal/dextran treated FBS and 1% non-essential amino acid solution (Sigma-Aldrich). The medium was replaced with fresh medium, cells were cultured for another 24 h, and then treated for 24 h with test compounds dissolved in fresh medium. E2 (100 pM) was used as model inducer of ER target gene - trefoil factor 1 (TFF1; pS2); E2 (10 pM) was used for determination of ER antagonist activity of PCBs.

Human prostate carcinoma LNCaP cells (passage numbers 4 – 6; Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) were cultivated in RPMI-1640 medium (Invitrogen) supplemented with 10 % FBS (Sigma-Aldrich), as previously described (Hrubá et al., 2011, 2014). For exposure, cells were maintained in phenol red-free RPMI-1640 supplemented with 5% dextran/charcoal-treated serum (Hrubá et al., 2014). The medium was replaced with fresh medium, cells were cultured for another 24 h, and then treated for 24 h with test compounds. DHT (10 nM) was used as a model inducer of AR target gene prostate-specific antigen (PSA) mRNA induction; DHT (1 nM) was used for determination of AR antagonist activity of PCBs.

Human hepatic HepaRG® cells (passage numbers 7 – 9; Biopredic, Rennes, France) were cultivated, based on the protocol of the cell supplier, in HepaRG® growth medium (Biopredic) for 14 days. Then, the cells were cultivated for an additional 14 days in HepaRG® differentiation medium (Biopredic). The medium was renewed every 2 to 3 days. Three days before PCB exposure, the differentiation medium was replaced with growth medium, and cells were treated for 24 h with test compounds. PB (2 mM; Sigma-Aldrich) and RIF (50 μM; Sigma-Aldrich) were used as model inducers of CAR and PXR target genes, CYP3A4 and CYP2B6, respectively.

Maximum concentrations of DMSO, which was also used as a negative control in all experiments, did not exceed 0.5% (v/v). The selected concentrations range of PCBs was between 0.01 to 25 μM, in order to include both sub-micromolar and micromolar concentrations.

ER-CALUX assay

Human breast carcinoma T47D.Luc cell line (passage numbers 22 – 25), stably transfected with pEREtataLuc plasmid (Legler et al. 1999), was used to determine ER-mediated activity

following PCB 136 exposure. The cells were kindly supplied by BioDetection Systems BV (Amsterdam, The Netherlands). Cells were grown in a 1:1 mixture of DMEM (Thermo Fisher Scientific, Waltham, MA, USA) and Ham's F-12 nutrient mixture (Gibco) supplemented with 7.5% FBS (GE Healthcare Life Sciences). For the exposure, cells were maintained in phenol-red free DMEM/F12 (Gibco) containing 5% charcoal/dextran-treated FBS and 1% non-essential amino acid solution (Sigma-Aldrich). The medium was replaced with fresh medium and after 24 h the cells were treated for 24 h with test compounds dissolved in fresh medium.

Assays were performed in 96-well cell culture plates (Costar, Cambridge, MA, USA). Briefly, 24 h after seeding (20,000 cells/cm²) (after reaching 80–90% confluence), the cells were exposed to test or reference compounds dissolved in dimethyl sulfoxide (DMSO). Maximum concentration of DMSO did not exceed 0.5% (v/v). Following 24 h exposure, the medium was removed, the cells were washed with phosphate buffered saline, luciferase was extracted with low salt lysis buffer (10 mM Tris, 2 mM DTT, 2 mM EGTA, pH 7.8) and the plates were frozen at –80 °C. The luciferase activity was measured with a Labsystems Luminoscan RS luminometer using a Luciferase Assay Kit (BioThema, Handen, Sweden). Luciferase activity was expressed in relative light units (RLU) and fold induction was calculated relative to the maximal induction by E2 (15 pM), after subtracting the value of negative control (DMSO). For estimation of antagonist activity of (±)-PCB 136 and its atropisomers, we used their co-exposure with E2 (5 pM).

Real-time RT-PCR assay

Following the exposure, cells were washed twice with PBS and harvested in the cell lysis buffer provided with the NucleoSpin RNA II Purification Kit (Macherey Nagel, Düren, Germany). Total RNA was then isolated according to the manufacturer's instructions. The levels of TFF1 and PSA mRNAs in MCF-7 cells and LNCaP cells, respectively, and CYP2B6 and CYP3A4 mRNAs in differentiated HepaRG cells were determined by quantitative real-time RT-PCR. Porphobilinogen deaminase (PBGD, NM_000190) was used as a reference gene. The primers were designed to flank the exon junctions of transcripts for amplification of cDNA only. Primers and probes for human TFF1, PSA and PBGD were synthesized by Generi Biotech (Hradec Králové, Czech Republic), primers and probes for human CYP2B6 and CYP3A4 were synthesized by Roche (Mannheim, Germany); for CYP2B6 and CYP3A4 detection, we used probes from the Universal Probe Library (Roche). The primers and probes are listed in Table 1. The amplifications were carried out in 20 µl reaction mixture containing: 10 µl of QuantiTect Probe RT-PCR Master Mix, 0.2 µl of QuantiTect RT mix (Qiagen GmbH, Hilden, Germany), 2 µl of solution of primers and probe, 5.8 µl of water and 2 µl of sample. The amplifications were run on the LightCycler (Roche) using the following program: reverse transcription at 50°C for 20 min and initial activation step at 95°C for 15 min, followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. Changes in gene expression were calculated using the comparative threshold cycle method (Schmittgen and Livak, 2008). For calculation of relative mRNA levels, the fold induction of respective mRNA after exposure to racemic PCB 136 or respective atropisomer was expressed relative to the fold-change elicited by the respective reference inducer alone.

Cytotoxicity and data analysis

For all cell lines, cytotoxicity of ($\pm$)-PCB 136 or its atropisomers was checked using the neutral red assay; no cytotoxicity was observed up to 25 μ M concentration. Using dose-response curves, the EC25 and EC50 values of individual PCB atropisomers were calculated as concentrations inducing the same level of luciferase activity or specific gene expression as the respective reference agonist (25 and 50% of maximum induction, respectively). For determination of antagonistic activities, co-treatment with the respective reference agonist (at the concentration of agonist, which induced lower than maximum activity) and PCB was used, and inhibitory concentrations IC25 and IC50 (concentrations inhibiting the agonist-induced luciferase activity or specific gene expression by 25 and 50%, respectively) were then calculated based on full dose-response curves. Data are presented as means derived from three independent experiments performed in triplicates and were analyzed by Student's *t*-test; a *p* value < 0.05 was considered significant.

Results

Effects of PCB136 atropisomers on ER-mediated activity

We used the ER-CALUX assay, employing T47D human breast carcinoma cells stably transfected with luciferase reporter gene under the control of estrogen responsive elements (Legler et al. 1999), in order to estimate the ER-mediated activity of ($\pm$)-PCB 136 and its two atropisomers, (+)-PCB 136 and (–)-PCB 136. E2 was used as a reference ER inducer. (+)-PCB 136 exhibited a strong ER-mediated activity, while (–)-PCB 136 did not induce a significant increase of luciferase activity (Fig. 1A). ($\pm$)-PCB 136 caused an intermediate effect. The respective EC25 and EC50 values are listed in Table 2. The ER-mediated responses to a combined exposure with E2 (5 pM) and ($\pm$)-PCB 136 or to its individual atropisomers showed also significant differences. (+)-PCB 136 slightly potentiated E2-dependent induction of luciferase activity, while (–)-PCB 136 exhibited antiestrogenic effects, especially at higher concentrations (Fig. 1B). The IC25 and IC50 values for antiestrogenic activities are presented in Table 2.

In order to confirm these observations, we next evaluated the effects of ($\pm$)-PCB 136 and its two atropisomers on the expression of *TFF1* mRNA, an endogenous ER-regulated gene, in human breast carcinoma MCF-7 cells. We again observed relatively high estrogenicity of (+)-PCB 136 and clear differences between ER-dependent responses towards (+)- vs. (–)-PCB 136 atropisomers (Fig. 2A). Moreover, both ($\pm$)-PCB 136 and (+)-PCB 136 significantly potentiated the E2-induced *TFF1* gene expression, while (–)-PCB 136 was inactive in this assay (Fig. 2B). These results confirmed that atropisomers of PCB 136 exhibit atropispecific effects on human ER activation.

(Anti-)androgenic effects of PCB 136 atropisomers

The androgenic and anti-androgenic activities of PCB 136 and its atropisomers were investigated in human prostate carcinoma LNCaP cells, a frequently used model for evaluation of androgen-dependent gene expression (Morrow et al. 2004, Hrubá et al. 2011). Expression of PSA, AR target gene, was used as an end-point for measuring potential androgenic/antiandrogenic effects (Xing et al., 2001). None of the test compounds induced

PSA mRNA at the concentration of 25 μM and, therefore, they exhibited no androgenicity (data not shown). In contrast, ($\pm$)-PCB 136 and both its atropisomers showed significant antiandrogenic activity, when PCB exposure was combined with exposure to the endogenous AR agonist, DHT. However, no stereoselective effects were observed (Fig. 3).

Effects of ($\pm$)-PCB 136 and its atropisomers on CAR- and PXR-dependent gene expression

The differentiated human HepaRG cell line has been shown to respond both to phenobarbital (PB) and rifampicin (RIF), prototypical inducers of CAR and PXR, respectively (Anthérieu et al., 2010). Here, we used this cell model to assess the effects of PCB 136 atropisomers on CAR- and PXR-dependent gene expression; induction of CYP2B6 and CYP3A4 mRNAs was used as a respective end-point. PB and RIF were used as reference agonists. Both PCB 136 atropisomers were found to be potent, but only partial agonists of CAR-dependent CYP2B6 induction, with no stereoselectivity being observed (Fig. 4). EC₂₅ values for (+)-PCB 136 and (–)-PCB 136 were 1.0 and 0.7 μM , respectively. EC₅₀ value for (+)-PCB 136 was 6.6 μM . The PXR-dependent gene expression was only partially induced by PCB atropisomers, as compared with the reference inducer, RIF. However, in contrast to CAR, we found that (–)-PCB 136 was a significantly more potent inducer of CYP3A4 mRNA than (+)-PCB 136 (Fig. 5), with EC₂₅ value being 7.4 μM for (–)-PCB 136. The data on CAR-agonistic and PXR-agonistic responses of PCB 136 atropisomers are summarized in Table 3.

Discussion

Chirality is a growing aspect of environmental research, since individual enantiomers may interact differentially with other chiral molecules, such as enzymes or biological receptors, leading to different biological and toxicological effects. Since a number of PCB congeners are axially chiral, understanding of enantiomer-specific PCB toxicity becomes increasingly recognized as being of significant importance (Lehmle et al., 2010). Chiral PCBs, including PCB 136, have been shown to display atropisomeric enrichment in animal organism, especially at higher trophic levels, and undergo atropselective biotransformation (ref. in Kania-Korwel and Lehmler, 2016a). Chiral PCBs are toxicologically important congeners (Robertson and Hansen, 2001, Pessah et al. 2010), and the identification of their mechanisms of action, determination of relative effective potencies and stereoselective effects are important steps in hazard assessment. Recently, (–)-PCB 136 has been reported to activate RyRs, Ca²⁺ channels widely expressed in brain and muscle tissues, while (+)-PCB 136 exhibited no significant activity towards the RyR (Pessah et al. 2009). PCB 136 atropselectively alters morphometric and functional parameters of neuronal connectivity in cultured rat hippocampal neurons via RyR-dependent mechanisms (Yang et al. 2014). This indicates that PCB 136 might interact in an atropselective manner with other types of receptors, including nuclear receptors. Importantly, PCB 136 atropisomers have been found to exhibit differential inducibility of aminopyrine-N-demethylase and aldrin epoxidase activities, which can be attributed to activities of CYP2B and/or CYP3A monooxygenases (Püttman et al., 1989). Both PCB 95, which is structurally similar to PCB 136, and PCB 136 have been found to alter the expression of related CYPs in mice (Kania-Korwel et al., 2012; Kania-Korwel et al., 2017). An atropselective induction of CAR or PXR (or other transcription factors) controlling the expression of xenobiotic-metabolizing enzymes may

alter also levels of endogenous signaling compounds, such as steroid hormones, since xenobiotic-metabolizing enzymes are involved not only in detoxification of xenobiotics entering the body, but also in catabolism of endogenous lipid compounds, including steroids.

A number of PCB congeners have been proposed to elicit endocrine-disrupting effects via modulation of transcription activity of steroid receptors or indirectly via modulations of xenobiotic/steroid hormone metabolism. Racemic ($\pm$)-PCB 136 exerts ER-agonistic and AR-antagonistic activities in luciferase reporter gene assays (Hamers et al. 2011); however, effects of its individual atropisomers remain unknown. We hypothesized that the effects of chiral PCBs on nuclear receptors, including both steroid receptors (AR, ER) and receptors regulating enzymes involved in metabolism of steroid hormones might be atropselective. Therefore, the aim of the present study was to investigate whether enantiospecific modulations of selected nuclear receptors may occur in cell models derived from tissues expressing significant levels of the respective receptors, including breast, prostate and liver cells. In this study, we examined the ability of PCB 136 atropisomers to activate or suppress transcriptional activities of human ER, AR, CAR and PXR.

We observed a significant stereoselectivity of ER responses, with (+)-PCB 136 being a potent estrogen ($EC_{25} = 1.3 \mu M$), while (–)-PCB 136 was antiestrogenic ($IC_{25} = 7.5 \mu M$). Similarly, but to a lesser extent, PCB 136 atropisomers exhibited different potencies to induce PXR-mediated gene expression, (–)-PCB 136 being much more potent, although only a partial PXR agonist ($EC_{25} = 7.4 \mu M$), than (+)-PCB 136. In contrast, CAR activation as well as the suppression of AR-mediated gene expression, which represent other important mechanisms of action of PCB 136 linked with endocrine disruption, were similarly affected by both atropisomers (EC_{25} being 1.0 and 0.7 μM for (+)-PCB 136 and (–)-PCB 136, respectively). Comparison of EC_{25} , EC_{50} and/or IC_{25} and IC_{50} values suggests that ER and CAR activation were the most sensitive endpoints reflecting the PCB 136-elicited modulations of selected nuclear receptors. Our results are in line with the evidence showing that purified PCB 136 exhibits primarily AR-antagonistic and ER-agonistic activities as determined by reporter gene assays (Hamers et al., 2011). Importantly, the modulations of the ER and PXR were stereospecific, which implies that atropselective alterations of activities of nuclear receptors might contribute, in a stereospecific manner, to neurodevelopmental and reproduction effects of chiral PCB congeners. This might be relevant especially for ER-mediated activities of chiral PCBs, since (+)-PCB 136 and (–)-PCB 136 exhibited opposite activities towards ER. Until recently, little attention has been paid to atropselective effects of environmental contaminants, such as PCBs, despite the fact that the enantioselective enrichment of PCB atropisomers observed in both animal and human tissues might have significant consequences for endocrine-disrupting effects of chiral *ortho*-substituted PCB congeners. This is in a sharp contrast with the attention given e.g. to chiral pharmaceuticals. Future studies should thus focus on the relevance of the observed stereospecific action of chiral PCB on nuclear receptors *in vivo*.

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Fig. 1.

ER-agonistic and antagonistic responses to (±)-PCB 136 and its atropisomers. **A**, induction of luciferase reporter gene activity after 24 h exposure; **B**, modulation of 5 pM E2-mediated luciferase gene expression by (±)-PCB 136 and its atropisomers (24 h exposure, ER-CALUX assay). The induction of luciferase activity was expressed relative to maximal induction by E2 (100 pM). The data obtained in three independent experiments are shown as means $\pm$ S.D. (n=9). The asterisks denote a significant difference between the effects of respective concentrations of (+)-PCB 136 and (-)-PCB 136 (** p <0.01, *** p <0.001).

Fig. 2.

Induction of TFF1 mRNA in human breast carcinoma MCF-7 cells exposed to 25 μ M PCB 136 and its two atropisomers for 24 h. **A**, estrogenicity of (±)-PCB 136 and its atropisomers; data are expressed relative to the maximal induction of TFF1 mRNA by E2 (15 pM); **B**, potentiation of E2-induced estrogenic activity by (±)-PCB 136 and (+)-PCB 136 atropisomer after the co-exposure of cells with test PCB (25 μ M) and E2 (5 pM). The data obtained in three independent experiments are presented as means $\pm$ S.D. (n=6). The asterisks denote a significant difference between the effects of test compound and the respective control (* p <0.05, ** p <0.01, *** p <0.001).

Fig. 3.

Suppression of induction of PSA mRNA in LNCaP cells by 25 μM ($\pm$)-PCB 136 and its atropisomers after 24-h co-exposure to 1 nM DHT. Following the exposure, total RNA was isolated and PSA mRNA levels were determined by qRT-PCR as described in Materials and methods, and expressed relative to DHT. The data obtained in three independent experiments are presented as means $\pm$ S.D. (n=6). The asterisks denote a significant difference between the effects of test compound + DHT and DHT alone (** $p < 0.01$, *** $p < 0.001$).

Fig. 4. Induction of CYP2B6 mRNA in differentiated HepaRG cells after 24-h exposure to PCB 136 atropoisomers. Following the exposure, total RNA was isolated and CYP2B6 mRNA levels were determined by qRT-PCR as described in Materials and methods. The data obtained in three independent experiments are expressed relative to phenobarbital (PB, 2 mM) and presented as means $\pm$ S.D. (n=6).

Fig. 5.

Induction of CYP3A4 mRNA in differentiated HepaRG cells after 24-h exposure to PCB 136 atropoisomers. Following the exposure, total RNA was isolated and CYP3A4 mRNA levels were determined by qRT-PCR as described in Materials and methods. The data obtained in three independent experiments are expressed relative to rifampicin (RIF, 50 μM) and presented as means $\pm$ S.D. (n=6). The asterisks denote a significant difference between the effects of respective concentrations of (+)-PCB 136 and (-)-PCB 136 (* p <0.05).

Table 1

Primers and probes used for qRT-PCR

Gene	Accession number	Sequence
CYP2B6	NM_00767.4	F: 5'-CAG CAC CAC TCT CCG CTA C-3'
		R: 5'-TCA ATC TCC CTG TAG ACT CTC TCT G-3'
		P: UPL # 4 (Roche, Mannheim, Germany)
CYP3A4	NM_001202855.2	F: 5'-GAT GGC TCT CAT CCC AGA CTT-3'
		R: 5'-AGT CCA TGT GAA TGG GTT CC-3'
		P: UPL # 64 (Roche, Mannheim, Germany)
TTF1	NM_003225	F: 5'-CAAATAAGGGCTGCTGTTTCG-3'
		R: 5'-GCTCTGGGACTAATCACCGT-3'
		P: 5'-CCCCTGGTGCTTCTATCCTAATACC-3'
PSA	NM_001648	F: 5'-CCTGAGCACCCCTATCAAC-3'
		R: 5'-CCTGGACCTCACACCTAAG-3'
		P: 5'-CTCAAGCCTCCCCAGTTCTACTGACC-3'

F: forward primer; R: reverse primer; P: probe

ER-agonistic and antagonistic responses of ($\pm$)-PCB136 and its atropisomers expressed relative to reference compound 17 β -estradiol (E2)

Table 2

Compound	ER-agonistic			ER-antagonistic		
	EC ₂₅	EC ₅₀	Response at 10 μ M (% of E2max)	IC ₂₅	IC ₅₀	Response at 25 μ M (% of E2 response)
($\pm$)-PCB136	2.6	6.8	63	ND	ND	81
(+)-PCB136	1.3	2.9	89	ND	ND	123
(-)-PCB136	8.7	ND	27	7.5	15.9	27

Note: ER-agonistic activities are expressed relative to the effect of E2 (15 pM); ER-antagonistic effects of test compounds were determined in the presence of E2 (5 pM); n = 3; ND, not determined.

Table 3

CAR- and PXR-agonistic responses of (+)- and (-)-PCB 136 expressed relative to reference inducers phenobarbital (PB) and rifampicin (RIF)

Compound	CAR-agonistic response			PXR-agonistic response		
	EC25 (µM)	EC50 (µM)	Maximum response at 5 µM (% of PB max)	EC25 (µM)	EC50 (µM)	Maximum response at 25 µM (% of RIF max)
(+)-PCB 136	1.0	ND	38	ND	ND	17
(-)-PCB 136	0.7	6.6	51	7.4	ND	38

Note: CAR-agonistic activities (induction of CYP2B6 mRNA) are expressed relative to the effect of PB (2 mM); PXR-agonistic activities (induction of CYP3A4 mRNA) are expressed relative to the effect of RIF (50 µM); n = 3; ND, not determined.