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In vitro profiling of toxic effects of prominent environmental lower-chlorinated PCB congeners linked with endocrine disruption and tumor promotion

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Abstract

The mechanisms contributing to toxic effects of airborne lower-chlorinated PCB congeners (LC-PCBs) remain poorly characterized. We evaluated *in vitro* toxicities of environmental LC-PCBs found in both indoor and outdoor air (PCB 4, 8, 11, 18, 28 and 31), and selected hydroxylated metabolites of PCB 8, 11 and 18, using reporter gene assays, as well as other functional cellular bioassays. We focused on processes linked with endocrine disruption, tumor promotion and/or regulation of transcription factors controlling metabolism of both endogenous compounds and xenobiotics. The tested LC-PCBs were found to be mostly efficient anti-androgenic (within nanomolar – micromolar range) and estrogenic (at micromolar concentrations) compounds, as well as inhibitors of gap junctional intercellular communication (GJIC) at micromolar concentrations. PCB 8, 28 and 31 were found to partially inhibit the aryl hydrocarbon receptor (AhR)-mediated activity. The tested LC-PCBs were also partial constitutive androstane receptor (CAR) and pregnane X receptor (PXR) agonists, with PCB 4, 8 and 18 being the most active compounds. They were inactive towards other nuclear receptors, such as vitamin D receptor, thyroid receptor α , glucocorticoid receptor or peroxisome proliferator-activated receptor γ . We found that only PCB 8 contributed to generation of oxidative stress, while all tested LC-PCBs

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Conflict of interest

The authors declare no conflict of interest.

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induced arachidonic acid release (albeit without further modulations of arachidonic acid metabolism) in human lung epithelial cells. Importantly, estrogenic effects of hydroxylated (OH-PCB) metabolites of LC-PCBs (4-OH-PCB 8, 4-OH-PCB 11 and 4'-OH-PCB 18) were higher than those of the parent PCBs, while their other toxic effects were only slightly altered or suppressed. This suggested that metabolism may alter toxicity profiles of LC-PCBs in a receptor-specific manner. In summary, anti-androgenic and estrogenic activities, acute inhibition of GJIC and suppression of the AhR-mediated activity were found to be the most relevant modes of action of airborne LC-PCBs, although they partially affected also additional cellular targets.

Graphical Abstract

Keywords

airborne polychlorinated biphenyls; endocrine disruption; metabolism of xenobiotics; tumor promotion; hydroxylated PCBs

1. Introduction

Polychlorinated biphenyls (PCBs) are widely distributed organic pollutants, which have been used as electrical insulating fluids, in building construction materials or added as plasticizers and flame retardants to a variety of consumer products. Despite the ban on their production and their declining levels in the environment, PCB contamination remains a major environmental problem in many parts of the world, as they continue to be released into the environment through the use or disposal of various PCB-containing products (Lehmann et al., 2015). In particular, the volatile PCB congeners continue to present a threat to human health, since significant levels of the airborne lower-chlorinated PCB congeners (LC-PCBs) are found in PCB-contaminated buildings or within industrial and densely populated urban areas (Herrick et al., 2004; Lehmann et al., 2015). Their indoor concentrations are often higher than in the respective outdoor areas (Jamshidi et al., 2007). A recent study comparing indoor versus outdoor levels of PCBs in PCB-contaminated U.S. schools revealed median indoor air levels of Σ PCBs ranging from 2.05 to 111.83 ng/m³

(95th percentile 5.14 to 187.92 ng/m³) (Marek et al., 2017). In contrast, outdoor air levels in the same study were much lower and ranged from 0.16 to 0.58 ng/m³ (95th percentile: 0.50 to 2.38 ng/m³). LC-PCBs have been also identified in blood samples of exposed human populations (Fitzgerald et al., 2011; Gabrio et al., 2000). During recent years, LC-PCBs have become a point of a renewed interest, as several PCBs, such as PCB 11, have been discovered to be byproducts of pigment production (Hu and Hornbuckle, 2010; Anezaki et al., 2015). These so-called non-legacy or unintentional PCBs are found, together with other LC-PCB congeners, in consumer products, as well as in the environment (Grimm et al., 2015).

Based on their toxicological mechanism of action, PCBs can be divided into two groups. The dioxin-like coplanar PCBs (DL-PCBs) activate the aryl hydrocarbon receptor (AhR) and this is considered their principle mode of action (Van den Berg et al., 2006). In contrast, the most abundant environmental non-dioxin-like PCBs (NDL-PCBs) do not activate the AhR, and there is no generally accepted risk concept for these compounds (Hamers et al., 2011), although some NDL-PCBs have been shown to inhibit the AhR transcriptional activity (Brennerová et al., 2016; Ovesen et al., 2011). Nevertheless, NDL-PCBs exert numerous specific modes of action linked with carcinogenicity, neurotoxicity or endocrine disruption (Gore et al., 2015; Grandjean and Landrigan, 2006; Lauby-Secretan et al., 2013). They have been shown: i) to interact with nuclear receptors controlling metabolism of both endogenous and exogenous compounds (including hormones), such as constitutive androstane receptor (CAR) or pregnane X receptor (PXR); ii) to directly modulate gene expression dependent on activation of estrogen (ER), androgen (AR) or thyroid receptors; iii) to exert effects linked with carcinogenesis/tumor promotion, such as generation of reactive oxygen species (ROS) or inhibition of gap junctional intercellular communication (GJIC); or to produce neurotoxic effects (Gore et al., 2015; Grimm et al., 2015; Hamers et al., 2011; Knerr and Schrenk, 2006; Machala et al., 2003; Pessah et al., 2006; Simon et al., 2007). Effects of NDL-PCBs can also be associated with further alterations of physiological processes within the body, as illustrated e.g. in case of disruption of CAR/PXR signaling by PCBs, linked with deregulation of energy metabolism, inflammation and liver diseases (Wahlang et al., 2016).

Therefore, both DL- and NDL-PCBs may act through multiple mechanisms contributing to their toxic action, which can be used to derive their respective relative effective potency values. Such data may aid the risk assessment of both individual PCB congeners and their mixtures (Hamers et al., 2011). However, the available information about specific toxicities of LC-PCBs is very limited. Just recently, a study comparing activities of LC-PCBs towards several hormonal receptors and AhR has suggested that LC-PCBs may act as agonists or antagonists of ER, AR, glucocorticoid receptor (GR) or AhR (Takeuchi et al., 2017). However, this study used ectopic expression of specific nuclear receptors together with the respective reporter genes in rodent, not human, cells. Importantly, it also did not investigate some LC-PCBs found at significant levels in both indoor and outdoor air, such as PCB 18, 28 and 31 (Carpenter, 2015; Marek et al., 2017). Therefore, we still have only a limited information about the activity of environmental LC-PCBs within the context of endogenous nuclear receptor expression in human cell models. Furthermore, little is known about their further impact on cells, including their potential effects linked with tumor promotion. The rapid metabolism of abundant LC-PCB congeners may lead to formation of hydroxylated

metabolites with specific toxicity profiles (Grimm et al., 2015); however, the toxicities of hydroxylated LC-PCBs are less known than those of their parent compounds, although hydroxylation significantly alters the activities of a number of PCB congeners (Dhakal et al., 2017; Machala et al., 2004), and some of them could be highly active towards ER or AR (Takeuchi et al., 2011).

Therefore, we employed a series of *in vitro* assays to determine toxicity profiles of six abundant LC-PCBs that are present at high levels in Aroclor 1260 vapor, and/or that are among the most abundant LC-PCB congeners found in contaminated school buildings – PCB 4, 8, 11, 18, 28 and 31 (Carpenter, 2015; Marek et al., 2017). Next to these LC-PCBs, we determined *in vitro* toxicities of major hydroxylated metabolites of PCB 8, 11 and 18: 4-OH-PCB 8, 4-OH-PCB 11, 5-OH-PCB 11 and 4'-OH-PCB 18. As impurities may alter cellular responses to NDL-PCBs, the purity of all test compounds was rigorously tested by gas chromatography-mass spectrometry (GC-MS). We tested the potencies of selected LC-PCBs and OH-LC-PCBs to interact with AhR, ER, AR, GR, thyroid receptor (TR), vitamin D receptor (VDR), peroxisomal-proliferator activated receptor γ (PPAR γ), using human cell lines endogenously expressing the receptors and stably transfected with the respective reporter vectors. Activation of CAR/PXR was estimated using determination of expression of their respective endogenous gene targets - cytochrome P450 2B6 (CYP2B6) and CYP3A4 in differentiated HepaRG cells (Anthérieu et al., 2010). The estimation of receptor activities was further complemented with functional cell bioassays, focusing on the effects leading to generation of oxidative stress and oxidative DNA damage, deregulation of ER-dependent cell proliferation, stimulation of arachidonic acid (AA) release and disruption of GJIC.

2. Materials and methods

2.1 Reagents

2,2'-Dichlorobiphenyl (PCB 4), 2,4'-dichlorobiphenyl (PCB 8), 3,3'-dichlorobiphenyl (PCB 11), 2,2',5-trichlorobiphenyl (PCB 18), 2,4',5-trichlorobiphenyl (PCB 31) and 2,2',5-trichlorobiphenyl-4'-ol (4'-OH-PCB 18) were purchased from AccuStandard (New Haven, USA). 2,4,4'-Trichlorobiphenyl (PCB 28) was from Dr. Ehrenstorfer (LGC Standards, Teddington, UK). 2,4'-Dichlorobiphenyl-4-ol (4-OH-PCB 8), 3,3'-dichlorobiphenyl-4-ol (4-OH-PCB 11) and 3,3'-dichlorobiphenyl-5-ol (5-OH-PCB 11) were synthesized as described in Supplementary Experimental Procedures. The purities of all LC-PCB congeners and trace impurities are reported in Supplementary Tables 1 and 2. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), 99% purity, was from Cambridge Isotope Laboratories (Andover, USA). ICI 182,780 was from Tocris Bioscience (Bristol, UK). Mifepristone was purchased from (Cayman Chemical). Cyproterone acetate (CPTA) was from Santa Cruz Biotechnology (Santa Cruz, USA). 17 β -Estradiol (E2), 5 α -dihydrotestosterone (DHT), triiodothyronine (T3), rosiglitazone (RGS), phenobarbital (PB) and rifampicin (RIF) were from Sigma-Aldrich (Prague, Czech Republic). All other chemicals not specified above were of the highest purity available; they were purchased from Sigma-Aldrich, if not indicated otherwise.

2.2 Reporter gene assays

The following cell lines, endogenously expressing the respective receptors and stably transfected with corresponding luciferase reporter genes were used to estimate specific receptor activation: AZ-AHR cell line was used to quantify the AhR- and anti-AhR-mediated activity (Novotná et al., 2011); AZ-GR cell line was used to determine the GR-mediated activity (Novotná et al., 2012); IZ-VDRE and IZ-CYP24 cell lines were used for detection of VDR activation (Bartošková et al., 2016); PZ-TR cell line was used for determination of TR-mediated activity (Illés et al., 2015); T47D.luc cell line, kindly provided by BioDetection Systems BV (BDS, Amsterdam, The Netherlands), was used to estimate the ER-mediated activity (Legler et al., 1999); AIZ-AR cell line was used for determination of androgen receptor (AR) activation (Bartošková et al., 2015); HT-29 cell stably transfected with PPAR γ reporter were used for determination of PPAR γ -mediated activity (Tylichová et al., 2017). For detection of PXR-mediated activity, we employed human colon adenocarcinoma LS180 (purchased from the European Collection of Cell Cultures, Salisbury, UK) transiently transfected with PXR-regulated reporter gene, as described previously (Kubešová et al., 2016). Description of all individual assays is provided in Supplemental Experimental Procedures.

2.3 Real-time quantitative reverse transcription PCR (qRT-PCR)

HepaRGTM cells (passage numbers 7–9; Biopredic, Rennes, France) were cultivated and differentiated according to the protocol of the provider, as described previously (P n íková et al., 2017). The differentiation medium was exchanged with cultivation medium 3 days before treatment, and the cells were treated for 24 h with test compounds. PB (10 – 2000 μ M) and RIF (1 – 100 μ M) were used as model inducers of CAR and PXR, respectively. Cells were collected, total RNA was isolated, and mRNA levels of CYP2B6 and CYP3A4 were determined by qRT-PCR, using QuantiTect Probe RT-PCR kit (Qiagen GmbH, Hilden, Germany). The qRT-PCR conditions, sequences of primers and numbers of Universal Probe Library probes (Roche LifeScience, Mannheim, Germany) for CYP2B6 and CYP3A4 have been reported previously (P n íková et al., 2017). The primers and probe for reference gene PBGD were provided by Generi Biotech Hradec Králové, Czech Republic).

2.4 Cell cycle analysis

Human breast cancer MCF-7 cells, (American Type Culture Collection, Rockville, MD, USA) were cultivated in Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DMEM/F12; Gibco, Thermo Fisher Scientific, Waltham, USA) supplemented with antibiotics and 5% (v/v) fetal bovine serum (FBS), respectively (Gibco). Cells were synchronized using phenol red-free DMEM/F12, supplemented with 5% dextran/charcoal-treated FBS for 48 hours. Cells were exposed to test compounds for 24 h, using DMSO and E2 as negative and positive controls, respectively. Following the treatment, cells were harvested by trypsinization, washed twice in PBS, and fixed in 70% ethanol. DNA was stained (37°C; 30 min) with Vindelov solution (10 mM Tris buffer, pH 8; 0.7 mg/ml RNase; 50 μ g/ml propidium iodide; 0.1% Triton-X100). The DNA content was analyzed by flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA, USA). 15,000 events were

acquired per each sample and cell cycle was analyzed using ModFit 3.0 software (Verity Software House, Topsham, USA).

2.5 The scrape loading-dye transfer (SL-DT) detection of GJIC

The SL-DT method was performed as previously described (Machala et al., 2003). Briefly, WB-F344 rat liver epithelial cells, kindly provided by James E. Trosko (Michigan State University, East Lansing, USA) were cultivated in the Dulbecco's modified Eagle medium (DMEM; Gibco), supplemented with 5% FBS, 24 mM NaHCO₃, 10 mM HEPES and antibiotics. Confluent cells were exposed to test compounds, fluoranthene (Fla; 100 µM; positive control) or DMSO (0.5% (v/v); negative control) for 1 h. After the exposure, a modified protocol of SL-DT assay was used to assess GJIC. The cells were washed twice with phosphate-buffered saline solution (PBS), fluorescent dye was added (Lucifer Yellow, Sigma; 0.05% w/v in PBS), and the cells were scraped using surgical steel blade. After 2 min, the cells were washed twice with 0.5 ml PBS and fixed with 4% formaldehyde (v/v). The migration of the dye from the scrape line was measured with epifluorescence microscope. At least 3 scrapes per well were evaluated. The GJIC inhibition was expressed relative to negative control (% DMSO).

2.6 Detection of oxidative stress and AA release

Human adenocarcinoma A549 cells were grown in DMEM supplemented with 1.0 g/l glucose and pyruvate, 10% fetal bovine serum (FBS), 200 mM glutamine and antibiotics. Cells were seeded in cell-culture dishes with 35 mm diameter at density of 17,000 cells/cm². Test compounds were diluted with DMSO and added to the medium at 25 µM concentration. The final concentration of DMSO was less than 0.1% (v/v). For detection of ROS generation, cells were exposed to test compounds for 24 h. Exposure to hydrogen peroxide (400 µM; 5 min) was used as a positive control. At the end of exposure, cells were washed twice with PBS, trypsinized, centrifuged, and re-suspended in Hank's balanced salt solution (PAN BioTech GmbH, Aidenbach, Germany) with 5% FBS. The cell suspension was incubated for 15 min with the fluorogenic probe dihydrorhodamine 123 (Sigma-Aldrich; 2 µM). Cells were then washed once, centrifuged, and analyzed with flow cytometer (FACSCalibur, Becton Dickinson), using 488 nm argon ion laser for excitation and the CellQuestTM software (Becton Dickinson) for analysis. For detection of 8-hydroxy-2'-deoxyguanosine (8-OHdG), cells were exposed to 25 µM of test compounds for 6 h, or to 2 mM KBrO₃ (positive control) for 24 h. After exposure, cells were washed twice by ice-cold PBS and harvested in PBS. The cell pellets were frozen at -20 °C. DNA was isolated from cells by DNeasy Blood and Tissue Kit (Qiagen) and 8-OHdG levels were determined using liquid chromatography/tandem mass spectrometry (LC/MS-MS) using Agilent 1200 chromatographic system (Agilent Technologies, Santa Clara, USA), as described previously (Trilecová et al., 2011). For AA release analysis, we used a modification of the method described previously (Umannová et al., 2008). Briefly, cells were exposed to test compounds for 6 h, cell culture medium was extracted using solid-phase-extraction with SELECT HLB SPE 1 ml (30 mg) cartridges (Supelco, Prague, Czech Republic) and levels of AA and AA metabolites were determined by LC/MS-MS using Agilent 1200 chromatographic system. A triple quadrupole mass spectrometer Agilent 6410 Triple Quad LC/MS-MS (Agilent

Technologies) with an electrospray interface (ESI) was used for detection of the analytes. Description of LC/MS-MS methods is provided in Supplemental Experimental Procedures.

2.7 Cytotoxicity assays

In order to exclude any non-specific cytotoxicity-related effects of LC-PCBs from our analyses (Marin-Kuan et al., 2017), cytotoxicity was checked in all cell lines using the neutral red (NR) uptake or MTT assays. For additional information on methods, see Supplemental Experimental Procedures, where the results of cytotoxicity assays are also summarized in Supplementary Table 4.

2.8 Data Analysis

All experiments were performed independently at least three times, and the data were quantitatively expressed as means $\pm$ SD and analyzed by t-test or one-way ANOVA. A p value of less than 0.05 was considered significant. Using dose-response curves, the EC25 and EC50 values of individual PCB congeners were calculated as concentrations inducing the same level of luciferase activity 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 that induced lower than maximum activity) and PCB was used, and inhibitory concentrations IC25 and IC50 (concentrations inhibiting the agonist-induced luciferase activity by 25 and 50%, respectively) were again calculated based on full dose-response curves, using GraphPad Prism for curve fitting (GraphPad Software, San Diego, USA).

3. Results

3.1 LC-PCBs exert partial anti-AhR activities and partial CAR/PXR-mediated activities

A previous study has indicated that several LC-PCBs may act as weak AhR agonists (Takeuchi et al., 2017). Therefore, we first analyzed the AhR-mediated activities of PCB 4, 8, 11, 18, 28 and 31 in reporter gene assay employing human AZ-AhR cell line. However, none of the tested compounds induced a significant AhR-mediated activity (data not shown). In contrast, three of the tested PCB congeners – PCB 8, 28 and 31, were found to inhibit the TCDD-induced AhR activation in a dose-dependent manner at micromolar concentrations (Fig. 1A, Table 1). A similar trend was observed also for PCB 11 (Fig 1A). Two LC-PCBs bearing chlorine substitutions at both 2 and 2' positions, PCB 4 and 18, did not modulate AhR activity up to a concentration of 40 μ M.

All LC-PCBs acted as partial CAR agonists inducing CYP2B6 mRNA levels in differentiated human liver HepaRG cells (Fig. 1B). Maximum induction levels did not reach 50% of maximum activity induced by CAR reference inducer, PB. Based on EC25 values, the order of activities of individual compounds was PCB 18 ~ PCB 4 ~ PCB 31 > PCB 8 > PCB 11 > PCB 28 (Table 1). The tested LC-PCBs were also weak PXR inducers. PCB 4, 8 and 18 induced lower than 20% level of CYP3A4 mRNA as compared with maximum CYP3A4 mRNA level induced by reference PXR agonist RIF (Fig. 1C). The other LC-PCBs had only very limited or no effects on CYP3A4 induction. When tested in the reporter gene assay using human colon carcinoma cells transiently transfected with PXR-dependent

reporter, only PCB 18 induced major PXR activity, with a response of more than 50% relative to RIF. All other compounds induced only a minor increase of PXR-mediated activity at high micromolar concentrations (Suppl. Fig. 1).

3.2 Anti-androgenic and estrogenic activities of LC-PCBs

Some previous studies have indicated that both anti-androgenic and estrogenic activities of NDL-PCBs, including LC-PCB congeners, are among their most prominent *in vitro* effects (Hamers et al., 2011; Takeuchi et al., 2017). All tested PCB congeners inhibited the DHT-induced activity of the AR-regulated reporter gene, with some compounds being partially active already at nanomolar range (Fig. 2A, Table 1). Based on their IC50 values, the order of inhibitory activities of individual compounds was PCB 8 > PCB 11 ~ PCB 28 > PCB 4 > PCB 18 ~ PCB 31 (Table 1).

There were larger differences among the estrogenic activities of LC-PCBs. PCB 11 and 31 were only weakly active, both inducing less than 20% of the maximum response induced by E2 (Fig. 2B). In contrast, PCB 4, 8 and 18 behaved as full ER agonists, with PCB 28 being somewhat less active (Fig. 2B). Based on their EC25 values and maximum induced responses, the order of estrogenic activities of active compounds was PCB 4 ~ PCB 18 > PCB 8 > PCB 28 >> PCB 31 ~ PCB 11 (Table 1). Since induction of reporter gene may not always reflect the transcriptional activation of endogenous estrogen gene targets (Gozgit et al., 2004), we also evaluated LC-PCB-induced stimulation of estrogen-dependent cell cycle progression in human breast cancer MCF-7 cells (Plíšková et al., 2005). As shown in Fig. 2C, all tested LC-PCBs (including PCB 11) increased percentage of cells in the S-phase of the cell cycle, i.e. stimulated cell cycle progression, in synchronized MCF-7 cells, with PCB 18 and 28 being already active at 10 µM concentration.

In contrast to their anti-AR- or ER-mediated activities, LC-PCBs exerted no major effects on activation of other nuclear receptors analyzed in this study (when tested in either agonist or antagonist mode) – GR, VDR or PPARγ (Suppl. Table 3). We observed a marginal increase of TRα-mediated activity to be induced by PCB 18; however, other LC-PCBs did not affect the TRα activation (Suppl. Table 3).

3.3 Effects of LC-PCBs linked with carcinogenesis and/or tumor promotion

Induction of ROS production and induction of AA release have been suggested to be closely associated with disruption of cellular control mechanism linked both with their neurotoxicity and with carcinogenicity/tumor promotion (De et al., 2010; Chaudhuri et al., 2010; Kodavanti and Derr-Yellin, 2002; Tithof et al., 1996; Zhu et al., 2013). Therefore, we next analyzed the impact of LC-PCBs on these events in human lung carcinoma A549 cells. This cell line is a suitable model of respiratory epithelium (a primary target of airborne LC-PCBs), which can be used to assess the activities of phospholipases, as well as AA metabolism (Choudhury et al., 2000). We screened the effects of LC-PCBs at 25 µM concentration. As shown in Fig. 3A, only PCB 8 was found to significantly increase ROS production and to induce oxidative DNA damage, detected as 8-OHdG formation (Fig. 3B). All six tested LC-PCBs increased AA release in A549 cells to a similar extent, with exception of PCB 4, which was only marginally active. Using the same experimental system,

we also evaluated the impact of LC-PCBs on formation of principal AA metabolites, in particular on production of prostaglandins; however, we found no significant impact of LC-PCBs on AA metabolism (data not shown). Finally, using rat liver epithelial WB-F344 to estimate disruption of GJIC, another parameter closely linked with tumor promoting effects of PCBs (Machala et al., 2004; Machala et al., 2003; Šimeková et al., 2009), we found that all six LC-PCBs inhibited GJIC in a dose-dependent manner, with PCB 18 being the most effective compound (Fig. 3D, Table 1).

3.4 Hydroxylation may promote the toxic effects of LC-PCBs, in particular their estrogenicity

Next, we evaluated the impact of metabolism of LC-PCBs to hydroxylate metabolites on their toxic effects. For this, we selected the toxic endpoints that were found to be significantly altered by parent LC-PCBs. The anti-AhR activity was mostly not affected by hydroxylation of parent LC-PCBs, as the effects of 4-OH-PCB 8, 4-OH-PCB 11, 5-OH-PCB 11 were similar to their parent compounds (Fig. 4A; Table 2). Interestingly, 4'-OH-PCB 18 weakly potentiated the AhR-mediated activity, while the parent compound was inactive in this assay. Hydroxylated LC-PCBs did not induce a full suppression of the AhR-mediated activity, and both their IC₂₅ and IC₅₀ values were similar to parent compounds (Table 1). Hydroxylated LC-PCBs (with exception of 4-OH-PCB 8 and 4'-OH-PCB 18 which marginally induced CYP2B6 mRNA) did not activate either CAR or PXR in HepaRG cells (Table 2). Also the anti-androgenic activities were only slightly altered in case of 4-OH-PCB 8, 4-OH-PCB 11, 5-OH-PCB 11 and 4'-OH-PCB 18, as compared with their parent compounds (Fig. 4B, Table 2).

In contrast, the estrogenic activity of LC-PCBs was the significantly affected by hydroxylation. In particular, 4-OH-PCB 8 and 4'-OH-PCB 18 were more active than the respective parent LC-PCBs, with their EC₅₀ values being in nanomolar range and maximum luciferase induction levels exceeding those of E2 (Fig. 4C, Table 2). Although PCB 11 induced only a marginal ER-mediated activity (Fig. 2B, Table 1), its 4-OH-PCB 11 metabolite had significantly higher activity (Fig. 4C, Table 2), while 5-OH-PCB 11 was not estrogenic. Inhibition of GJIC was only partially modulated by hydroxylation. Hydroxylation at position 4 reduced GJIC inhibitory activity of PCB 11 (Fig. 4D, Table 2).

4. Discussion

Despite the evidence suggesting that LC-PCBs are a matter of public concern (Hu and Hornbuckle, 2010; Marek et al., 2017), their toxic modes of action remain mostly poorly characterized, as compared with more abundant higher-chlorinated PCBs (Grimm et al., 2015; Lehmann et al., 2015). Therefore, we aimed to characterize toxic action of selected environmental LC-PCBs and their hydroxylated metabolites *in vitro*, using a battery of assays covering both their interactions with intracellular receptors (linked with endocrine disruption and alterations of metabolism of both endogenous compounds and xenobiotics) and further toxic effects associated with carcinogenesis/tumor promotion, including generation of oxidative stress, disruption of GJIC or alterations of AA release and metabolism.

The toxicity profiles of individual LC-PCBs and hydroxylated PCBs are summarized in Table 3. The anti-androgenic activity is a major toxic mode of action of pure NDL-PCB congeners, with IC₅₀ values ranging from nanomolar to micromolar range (Hamers et al., 2011). A recent study, using rodent cells transfected with human androgen receptor, suggested that this might be also relevant for LC-PCBs (Takeuchi et al., 2017). Here, using a human cell model, we observed that environmentally-relevant PCBs 18 and 31, not evaluated in previous studies, inhibited the AR-mediated activity, with PCB 18 belonging among the most active compounds. All six tested PCB congeners significantly reduced AR-mediated activity in assay employing endogenous AR in human cell line, with their IC₅₀ values approaching some of the previously published data (Hamers et al., 2011; Takeuchi et al., 2017).

Estrogenicity was the second most prominent activity of LC-PCBs. Unlike higher chlorinated PCBs, which are anti-estrogenic, a number of lower chlorinated congeners have been reported to be estrogenic (Hamers et al., 2011; Plíšková et al., 2005; Zhang et al., 2014). The estrogenic effects were recently confirmed also for some additional LC-PCBs (Takeuchi et al., 2017), including some investigated here, such as PCB 4 and 8. Here, PCB 18 was identified as the most active congener, although EC₅₀ values for all six LC-PCBs fell within a narrow micromolar range. The only exception was PCB 11, thus confirming a lack of estrogenic effects of this congener (Takeuchi et al., 2017). Nevertheless, all six compounds stimulated ER-dependent cell cycle progression in synchronized human breast cancer MCF-7 cells, an endpoint relevant for activation of endogenous ER-mediated response (induction of cell proliferation) by PCBs (Zhang et al., 2014). LC-PCBs also increased percentage of cells in G2M phase of cell cycle in a similar manner as S-phase (data not shown). This was again most probably linked with their proliferative effects related to activation of ER α , which has been documented both in this study and in previous reports on estrogenic effects of some other tri- and tetrachlorinated PCB congeners in cell models derived from human breast carcinoma cells (Plíšková et al., 2005; Hamers et al., 2011).

LC-PCBs did not induce or inhibited the GR-, VDR- and PPAR- γ -mediated activities. This is in line with the evidence suggesting that although some higher chlorinated PCBs, such as PCB 101, may weakly activate GR or antagonize GR in yeast bioassay, this occurs mostly at very high concentrations (Bovee et al., 2011). No information is currently available for the other two receptors; however, our results suggest that LC-PCBs do not activate or inhibit VDR and PPAR- γ . We have observed a weak potentiation of T3-induced response and a weak TR α -mediated activity of PCB 18. This, however, did not reach a higher than 20 % activity of the reference agonist T3 activity. This observation supports the current view that thyroid-disrupting effects of NDL-PCBs are primarily linked to disruption of binding of thyroxine to thyroxine-binding protein, transthyretin (Hamers et al., 2011).

Environmental ligands of AhR, CAR and PXR contribute both to regulation of xenobiotic-metabolizing enzymes (XME) and control metabolism of endogenous substrates, including altered metabolism of hormones or other substances (Kretschmer and Baldwin, 2005; Moreau et al., 2008; Safe and Wormke, 2003). Inhibition of the AhR was the third most prominent effect of LC-PCBs, after their anti-androgenicity and estrogenicity (Table 3). A previous study has indicated that LC-PCBs are not major AhR ligands; however, their anti-

AhR activities were not addressed (Takeuchi et al., 2017). Here, we observed that, similar to some higher chlorinated PCB congeners (Brennerová et al., 2016), PCB 8, 11 and 31 inhibited that the AhR-mediated activity, although less efficiently than PCB 28 (Brennerová et al., 2016; this study).

Activation of CAR and PXR alters lipid metabolism, glucose homeostasis and inflammation primarily through interactions with further transcription factors, and may contribute to endocrine disrupting effects linked with metabolic diseases including obesity and diabetes (Moreau et al., 2008). Therefore, the potential impact of LC-PCBs on the activation of these two receptors is of particular interest. Previous studies have indicated that NDL-PCBs, including PCB 28, are primarily CAR inducers (Gähres et al., 2013), with only minor contribution to PXR activation. In addition, several LC-PCBs were found to be active in yeast CAR assay (Kamata et al., 2015). However, the information about activities of PCBs in human cells is limited to higher chlorinated PCB congeners (Al-Salman and Plant, 2012; Gähres et al., 2013). Interestingly, we found that all tested LC-PCBs were more potent CAR ligands than PCB 28 (Gähres et al., 2013), despite inducing only a partial CAR-mediated activity. For some of them, we observed significant stimulation of CAR-dependent CYP2B6 expression already at lower than micromolar concentrations in differentiated HepaRG cells, a well-defined model for analysis of CAR and PXR-dependent regulation of XMEs (Anthérieu et al., 2010). LC-PCBs induced less prominent PXR activity, with PCB 11 and 18 being most active in the PXR-dependent reporter gene assay. This supports recent findings suggesting that NDL-PCBs are less efficient PXR inducers (Gähres et al., 2013). Interestingly, an earlier study indicated that higher chlorinated PCBs may also act as PXR antagonists (Tabb et al., 2004). This suggests that chlorination pattern may define PXR activity in a similar manner as their estrogenic and anti-estrogenic effects (Hamers et al., 2011; Plíšková et al., 2005; Zhang et al., 2014). Together, our data suggest that LC-PCBs are moderately active towards receptors controlling XMEs and metabolism, AhR, CAR and PXR, which are also known to contribute to carcinogenic effects of PCBs (Knerr and Schrenk, 2006; Lauby-Secretan et al., 2013).

Finally, we screened toxic endpoints expected to be relevant for carcinogenic/tumor promoting (but also neurotoxic) effects of PCBs, including generation of oxidative stress, AA release and inhibition of GJIC. Only one tested congener, PCB 8, induced significant ROS production and oxidative DNA damage in a human lung epithelial cell model. This type of activity has been previously observed for hydroxylated or quinone metabolites of other dichlorobiphenyls, including PCB4 and 11, but not for individual NDL-PCBs or Aroclor 1254 mixture (Chaudhuri et al., 2010; Dreiem et al., 2009; Zhu et al., 2013). In contrast, all of the tested congeners stimulated AA release, although we did not observe any alterations of profiles of AA metabolites, such as prostaglandins, in lung cells (data not shown). All tested LC-PCBs were found to inhibit GJIC, an effect linked with tumor promotion, in a dose-dependent manner, as observed for a number of NDL-PCBs (Hamers et al., 2011; Machala et al., 2003). Here, we found that PCBs inhibited GJIC within a micromolar range corresponding well with values reported for purified NDL-PCBs (Hamers et al., 2011).

Importantly, we evaluated for the first time the impact of hydroxylated metabolites of two tested LC-PCBs, PCB 8 and 11, and we compared them with available data on 4'-OH-PCB 18 (Takeuchi et al., 2011), using the assays selected based on prominent activities of parent compounds. Several hydroxylated metabolites of LC-PCBs, in particular those of PCB 11, have been identified both in experimental animals and in human serum, and they have been shown to exert unique toxicities (Hu et al., 2013; Sethi et al., 2017; Zhu et al., 2013). We found that the estrogenic activity of LC-PCBs was the parameter most affected by hydroxylation. In particular, 4-OH-PCB 8, similar to 4'-OH-PCB 18, was a much stronger ER activator, with EC50 values within nanomolar range, which corresponded with previously reported EC20 value for 4'-OH-PCB 18. Similarly, while the non-Aroclor PCB 11 was a poor ER agonist, its 4-OH metabolite induced a significantly higher ER-mediated activity. Interestingly, 4-OH-PCB 8 and 4'-OH-PCB 18 were found to induce higher levels of ER-mediated activity than natural ER agonist, E2. This could be due to several reasons. Recently, it has been proposed that the superinduction of ER-mediated luciferase reporter gene activity may represent a post-transcriptional effect of some compounds on enzyme stability (Sotoca et al., 2010). An alternative explanation could be that hydroxylated LC-PCBs may activate another signaling pathway contributing to ER α activation through mechanisms that are not dependent on direct ligand binding, but which may instead affect activation function 1 of this receptor (Safe et al., 2002).

Other parameters (anti-AhR activity, anti-androgenicity or inhibition of GJIC) were only marginally affected by addition of OH group; however, we found that hydroxylated LC-PCBs were mostly inactive as CAR or PXR inducers, which seems to support the hypothesis that hydroxylation may modulate CAR-mediated activity of PCBs (Kamata et al., 2015).

In conclusion, we evaluated in vitro toxicity profiles of LC-PCBs suggesting that LC-PCBs induce primarily anti-androgenic and estrogenic effects, with some of them inhibiting AhR signaling and inducing moderate CAR/PXR-mediated activities. All tested LC-PCBs were found to inhibit GJIC and induce AA release, while a single compound, PCB 8 was found to induce oxidative stress. Importantly, hydroxylation of LC-PCBs altered their toxicities in both substitution- and receptor-specific manner, promoting their estrogenic effects, which was observed in particular for PCB 8 and 18, as well as for unconventional PCB 11, a unique PCB congener formed during pigment production. Our results, providing information about individual toxic potencies of environmentally relevant LC-PCB congeners, may in future contribute to refine the risk assessment of this poorly characterized group of PCBs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary Data

Extended information on methodology (Supplemental Experimental Procedures), individual tested compounds (Supplementary Tables 1 and 2), the effects of LC-PCBs in assays reflecting VDR, TR, GR and PPAR γ activation (Supplementary Table 3), an overview of cytotoxicity of PCBs in individual assays (Supplementary Table 4) and detection of PXR activation in LS180 cells transiently transfected with PXR-dependent luciferase reporter gene (Supplementary Figure 1), is provided in Supplementary Data file.

- In vitro toxicities of environmental LC-PCBs and hydroxylated LC-PCBs were estimated.
- LC-PCBs induced anti-androgenic and estrogenic effects, inhibition of AhR signaling and moderate CAR/PXR-mediated activities.
- LC-PCBs were found to inhibit gap junctional intercellular communication and induce AA release.
- Hydroxylation of LC-PCBs altered their toxicities in a receptor-specific manner, promoting their estrogenic effects.

Figure 1.

Anti-AhR-, CAR- and PXR-mediated activities of LC-PCBs in human liver cell models. **A)** Anti-AhR activities determined in AZ-AhR cells co-exposed to test compounds and 4.5 nM TCDD for 24 h. The results, expressed relative to TCDD-induced luciferase activity, represent means $\pm$ S.D. of three independent experiments. Symbols ‘*’ and ‘**’ denote significant difference ($p < 0.05$ and $p < 0.01$, respectively), as compared to TCDD-treated cells. **B)** and **C)** CAR- and PXR-mediated activities determined as induction of CYP2B6 and CYP3A4 mRNAs, respectively, in HepaRG cells exposed to LC-PCBs or reference inducers,

PB and RIF, for 24 h. The results, expressed relative to maximum PB-induced CYP2B6 and RIF-induced CYP3A4 mRNA levels, represent means $\pm$ S.D. of three independent experiments. Symbols ‘*’ and ‘**’ denote significant difference ($p < 0.05$ and $p < 0.01$, respectively), as compared to vehicle (DMSO).

Figure 2.

Anti-androgenic and estrogenic effects of LC-PCBs. **A)** Anti-androgenic activities determined in AIZ-AR cell line co-exposed to LC-PCBs and 2 nM DHT for 24 h. CPTA was used as a control compound suppressing AR activity. The results, expressed relative to DHT-induced luciferase activity, represent means $\pm$ S.D. of three independent experiments. Symbols ‘*’ and ‘**’ denote significant difference ($p < 0.05$ and $p < 0.01$, respectively), as compared to DHT-treated cells. **B)** Estrogenic activities determined in T47D.luc cells exposed to LC-PCBs or E2, as a reference compound, for 24 h. The results, expressed

relative to maximum E2-induced luciferase activity, represent means $\pm$ S.D. of three independent experiments. Symbols '*' and '**' denote significant difference ($p < 0.05$ and $p < 0.01$, respectively), as compared to DMSO-treated cells. C) Induction of cell cycle progression in MCF-7 cells by LC-PCBs. Synchronized MCF-7 cells were exposed to test compounds or vehicle (DMSO) for 24 h and cell cycle was analyzed by flow cytometry. Percentage of cells in S-phase was expressed relative to DMSO-treated (control) cells. The results represent means $\pm$ S.D. of three independent experiments. Symbols '*' and '**' denote significant difference ($p < 0.05$ and $p < 0.01$, respectively), as compared to DMSO-treated cells.

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Figure 3.

Effects of LC-PCBs on induction of oxidative stress, release of AA and inhibition of GJIC.

A) Generation of ROS and formation of 8-OHdG in human lung A549 cells. Cells were exposed to LC-PCBs (25 μM) for 24 h or to indicated reference oxidative stress inducers, collected and analyzed by flow cytometry and LC/MS-MS, respectively. The results, expressed relative to control, represent means $\pm$ S.D. of three independent experiments. Symbols '*' and '**' denote significant difference ($p < 0.05$ and $p < 0.01$, respectively), as compared to DMSO-treated cells. **B)** Levels of AA release in culture medium collected from

A549 cells exposed to LC-PCBs (25 μ M) or AA (10 μ M) for 24 h. The results, expressed relative to control, represent means $\pm$ S.D. of three independent experiments. Symbols '*' and '**' denote significant difference ($p < 0.05$ and $p < 0.01$, respectively), as compared to DMSO-treated cells. C) Inhibition of GJIC in rat liver epithelial WB-F344 cells. Cells were exposed to LC-PCBs, fluoranthene (Fla; 100 μ M; positive control) or vehicle (DMSO) for 1 h, and GJIC was determined using SL-DT assay. The results, expressed relative to DMSO, represent means $\pm$ S.D. of three independent experiments. Symbols '*' and '**' denote significant difference ($p < 0.05$ and $p < 0.01$, respectively), as compared to DMSO-treated cells.

Figure 4.

Anti-AhR, anti-androgenic or estrogenic effects of hydroxylated LC-PCBs, and their impact on GJIC. **A)** Anti-AhR activities were determined in AZ-AhR cells as in Fig. 1A. The results, expressed relative to TCDD-induced luciferase activity, represent means $\pm$ S.D. of three independent experiments. Symbols '*' and '**' denote significant difference ($p < 0.05$ and $p < 0.01$, respectively), as compared to TCDD-treated cells. **B)** Anti-androgenic activities were determined in AIZ-AR cells as in Fig. 2A. The results, expressed relative to DHT-induced luciferase activity, represent means $\pm$ S.D. of three independent experiments.

Symbols ‘*’ and ‘***’ denote significant difference ($p < 0.05$ and $p < 0.01$, respectively), as compared to DHT-treated cells. **C)** Estrogenic activities were determined in T47D.luc cells as in Fig. 2B. The results, expressed relative to maximum E2-induced luciferase activity, represent means $\pm$ S.D. of three independent experiments performed in triplicates. Symbols ‘*’ and ‘***’ denote significant difference ($p < 0.05$ and $p < 0.01$, respectively), as compared to DMSO-treated cells. **D)** Inhibition of GJIC in rat liver epithelial WB-F344 cells was determined as in Fig. 3C. The results, expressed relative to DMSO, represent means $\pm$ S.D. of three independent experiments. Symbols ‘*’ and ‘***’ denote significant difference ($p < 0.05$ and $p < 0.01$, respectively), as compared to DMSO-treated cells.

Table 1

Comparison of agonistic and/or inhibitory activities of LC-PCBs towards AhR, CAR, PXR, AR, ER, and inhibition of GJIC

Compound	anti-AhR activity			CAR-mediated activity			PXR-mediated activity		
	IC25 (µM)	IC50 (µM)	maximum activity (%TCDD) ^a	EC25 (µM)	EC50 (µM)	maximum activity (%PB) ^b	EC25 (µM)	EC50 (µM)	maximum activity (%RIF) ^c
PCB 4	-	-	100	3	-	29 ^g	-	-	15 ^h
PCB 8	14	-	58	9	-	30 ^h	-	-	12 ^h
PCB 11	17	37	50	26	-	26 ^h	-	-	< 5 ^h
PCB 18	-	-	100	2	-	42 ^h	-	-	15 ^h
PCB 28	7	17	24	-	-	15 ^h	-	-	7 ^h
PCB 31	16	39	45	5	-	44 ^h	-	-	6 ^h

Compound	anti-AR activity			ER-mediated activity			GJIC inhibition		
	IC25 (µM)	IC50 (µM)	maximum activity (%DHT) ^d	EC25 (µM)	EC50 (µM)	maximum activity (%E2) ^e	IC25 (µM)	IC50 (µM)	maximum activity (%DMSO) ^f
PCB 4	0.7	7.4	25	6	11	103	15	23	19
PCB 8	0.04	0.9	18	10	20	81	17	27	18
PCB 11	1.3	4.4	14	-	-	18	18	27	16
PCB 18	0.4	9.5	28	5	9	116	8	12	0 ^j
PCB 28	1.4	4.5	12	15	27	66	14	22	17
PCB 31	3.1	11.5	23	-	-	24	13	21	12

^a anti-AhR activity is expressed relative to the activity induced by 4.5 nM TCDD;

^b CAR-mediated activity is expressed relative to CYP2B6 mRNA levels induced by 2 mM PB;

^c PXR-mediated activity is expressed relative to CYP3A4 mRNA levels induced by 50 µM RIF;

^d anti-AR activity is expressed relative to the activity induced by 2 nM DHT;

^e ER-mediated activity is expressed relative to the activity induced by 3.5 pM E2;

^f GJIC (migration of Lucifer Yellow dye) is expressed relative to the dye migration in control (DMSO-treated) cells; the maximum activities were determined at 40 µM concentration of a given compound, if not indicated otherwise;

j_g maximum activity at 5 μM ;
 j_f maximum activity at 25 μM ;
 j_i maximum activity at 20 μM ;

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Table 2

Comparison of agonistic and/or inhibitory activities of hydroxylated LC-PCBs towards AhR, CAR, PXR, AR, ER, and inhibition of GJIC

Compound	anti-AhR activity			CAR-mediated activity			PXR-mediated activity		
	IC25 (µM)	IC50 (µM)	maximum activity (%TCDD) ^a	EC25 (µM)	EC50 (µM)	maximum activity (%PB) ^b	EC25 (µM)	EC50 (µM)	maximum activity (%RIF) ^c
4-OH PCB 8	18	-	63 ^g	-	-	9 ^g	-	-	< 5 ^g
4-OH PCB 11	10	20	25 ^g	-	-	< 5 ^g	-	-	< 5 ^g
5-OH PCB 11	9	18	31 ^g	-	-	< 5 ^g	-	-	< 5 ^g
4'-OH-PCB 18	-	-	134 ^g	-	-	16 ^g	-	-	< 5 ^g

Compound	anti-AR activity			ER-mediated activity			GJIC inhibition		
	IC25 (µM)	IC50 (µM)	maximum activity (%DHT) ^d	EC25 (µM)	EC50 (µM)	maximum activity (%E2) ^e	IC25 (µM)	IC50 (µM)	maximum activity (%DMSO) ^f
4-OH PCB 8	0.3	0.7	9 ^h	0.1	0.1	175 ^j	15	19	0
4-OH PCB 11	1.0	2.1	24 ^h	5.0	8.2	65 ^g	43	-	76
5-OH PCB 11	0.7	1.6	18 ^h	-	-	10 ^j	25	33	22
4'-OH-PCB 18	1.5	3.9	41 ^h	0.1	0.2	136 ^j	7	11	0

^a anti-AhR activity is expressed relative to the activity induced by 4.5 nM TCDD;

^b CAR-mediated activity is expressed relative to CYP2B6 mRNA levels induced by 2 mM PB;

^c PXR-mediated activity is expressed relative to CYP3A4 mRNA levels induced by 50 µM RIF;

^d anti-AR activity is expressed relative to the activity induced by 2 nM DHT;

^e ER-mediated activity is expressed relative to the activity induced by 3.5 pM E2;

^f GJIC (migration of Lucifer Yellow dye) is expressed relative to the dye migration in control (DMSO-treated) cells; the maximum activities were determined at 40 µM concentration of a given compound, if not indicated otherwise;

^g maximum activity at 25 µM;

^h maximum activity at 5 µM;

ⁱ maximum activity at 10 µM;

f_{maximum} activity at 750 nM;

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Table 3

An overview of toxicity profiles of LC-PCBs and hydroxylated LC-PCBs

PCB congener	PCB 4	PCB 8	PCB 11	PCB 18	PCB 28	PCB 31	4-OH-PCB 8	4-OH-PCB 11	5-OH-PCB 11	4'-OH-PCB 18
<i>Effects linked with disruption of transcription factors controlling metabolism of endogenous compounds and xenobiotics</i>										
AhR activity	0	0	0	0	0	0	0	0	0	0
anti-AhR activity	0	1	2	0	3	2	1	2	3	0
CAR activity	1	1	1	1	0	1	0	0	0	0
PXR activity ^a	0	0	1	1	0	0				
<i>Effects linked with endocrine disruption and modulation of other nuclear receptors</i>										
ER activity	3	3	0	4	2	0	5	4	0	5
anti-ER activity	0	0	0	0	0	0	0	0	1	0
AR activity	0	0	0	0	0	0	0	0	0	0
anti-AR activity	4	5	4	4	4	3	5	4	4	4
GR activity	0	0	0	0	0	0				
anti-GR activity	0	0	0	0	0	0				
TR activity	0	0	0	0	0	0				
anti-TR activity	0	0	0	0	0	0				
PPAR γ activity	0	0	0	0	0	0				
anti-PPAR γ activity	0	0	0	0	0	0				
VD3R activity	0	0	0	0	0	0				
anti-VD3R activity	0	0	0	0	0	0				
<i>Effects linked with tumor promotion/carcinogenesis</i>										
inhibition of GJIC	2	2	2	3	2	2	2	0	2	4
ROS production ^b	0	2	0	0	0	0				
oxidative DNA damage ^b	0	1	0	0	0	0				
AA release ^b	0	1	1	2	1	2				

0 no or lower than 25% induction/suppression relative to reference compound; did not allow IC₂₅/EC₂₅ calculation;

■ 25 – 50% induction/suppression relative to reference compound; did not allow IC₅₀/EC₅₀ calculation;

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IC50 or EC50 reached between 20 and 40 μM concentration;
IC50 or EC50 reached between 10 and 20 μM concentration;
IC50 or EC50 reached between 1 and 10 μM concentration;
IC50 or EC50 reached at lower than 1 μM concentration;
for approximation of PXR activity, a combination of data from the reporter gene assay and induction of CYP3A4 mRNA were used;
for these parameters, only activities at 25 μM concentrations were screened;