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Atropselective Partitioning of Polychlorinated Biphenyls in a HepG2 Cell Culture System: Experimental and Modeling Results

Chun-Yun Zhang, Susanne Flor, Gabriele Ludewig, Hans-Joachim Lehmler

Department of Occupational and Environmental Health, The University of Iowa, Iowa City, Iowa 52242, United States

Abstract

Cell culture models are used to study the toxicity of polychlorinated biphenyls (PCBs); however, it is typically unknown how much PCB enters the cells and, for chiral PCBs, if the partitioning is atropselective. We investigated the partitioning of racemic PCB 91, PCB 95, PCB 132 and PCB 136 in HepG2 cells following a 72-h incubation. PCBs were present in cell culture medium (60.7–88.8 %), cells (8.0–14.6 %), and dishes (2.3–7.8 %), and displayed atropisomeric enrichment in cells (enantiomeric fraction [EF]=0.55–0.77) and dishes (EF=0.53–0.68). Polyparameter linear free energy relationships coupled with a composition-based model provided a good estimate of the PCB levels in cells and cell culture medium. The free concentration was subsequently used to extrapolate from the nominal cell culture concentration to PCB tissue levels and *vice versa*. This approach can be used for *in vitro-in vivo* extrapolations for all 209 PCB congeners. However, this model (and modified models based on descriptors incorporating atropselective interactions, i.e., relative retention time on a chiral column) did not predict the atropselective partitioning in the cell culture system. Improved chemical descriptors that account for the atropselective binding of PCBs to biological macromolecules are, therefore, needed to predict the atropselective partitioning of PCBs in biological systems.

Graphical Abstract

Corresponding Author: Dr. Hans-Joachim Lehmler, The University of Iowa, Department of Occupational and Environmental Health, University of Iowa Research Park, B164 MTF, Iowa City, IA 52242-5000, Phone: (319) 335-4981, Fax: (319) 335-4290, hans-joachim-lehmler@uiowa.edu.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interest.

SUPPORTING INFORMATION

Details regarding the deconjugation procedures, untargeted analysis, mathematic details of the partitioning models, chemical standards, enantiomeric fractions, quality assurance/quality control data, biological compositions, estimated ASPs of PCB atropisomers, predicted EFs, cytotoxicity data, overall extraction workflow, sorptive capacities of chemicals, schematic partitioning in cell culture model, partitioning of PCBs atropisomers in cell-free incubations, and PP-LFERs for relative retention times on chiral columns. This material is available free of charge via the Internet at <http://pubs.acs.org>

INTRODUCTION

The use of PCBs in technical applications is expected to end by 2025 under the Stockholm Convention on Persistent Organic Pollutants.¹ Moreover, legacy PCBs are supposed to be disposed of in an environmentally sound manner by 2028. However, some PCBs are still produced unintentionally and can be found in consumer products, such as paint and resins.^{2–4} Biomonitoring studies demonstrate that PCB levels in human food, human serum, and environmental samples, such as fish, only slightly decreased over the last two decades,^{5–7} indicating PCBs exposure still represents a significant and current public health concern. Exposure to PCBs has been linked to a range of adverse human health outcomes, including cancer, immunotoxicity, cardiovascular disease, and developmental neurotoxicity.⁸

PCB congeners with multiple *ortho* substituents are implicated in the developmental neurotoxicity of PCBs.⁹ Several of these neurotoxic PCB congeners display axial chirality. These congeners exist as stable rotational isomers, or atropisomers, which are non-superimposable mirror images of each other. Chiral PCB congeners are atropselectively metabolized to potentially neurotoxic hydroxylated and sulfated metabolites,^{10, 11} resulting in an atropisomeric enrichment of both the parent PCB and its metabolites in environmental samples, wildlife, and humans.^{10, 12} PCB atropisomers atropselectively affect the expression of drug-metabolizing enzymes in the rat liver,^{13, 14} and bind atropselectively to cytochrome P450 enzymes.¹⁵ Pure atropisomers of PCB 95 and 136 selectively affect endpoints involved in PCB developmental neurotoxicity by mechanisms involving ryanodine receptors (RyRs).^{16–18}

In vitro models, such as cells and tissue fractions in culture, are a powerful tool to study the toxicity of these PCB congeners. Studies with cells in culture typically report only the nominal concentrations of a chemical, such as chiral PCBs. Only a few studies have measured the fraction of PCBs that are associated with the cells;^{17, 19} however, it remains unknown to which extent these measurements reflect PCB levels inside the cells and how these levels relate to PCB tissue levels reported by animal and human biomonitoring studies. Composition-based models have been developed to predict the partitioning of different chemicals in microsomal incubations²⁰ and cell culture models,²¹ and to relate nominal concentrations to the free concentration (C_{free}) *in vitro*. Because only the C_{free} of a chemical is considered available for cell membrane uptake and partitioning into or out of cells, composition-based models have also used the C_{free} for *in vitro-in vivo* extrapolation (IVIVE)

or interassay results comparisons.^{21–23} Studies also showed that C_{free} can be used to explain biological effects under different exposure scenarios.^{24, 25}

The C_{free} in cell culture assays can be determined experimentally with solid-phase microextraction techniques.^{24, 26, 27} Alternatively, the C_{free} can be calculated using the quantities and sorptive capacities of each biological components (i.e., protein, lipid, and water) in the bioassay system.^{20, 21, 28, 29} Briefly, C_{free} in the aqueous phase can be determined from the nominal concentration ($C_{nominal}$) using the following general mass balance equation:

$$C_{free} = \frac{C_{nominal} V_{medium}}{\sum K_{i/water} V_i} \quad (1)$$

where i represents the sorptive components, including protein, lipid, water, etc. When the sorptive component i is water, the partitioning coefficient $K_{i/water}$ equals 1. V_{medium} is the volume of medium, and V_i is the volume of component i . The C_{free} calculated for an *in vitro* bioassay can subsequently be used for IVIVE to estimate *in vivo* tissue levels (C_{tissue}) with the following equation:

$$C_{tissue} = C_{free} \sum K_{i/water} f_i \quad (2)$$

where f_i is the volume fraction of the biological component i *in vivo*. The quantities of the biological components *in vitro* and *in vivo* can be determined experimentally or are available from the literature.^{21, 30} In addition, $K_{i/water}$ can be measured or estimated from the octanol/water partition coefficient (K_{ow})^{22, 28} or polyparameter-linear free energy relationships (PP-LFERs).^{21, 30} These data are readily available for all 209 PCB congeners.³¹ Although the composition-based models coupled with PP-LFERs have been developed in both *in vitro* and *in vivo* systems,^{21, 30} no such model has been linked together to extrapolate from nominal concentrations in *in vitro* assays to PCB tissue concentrations and *vice versa*.

This study characterized the partitioning of PCB 91 (2,2',3,4',6-pentachlorobiphenyl), PCB 95 (2,2',3,5',6-oentachlorobiphenyl), PCB 132 (2,2',3,3',4,6'-hexachlorobiphenyl), and PCB 136 (2,2',3,3',6,6'-hexachlorobiphenyl), four environmentally and toxicologically relevant chiral PCBs,^{10, 11} in a HepG2 cell culture model in the absence of detectable PCB metabolism. The C_{free} and PCBs levels in both medium and cells in the cell culture model was estimated using a PP-LFER composition-based model. Subsequently, different PP-LFER-assisted composition-based models for *in vitro* or *in vivo* systems were linked via the C_{free} . This approach enabled us to extrapolate from the nominal PCB concentration in the cell culture experiments to tissue PCB levels and *vice versa*. Because the PCB congeners investigated are chiral, their atropisomeric enrichment was assessed in medium, cell, and culture plates. Overall, the composition-based model allowed a straightforward IVIVE useful for toxicologists. However, this model, or a modified model that was based on descriptors incorporating atropselective interactions (i.e., retention time on a chiral column), did not account for the atropselective partitioning of chiral PCBs between medium and HepG2 cells.

EXPERIMENTAL SECTION

Materials.

PCB 95, PCB 136, 4'-chloro-3'-fluoro-4-sulfooxy-biphenyl (3-F,4'-PCB 3 sulfate, surrogate of PCB metabolites), and 4'-chloro-3'-fluoro-4-hydroxy-biphenyl (3-F,4'-OH-PCB 3, surrogate of PCB metabolites) were synthesized and authenticated as previously described.^{32, 33} PCB 91, PCB 132, 2,3,4',5,6-pentachlorobiphenyl (PCB 117, recovery standard for PCBs), 2,3,3',4,5,5'-hexachlorobiphenyl-4'-ol (4'-OH-PCB 159, recovery standard for OH-PCBs) and 2,2',3,4,4',5,6,6'-octachlorobiphenyl (PCB 204, internal standard) were purchased from AccuStandard, Inc. (New Haven, CT, USA). Analytical standards of OH-PCBs and methylated derivatives of OH-PCBs were synthesized and authenticated as reported earlier (for additional information, see Table S1).³⁴ Solutions of diazomethane in diethyl ether were prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald) using an Aldrich mini Diazald apparatus (Milwaukee, WI, USA).

Sulfatase (type H-2 from *Helix pomatia*, 2000 units/mL) for the deconjugating of potential hydroxylated PCB conjugates and resazurin sodium salt were purchased from Sigma-Aldrich (St Louis, MO, USA). Phenol red-free minimum essential medium (MEM), fetal bovine serum (FBS), L-glutamine, glucose solution, penicillin/streptomycin (P/S), Dulbecco's phosphate-buffered saline (PBS), trypsin-EDTA, Costar 6- and 24-well plates, as well as dimethyl sulfoxide (DMSO), were obtained from Thermo Fisher Scientific (Radnor, PA, USA).

HepG2 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The authenticity of the human hepatocellular carcinoma cell line HepG2 was confirmed by analysis of genomic DNA conducted by the University of Arizona Genetics Core (Arizona Research laboratories, Tucson, AZ, USA). The HepG2 cells used in this study were between passages 18 through 35. Cells were maintained in complete medium (MEM supplemented with 10 % FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM l-glutamine) in a humidified incubator with 5% CO₂ at 37 °C. The exposure medium contained MEM with 10 % FBS was supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM l-glutamine. PCBs (i.e., PCB 91, PCB 95, PCB 132, and PCB 136) were dissolved in DMSO. The final concentration of DMSO in the medium did not exceed 0.1 % (v/v). This DMSO concentration did not affect cell viability compared to unexposed HepG2 cells.

PCBs exposure of HepG2 cells.

HepG2 cells (6×10⁶/well) were seeded into 6-well plates with 3 mL complete medium per well. After 48 h attachment, cells were exposed in triplicates to 0.25, 1 or 10 µM of the racemic PCB 91, PCB 95, PCB 132, and PCB 136 (0.1 % DMSO) in exposure medium (3 mL). Comparatively high PCB concentrations of 1 or 10 µM were used in preliminary experiments to assess if these PCBs are metabolized to an appreciable extent by HepG2 cells. Studies of the partitioning of PCBs were performed at 0.25 µM (245 and 270 ng for penta- and hexa-chlorinated PCBs, respectively). The amount of PCB used in these studies was selected based on earlier *in vitro* metabolism studies to ensure that we can detect robust

changes in the EF values.^{35–38} The control group was exposed to 0.1 % DMSO in triplicates in the exposure medium. After a 72 h incubation (24 h for 1 μ M incubations), the medium was carefully transferred into weighted glass vials, and the cells were washed once with PBS (1 mL). This solution was combined with the exposure medium. The cells were harvested into PBS (1 mL) with a rubber policeman and added into a weighted glass vial. The wells were washed once with PBS (1 mL), and the washing solution was combined with the cell suspension. All vials were stored at -20°C until analysis. The plates were wrapped in aluminum foil and stored at 4°C until analysis. The cytotoxicities of PCBs at concentrations of 0.25 μ M and 10 μ M are summarized in Fig. S1.

In separate experiments, cell-free plates with 0.25 μ M of the racemic PCBs in exposure medium were also incubated for 72 h, and samples were processed as described above to study the partitioning of PCBs in the cell culture wells in the absence of cells.

Extraction of PCBs and their metabolites from media, cell pellets, and dishes.

Depending on the PCB concentrations, different extraction workflows were used for media, cell pellets, and dish samples (Fig. S2). The extraction of media samples (0.25 and 10 μ M) and cell pellets samples (0.25 μ M only) was described previously, with minor modification.^{39–41} Briefly, samples were spiked with PCB 117 (100 ng in isooctane) and 4'-OH-PCB 159 (50 ng in methanol) and acidified with hydrochloric acid to protonate OH-PCBs (6 M, 1 mL). The samples were extracted with hexane/MTBE (1:1 v/v, 5 mL), 2-propanol (5 mL) was added, and samples were re-extracted with hexane (3 mL). The combined organic phases were washed with a potassium chloride solution (1 % w/v, 4 mL), the organic phase was evaporated to dryness under a gentle stream of nitrogen, and the sample was reconstituted in hexane (1 mL). The extract was derivatized using diazomethane in ethyl ether (0.5 mL) overnight at 4°C ,⁴² and subjected to sulfur removal and sulfuric acid clean-up steps as described.^{43, 44} After extraction with hexane/MTBE, the medium samples (10 μ M PCB exposure groups only) were deconjugated with sulfatase (type H-2 from *Helix pomatia*) as described in the Supporting Information to assess the presence of sulfate or glucuronide conjugates in the cell culture media. PCB 204 (100 ng) was added as an internal standard (volume corrector) before the gas chromatographic analysis.^{43, 44}

Cell culture dishes were allowed to dry, PCB 117 (100 ng in isooctane) was added, and all dishes were washed twice with hexane (4 mL and 3 mL).¹⁷ The hexane phase was cleaned up as described above for media and cell pellets samples.

Untargeted analyses were performed with media samples from the 1 μ M PCB exposure groups. Briefly, media samples were extracted with acetonitrile, and the resulting extracts were screened for the presence of PCB metabolites by Ultra-Performance Liquid Chromatography-Quadrupole Time-of-flight Mass Spectrometry (UPLC-QToF-MS) as described in the Supporting Information.

Quantitative gas chromatographic analyses of PCBs and their metabolites.

Quantitative analysis of PCBs and OH-PCBs (as methylated derivatives) in sample extracts was carried out on an Agilent 7890A gas chromatography (GC) equipped with an SPB-1 capillary column (60 m length, 250 μ m inner diameter, 0.25 μ m film thickness; Supelco, St

Louis, MO, USA) and a ^{63}Ni -micro electron capture detector (μECD) as previously reported.^{39, 40} Helium was used as carrier gas with a constant flow rate of 2 mL/min. The temperature program was as follows: initial temperature 50 °C for 1 min, 30 °C/min to 200 °C, 1 °C/min to 250 °C, 10°C/min to 280 °C, and hold for 3 min. The PCBs were identified based on their retention time, with relative retention times (RRT) being within 0.5 % of the RRT of the respective PCBs standard.⁴⁵ PCBs were quantified with the internal standard using the relative response factors in both samples and the reference standard mixture. PCB levels were corrected for the recovery of the surrogate recovery standard to account for any loss of PCBs during the extraction. The hydroxylated PCB metabolites (as methylated derivatives) listed in Table S1 were not detected in any sample.

Atropselective gas chromatographic analyses of PCBs.

Atropselective analyses of PCBs were employed by an Agilent 6890 gas chromatography (GC) equipped with a ^{63}Ni - μECD detector and CP-Chirasil Dex CB (CD) (25 m length, 250 μm inner diameter, 0.25 μm film thickness; Agilent, Santa Clara, CA, USA) or Cyclosil-B (CB) (30 m length, 250 μm inner diameter, 0.25 μm film thickness; Agilent) capillary columns.^{37, 46, 47} The flow rate of the carrier gas, helium, was 3 mL/min.^{37, 38, 41, 46} The temperature program for the atropselective analysis of PCB 91, PCB 95, and PCB 136 was as follows: initial temperature 50 °C for 1 min, 10 °C/min to 140 °C, hold for 170 min, 15 °C/min to 200 °C, and hold for 20 min. The column temperature program for the atropselective analysis of PCB 132 was as follows: initial temperature 50 °C for 1 min, 10 °C/min to 160 °C, hold for 140 min, 15 °C/min to 200 °C, and hold for 20 min. The enantiomeric fraction (EF) was determined with the drop valley method and calculated as $\text{EF} = \text{Area } E_1 / (\text{Area } E_1 + \text{Area } E_2)$, where Area E_1 and Area E_2 are the peak areas of the atropisomers eluting first and second on the CD column.^{37, 38, 41, 46} A summary of the EF values on different columns is provided in Table S2.

Quality assurance/quality control (QA/QC).

The responses of the electron capture detector were linear from 1 to 1000 ng/mL for all the analytes. Method blanks and matrix blanks (media, cell pellets, and dishes) were analyzed in parallel with all samples. Besides, samples from control incubations were analyzed in parallel, including blank incubations with HepG2 cells exposed to 0.1 % DMSO only and blank, cell-free incubations containing only MEM medium and 0.1 % DMSO. The quality assurance/quality control (QA/QC) data, including the limits of detection (LODs) of PCBs, the PCB background levels calculated from the matrix blanks, the recoveries of surrogate standard (PCB 117) for all samples, resolution of the PCB atropisomers, and the EF values of the racemic standards, are summarized in Table S3.

Equilibrium partitioning model in the cell culture system.

We expanded a published composition-based model²¹ to predict the partitioning of the PCBs in the cell culture system by accounting for the well-documented differences in the partitioning of PCBs into different lipid classes (Fig. S3).^{48, 49} This model is based on several assumptions: First, both cell culture medium and cells contain four biological components (i.e., protein, storage lipid, membrane lipid, and water) (Fig. S4). Second, PCBs have reached a partition equilibrium between proteins and water and between lipids and

water. Third, the sorptive fractions (proteins, lipids, etc.) in medium change only insignificantly during the incubation. Based on these assumptions, the free concentration of PCBs can be calculated from the initial amount of PCB added to the incubation (m_0) and the sorptive capacities of the biological components in both medium and cells using the following equation (for more details, see the Supporting Information):

$$C_{free} = \frac{m_0}{K_{ap,w}(V_{m,ap} + V_{c,ap}) + K_{ml,w}(V_{m,ml} + V_{c,ml}) + K_{sl,w}(V_{m,sl} + V_{c,sl}) + V_{m,w} + V_{c,w}} \quad (3)$$

where $K_{ap,w}$, $K_{ml,w}$ and $K_{sl,w}$ are the partition coefficients of PCBs between protein and water, membrane lipid and water, and storage lipid and water, respectively; $V_{m,ap}$, $V_{m,ml}$, $V_{m,sl}$ and $V_{m,w}$ are the phase volumes of albumin protein, membrane lipid, storage lipid, and water in the medium; $V_{c,ap}$, $V_{c,ml}$, $V_{c,sl}$ and $V_{c,w}$ are the phase volumes of protein, membrane lipid, storage lipid, and water in cells.

The small contribution of plastic cell culture dishes on PCBs sorption has been observed in earlier cell culture studies.¹⁹ However, the chemical diffusion of PCBs into a solid phase, i.e., cell culture plastic (polystyrene) plate, is very slow compared to the partitioning to the biological components in medium and cells.⁵⁰ Moreover, the role of plastic dishes is considered minor in *in vitro* bioassays using protein- and lipid-rich media, such as 10 % FBS.^{23, 50} To account for the binding of PCBs to the cell culture dishes, the m_0 values used for the model calculations were corrected by subtracting the measured levels of PCBs absorbed to the cell culture dishes in this study. The partition coefficients of PCBs used for the calculations were obtained from established PP-LFERs.³⁰ The solvation parameters used for the PP-LFERs³¹ and other parameters needed for the model predictions were obtained from published literature values (Table S4).^{21, 51, 52} We employed different permutations of published experimental values for the biological components in cell culture medium and HepG2 cells because these values can show considerable variability (Table S4).

For comparison, the free concentration of PCBs in the cell culture system was calculated from the measured PCBs levels in medium (m_{medium}) and the sorptive capacities of the biological components in medium, i.e.,

$$C_{free} = \frac{m_{medium}}{K_{ap,w}V_{m,ap} + K_{ml,w}V_{m,ml} + K_{sl,w}V_{m,sl} + V_{m,w}} \quad (4)$$

For more details about the model prediction of the PCBs levels in cells and medium, see the Supporting Information.

***In vitro-in vivo* extrapolation of PCB levels.**

The C_{free} values from the cell culture experiments were used to calculate equivalent PCBs levels in the liver and plasma with a composition-based model for describing *in vivo* systems. In this model, the biological components in both plasma and liver are albumin proteins, muscle proteins, storage lipids, membrane lipids, and water.³⁰ The equivalent PCBs levels in liver and plasma can be expressed as (for more details, see the Supporting Information)

$$C_{liver} = C_{free}(K_{ap,w}f_{l,ap} + K_{mp,w}f_{l,mp} + K_{ml,w}f_{l,ml} + K_{sl,w}f_{l,sl} + f_{l,w}) \quad (5)$$

and

$$C_{plasma} = C_{free}(K_{ap,w}f_{p,ap} + K_{mp,w}f_{p,mp} + K_{ml,w}f_{p,ml} + K_{sl,w}f_{p,sl} + f_{p,w}) \quad (6)$$

respectively. $K_{mp,w}$ is the partition coefficient of PCBs between muscle protein and water. $f_{l,ap}$, $f_{l,mp}$, $f_{l,ml}$, $f_{l,sl}$ and $f_{l,w}$ are the volume fractions of albumin proteins, muscle proteins, membrane lipids, storage lipids, and water in the liver, respectively. $f_{p,ap}$, $f_{p,mp}$, $f_{p,ml}$, $f_{p,sl}$ and $f_{p,w}$ are the volume fractions of albumin proteins, muscle proteins, membrane lipids, storage lipids, and water in plasma, respectively. The partitioning coefficients of PCBs used for the calculations were obtained from established PP-LFERs.³⁰ All the volume fractions were obtained from literature data.³⁰

RESULTS AND DISCUSSION

Characterization of the PCBs metabolism in HepG2 cells.

Although HepG2 cells can metabolize environmental pollutants, such as PAHs and others,^{53–55} it is also well documented that, compared to primary human hepatocytes and hepatospheres, HepG2 cells express lower levels of xenobiotic processing enzymes,⁵⁶ including cytochrome P450 isoforms involved in the metabolism of chiral PCBs.³⁷ Cells were exposed for 24 h (1 μ M) or 72 h (10 μ M) to PCBs to assess if HepG2 cells can metabolize PCB 91, PCB 95, PCB 132 and PCB 136. Cell culture media were extracted and analyzed for PCB metabolites using targeted and untargeted approaches (Fig. S2). No hydroxylated, sulfated, glucuronidated, or other metabolites were detected in any sample. This finding is not surprising because PCB metabolism decreases with increasing degree of chlorination and is expected to be relatively low for the penta- and hexa-chlorinated PCB congeners investigated.⁵⁷

Quantitative distribution of PCBs in the HepG2 cell culture system.

We quantified the PCBs levels in cell culture media, cells, and dishes in the absence of detectable PCB metabolism after PCBs incubation with and without HepG2 cells for 72 h (Fig. 1). Large percentages (66.7 to 88.8 %) of PCBs were recovered from the cell culture medium supplemented with 10 % FBS. Only 8.0 to 14.6 % of the PCBs were associated with the cell pellets. Approximately 6 % (2.3 to 7.8 %) of PCBs were absorbed onto the cell culture dishes, both for incubations with and without cells. The percentage of the two hexachlorinated congeners was higher in the medium than the percentage of the pentachlorinated congeners. This finding is consistent with an increased affinity of PCBs for serum proteins, such as albumin, with an increasing degree of chlorination. Similarly, an *in vitro* study showed that the binding affinity of PCBs for binding site II of albumin, the major protein in FBS, increases with an increase in the number of chlorine atoms.⁵⁸ The PCB mass ratios between cell pellets : media : dishes were 1 : 4.9 : 0.54 for pentachlorinated congeners and 1 : 8.6 : 0.37 for hexachlorinated congeners, respectively. These ratios are comparable to earlier studies. For example, the PCB ratios between cell pellets : media : dishes for a lower chlorinated PCB congener, PCB 11, were 1 : 7.8 : 3.3 for 100 nM PCB 11, 1 : 8.5 : 2.3 for 1

μM PCB 11, and 1 : 5.0 : 1.2 for 10 μM PCB 11 in a primary cortical cell culture model.¹⁹ Another study with primary rat hippocampal neurons reported a mass ratio of PCB 136 between cells and media of 1 : 14.¹⁷

Modeling the partitioning of PCBs in the HepG2 cell culture system.

We used two approaches to estimate the C_{free} , a parameter that can be used for IVIVE,^{22, 23} and to assess the partitioning of PCBs in HepG2 cells. We theoretically estimated the C_{free} and the partitioning of the four PCB congeners under investigation using the initial mass of PCBs (m_0) and literature-derived information about the composition of biological components (BC) in cells and medium.^{21, 51, 52, 59, 60} These values were compared to the C_{free} calculated based on the measured PCB mass in medium (m_{medium}) coupled with the composition of the medium, and the experimental PCB levels in medium and cells, determined at the end of a 72 h exposure (Fig. 2).

The estimated C_{free} values for all four PCB congeners showed a good agreement between both methods (Fig. 2a). The model provided a good approximation of the experimental PCB levels in medium (Fig. 2b) but overestimated the PCB levels associated with the cell pellet by a factor of approximately 1.4–2.9 (0.15–0.47 log unit) for the four PCB congeners investigated. For comparisons, a similar model was recently validated and showed 3.0- and 3.4-fold differences between theoretical and experimental values for FBS:water and cell:water partitioning, respectively.²⁷ Another study considered deviations between experimental and calculated partitioning data < 1 log unit (10-fold) acceptable for composition-based mass balance models, both for *in vitro* and *in vivo* studies.³⁰

The differences between the theoretical model and the chemical measurements are likely due to uncertainties associated with literature-derived composition data, the evaporative loss of chemicals during the incubation, or biological effects limiting the cellular uptake of a compound. We used literature values, including data provided by the manufacturer, for relevant lipid and protein levels in HepG2 cells and cell culture media (Table S4) to assess the sensitivity of our model to the differences in the composition of the biological components. We used eight permutations of different biological composition values from various literature sources and observed relative standard deviations of 16.5 % and 4.7 % for predicting PCB levels in cells and media, respectively. Thus, the quantification of the biological components is a source of uncertainty in composition-based model predictions. Despite these uncertainties, our results suggest that at least at concentrations frequently used in toxicity and metabolism studies, the C_{free} of PCBs and the partitioning of PCBs in HepG2 cell culture assays can be calculated based on the nominal PCB concentration and published values for the biological components in the cell culture medium and the cells. Under these experimental conditions, the PCB concentration is typically below the saturation of the biological components.^{27, 61}

In vitro to *in vivo* extrapolation.

We used the free PCB concentrations from our *in vitro* predictions, determined with Eq. 3, to extrapolate from the nominal PCB concentrations to PCB tissue concentrations using a published IVIVE model structure²² with PP-LFERs for obtaining the partitioning

coefficients of PCBs. Based on this model, the PCB tissue levels equivalent to the *in vitro* concentrations (0.25 μM) were 550 ± 70 , 560 ± 70 , 650 ± 90 and 680 ± 100 ng/g plasma and $7,100\pm900$, $6,900\pm900$, $8,400\pm1,100$ and $8,900\pm1,300$ ng/g liver wet weight for PCB 91, PCB 95, PCB 132 and PCB 136 *in vivo*, respectively (Fig. 3a). Furthermore, IVIVE coefficients, defined as the ratio of the PCB level *in vivo* (i.e., plasma or liver levels as ng/g wet weight) over the nominal concentration (in μM) in the cell culture experiments, were calculated. The IVIVE coefficients were $2,300\pm300$, $2,200\pm300$, $2,600\pm400$ and $2,700\pm400$ for plasma and $28,000\pm3,700$, $28,000\pm3,600$, $33,000\pm4,600$ and $36,000\pm5,100$ for liver for PCB 91, PCB 95, PCB 132 and PCB 136, respectively. These plasma IVIVE coefficients demonstrate that blood PCB levels are a poor rationale for the selection of nominal concentration for cell culture-based toxicity studies.

The IVIVE model also allowed us to estimate nominal concentrations for cell culture experiments that are equivalent to the PCB tissue levels observed *in vivo*. For example, liver tissues levels of 1,800 ng/g for PCB 91, 1,200 ng/g for PCB 95 and 410 ng/g for PCB 136 observed in mice exposed orally to racemic PCBs^{62–64} correspond to nominal concentrations of 64 nM PCB 91, 43 nM PCB 95 and 11 nM PCB 136 in studies in HepG2 cells. Based on these estimations, the micromolar PCB concentrations employed in our study (0.25 μM) are 4 to 22-times higher than the equivalent levels of the *in vivo* observations, even though micromolar concentrations are commonly used in *in vitro* studies.^{53, 54} The model also enabled us to predict IVIVE extrapolation coefficients for all 209 PCBs for the experimental conditions employed in the present study (Fig. 3b). As expected, the extrapolation coefficients increased with an increasing degree of chlorination for plasma and liver. Relevant experimental parameters (e.g., cell number, medium volume, medium composition, etc.) are readily available from the published literature or can be determined experimentally. Thus, it is relatively easy to calculate similar extrapolation coefficients for other experimental systems using the spreadsheet provided in the Supporting Information.

Atropselective partitioning of PCBs in the HepG2 cell culture system.

The PCB congeners investigated in this project bind, for example, atropselectively to the active site of cytochrome P450 enzymes³⁷ and undergo atropselective metabolism in the liver.^{44, 63, 64} Although this has not been studied previously, it is likely that PCBs interact atropselectively with serum proteins, such as albumin. Based on our earlier binding studies with human serum albumin,⁵⁸ it is expected that PCB atropisomers differentially bind to major drug-binding sites of albumin. Therefore, we also assessed the atropselective partitioning of PCBs in our cell culture model.

Significant atropisomeric enrichment was detected in media, cells, and dishes for the four PCB congeners (Figs. 4&5). The atropisomer eluting first on a CD column (E_1) was significantly enriched in both cells and dishes for PCB 91 (i.e., (–)-PCB 91,⁶⁵; EF values of 0.77 for cells and 0.68 for dishes), PCB 132 (i.e., (–)-PCB 132⁶⁶; EF values of 0.69 for cells and 0.63 for dishes), and PCB 136 (i.e., (–)-PCB 136⁶⁶; EF values of 0.55 for cells and 0.68 for dishes). A slight enrichment of E_1 -PCB 95 (i.e., aR- or (–)-PCB 95⁶⁷) was observed, with EF values of 0.55 for cells and 0.53 for dishes. It is noteworthy that the direction of the atropisomeric enrichment in cells and dishes was identical for all four PCB congeners

investigated. The E₂-atropisomers of PCB 91, PCB 132, and PCB 136 were enriched in the cell culture medium, with EF values of 0.41, 0.44, and 0.47, respectively (Figs. 4&5). The PCB 95 residue was near racemic in the cell culture media samples, possibly because its high concentrations masked the atropisomeric enrichment. In parallel experiments, chiral analysis of samples from cell-free incubations revealed near-racemic chiral signatures in both dish and media samples (Fig. S5). These findings suggest that chiral PCBs atropselectively partition between medium and cells in the HepG2 cell culture system in the absence of detectable PCB metabolism. Besides, cells likely play a dominant role in driving the atropselective partitioning of PCBs.

Metabolism studies with human liver microsome showed a depletion of (+)-PCB 91,³⁸ (+)-PCB 95⁴¹ and (–)-PCB 132,⁶⁸ whereas (–)-PCB 91, (–)-PCB 95 and (–)-PCB 132 were enriched in HepG2 cells. These observations suggest that the atropselective partitioning of PCBs in biological systems is determined by the complex biological components and interactions of cells and tissues. In contrast, only a small portion of enzymes (i.e., the active site of cytochrome P450 enzymes) is responsible for the subsequent atropselective metabolism of PCBs observed in *in vitro* metabolism studies.^{37, 38, 41, 68} Consistent with this interpretation, we have demonstrated that the binding of PCB 136 atropisomers to rat cytochrome P450 enzymes¹⁵ does not predict the atropselective metabolism of racemic PCB 136 by rat liver microsomes.⁴⁰

Considerations for the modeling of the atropselective partitioning of PCBs *in vitro*.

The enantioselective interactions of chiral compounds with macromolecules are well studied, especially in the pharmaceutical sciences.^{69, 70} Such enantioselective interactions include the binding of a chiral molecule to cellular proteins, such as serum albumin and cytochrome P450 enzymes,^{37, 71} and, possibly, their interaction with the chiral glycerol backbone of membrane lipids. In contrast, the atropselective interactions of PCB atropisomers with protein, such as albumin, have received little attention to-date. Our binding studies reveal that the binding of PCB atropisomer to hepatic cytochrome P450 enzymes is complex.¹⁵ While PCBs are located near the glycerol backbone of model membranes,⁷² it remains unknown if the partitioning of chiral PCBs into lipid bilayers is atropselective. The complexity of the chemical and biological mechanism of atropselective interactions makes it challenging to model the partitioning of atropisomers with current composition-based models that use the same chemical descriptors for both atropisomers. For example, the Abraham solvation parameters (ASPs) of PCBs used in our model were derived with four chemical properties, including subcooled liquid vapor pressure, aqueous solubility, octanol/water partitioning coefficient, and gas to hexadecane partition coefficient at 298 K.³¹, and do not account for atropselective interactions.

To overcome these limitations, we explored if composition-based models can be modified by incorporating atropselective interactions into the determination of ASPs. Briefly, we established the PP-LFERs for a new chemical property (i.e., the relative retention times on three different chiral columns, Fig. S6) and determined the ASPs of the individual PCB atropisomers (for more details, see Supporting Information). The ASPs of the PCB atropisomers (Table S5) were subsequently used to estimate the EFs of the chiral PCBs in

the media and cells of our HepG2 cell culture system. For most relative retention time datasets, the respective model did not predict the enrichment of the PCB atropisomers in the cells (Table S6). Thus, composition-based models based on polarity-like chemical descriptors and insufficient information about system descriptors (e.g., unknown levels of biological components that are responsible for atropselective interactions) are unlikely to account for atropselective partitioning. Instead, other chemical descriptors, preferentially stereochemistry-derived parameters, and system descriptors reflecting the quantities of the lipids and proteins responsible for the atropselective interactions are needed to develop models that can predict the partitioning of PCB atropisomers in *in vitro* and *in vivo* models. However, to set up these new chemical and system descriptors for modeling atropselective partitioning, extensive experimental data are needed to determine $K_{i/water}$ (in Eq. (1)) values of a set of training compounds, including pure PCB atropisomers, and to establish correlations between chemical descriptors and $K_{i/water}$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

PCB 91, PCB 95, PCB 132, and PCB 136 were primarily present in the cell culture medium from experiments (a) with or (b) without HepG2 cells. Only a small percentage of the PCBs were recovered from the cell pellet (8.0 to 14.6 %) or the cell culture dish (2.3 to 7.8% for cell culture group and 3.7 to 7.2% for cell-free group). HepG2 cells (6×10^6 /well) were seeded into 6-well plates with complete MEM medium (3 mL) per well and allowed to attach for 48 h. Cells were exposed for 72 h to the individual PCB congeners ($0.25 \mu\text{M}$; 0.1 % DMSO) in exposure medium (3 mL) with 10 % FBS. Controls were exposed to 0.1% DMSO in the exposure medium. Parallel incubations were performed for cell-free groups. After the incubation, the media, cell pellets, and cell culture dishes were collected, and PCB levels were determined as described in the Experimental Section.

Fig. 2.

The four-phase composition-based model provided reasonable approximations of (a) C_{free} and (b) predicted PCBs levels in medium and cells showed a good agreement with measured PCB levels. The estimated C_{free} values in panel (a) were calculated either using the initial mass of PCBs (m_0) coupled with the biological components (BC) present in cells and medium or with the measured PCB mass in medium (m_{medium}) coupled with the BC in the medium. Eqs. (3) and (4) were used for the predictions of C_{free} , respectively. Experimental PCB levels in medium and cells were determined as described in the manuscript and are shown in Fig. 1. The predicted C_{free} values are presented as the average $\pm$ standard deviations of the results obtained with different values for the quantities of biological components in the cell culture system. The predicted PCB levels in medium and cells are

presented as the average of the results obtained with different data sources (for additional information, see Table S4).

Fig. 3.

The *in vitro-in vivo* extrapolation predicted (a) the equivalent *in vivo* plasma and liver levels of the four PCB congeners studied (PCB 91, PCB 95, PCB 132, and PCB 136) corresponding to 0.25 μ M dose level in cell culture and (b) the trend of plasma and liver extrapolation coefficients for 209 PCB congeners under the cell culture conditions employed in this study. Eqs. (5) and (6) were used for the predictions. The equivalent tissue levels of PCB 91, PCB 95, PCB 132, and PCB 136 are presented as the average $\pm$ standard deviations of the results obtained with different values for the quantities of biological components in the cell culture system (for additional information, see Table S4). The extrapolation coefficients of the 209 PCB congeners were given as the average value obtained from various permutations using the data sources listed in Table S4. The solvation parameters of PCBs and the quantities of the biological components in *in vivo* plasma and liver used for the predictions were obtained from the literature.^{30, 31}

Fig. 4.

Representative chromatograms show an atropisomeric enrichment in extracts from cell culture media, cell pellet, and cell culture dishes after incubation with racemic (a) PCB 91, (b) PCB 95, (c) PCB 132, and (d) PCB 136. The atropisomeric enrichments of these parent PCBs occur in the absence of any detectable metabolism. The racemic PCB standards are shown for comparison. HepG2 cells (6×10^6 /well) were exposed for 72 h to the individual, racemic PCB congeners ($0.25 \mu\text{M}$; 0.1% DMSO) in exposure medium (3 mL) with 10% FBS. After the incubation, the media, cells, and cell culture dishes were collected, and atropselective analyses were performed by GC- μ ECD using a CD capillary column as

described in the Experimental Section. E₁ and E₂ are the atropisomers eluting first and second on the CD capillary column.

Fig. 5.

The directions of the atropisomeric enrichment of (a) PCB 91, (b) PCB 95, (c) PCB 132, and (d) PCB 136 in HepG2 cells and cell culture dishes are opposite to the atropisomeric enrichment observed in the cell culture media. HepG2 cells (6×10^6 /well) were exposed for 72 h to the individual, racemic PCB congeners ($0.25 \mu\text{M}$; 0.1 % DMSO) in exposure medium (3 mL) with 10 % FBS. After the incubation, the media, cells, and cell culture dishes were collected, and atropselective analyses were performed by GC- μECD using a CD capillary column as described in the Experimental Section. The dotted line indicates the EF value of the respective racemic PCB congener.