Published in final edited form as:

Environ Sci Technol. 2020 October 06; 54(19): 12345–12357. doi:10.1021/acs.est.0c03476.

3,3'-Dichlorobiphenyl is Metabolized to a Complex Mixture of Oxidative Metabolites, Including Novel Methoxylated Metabolites, by HepG2 Cells

Chun-Yun Zhang¹, Susanne Flor¹, Patricia Ruiz³, Ram Dhakal¹, Xin Hu⁴, Lynn M. Teesch², Gabriele Ludewig¹, Hans-Joachim Lehmler¹

¹Department of Occupational and Environmental Health, The University of Iowa, Iowa City, Iowa 52242, United States

²High Resolution Mass Spectrometry Facility, The University of Iowa, Iowa City, Iowa 52242, United States

³Division of Toxicology and Human Health Sciences, Computational Toxicology and Methods Development Lab, Agency for Toxic Substances and Disease Registry, Atlanta, Georgia 30333, United States

⁴Department of Medicine, School of Medicine, Emory University, Atlanta, GA 30303, United States

Abstract

3,3'-Dichlorobiphenyl (PCB 11) is a byproduct of industrial processes and detected in environmental samples. PCB 11 and its metabolites are present in human serum, and emerging evidence demonstrates that PCB 11 is a developmental neurotoxicant. However, little is known about the metabolism of PCB 11 in humans. Here we investigated the metabolism of PCB 11 and the associated metabolomics changes in HepG2 cells using untargeted high-resolution mass spectrometry. HepG2 cells were exposed for 24 h to PCB 11 in DMSO or DMSO alone. Cell culture media were analyzed with ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry. Thirty different metabolites were formed by HepG2 cells exposed to 10 μ M PCB 11, including monohydroxylated, dihydroxylated, hydroxylated-methoxylated, and methoxylated-di-hydroxylated metabolites, and the corresponding sulfo and glucuronide conjugates. The methoxylated PCB metabolites were observed for the first time in a human-relevant model. 4-OH-PCB 11 (3,3'-dichlorobiphenyl-4-ol) and the corresponding catechol metabolite, 4,5-di-OH-PCB 11 (3',5-dichloro-3,4-dihydroxybiphenyl), were unambiguously

Corresponding Author: Dr. Hans-Joachim Lehmler, The University of Iowa, Department of Occupational and Environmental Health, University of Iowa Research Park, #164 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 synthesis of analytical standards; LC-QToF MS operation conditions and data; GC-ECD and GC-MS operation conditions and data; XCMS online processing parameters and results; estimated octanol-water partition coefficients of PCB 11 metabolites; cytotoxicity of PCB 11 toward HepG2 cells; the extracted ion chromatograms, MS and MS/MS data of PCB 11 metabolites; and structures of PCB 11 metabolites predicted by MetaDrug. This material is available free of charge via the Internet at <http://pubs.acs.org>.

identified based on liquid and gas chromatographic analyses. PCB 11 also altered several metabolic pathways, in particular vitamin B₆ metabolism. These results demonstrate that complex PCB 11 metabolite profiles are formed in HepG2 cells that warrant further toxicological investigation, particularly since catechol metabolites are likely reactive and toxic.

Graphical Abstract

INTRODUCTION

Polychlorinated biphenyls (PCBs) are persistent organic pollutants. They are banned under the Stockholm Convention, a treaty that protects the environment and humans from the adverse effects of PCBs and other harmful chemicals.¹ The use of PCBs is scheduled to cease worldwide by 2025, and all PCB wastes are expected to be disposed of in an environmentally sound manner by 2028.¹ Despite these global efforts, environmental and human biomonitoring studies demonstrate that industrial and consumer products, such as paints and epoxy resins,^{2–4} can be contaminated with unintentionally produced PCBs. 3,3'-Dichlorobiphenyl (PCB 11) is an unintentionally produced PCB congener that is not present in legacy PCB mixtures but has been detected in many parts of the world.^{5–14} This PCB congener is, for example, formed from dichlorinated benzidines during the production of yellow paint pigments.⁴ Because of its semi-volatile nature, humans may be exposed to PCB 11 by inhalation and via the diet. Because of its ubiquitous presence in the environment, PCB 11 and its metabolites, including 4-OH-PCB 11 and the corresponding sulfate, have been detected in human serum.^{15–19}

The metabolism of PCB 11 has received little attention to date, in part because PCB 11 is not present in technical PCB mixtures. PCB 11 has a short half-life in male rats following inhalation exposure and does not bioaccumulate, with 4-OH-PCB 11 being the major metabolite.^{20, 21} 4-OH-PCB 11 and a hydroxylated PCB 11 sulfate are also present in tissues and excreta from rats exposed intravenously to PCB 11 sulfate.²² Spectral binding studies suggest that PCB 11 binds to and is metabolized by cytochrome P450 enzymes to monohydroxylated and dihydrodiol metabolites.^{23, 24} 4-OH-PCB 11 is a substrate of human SULT2A1 and inhibits the sulfation of steroids, such as dehydroepiandrosterone (DHEA), by sulfotransferases (SULTs).^{25–27} Moreover, 4-OH-PCB 11 is likely a substrate for UDP-glucuronosyltransferases (UGTs) and, as with other hydroxylated PCBs,^{28, 29} likely metabolized to a glucuronide *in vivo*. As suggested from observations with polar bears,³⁰

hydroxylated, sulfated and glucuronidated PCB 11 metabolites may be further oxidized to diverse di- and tri-hydroxylated metabolites.

A growing number of studies indicate that PCB 11 and its metabolites are potentially toxic. An *in vitro* toxicity study demonstrate that PCB 11 is a weak sensitizer of ryanodine receptors (RyRs).³¹ PCB 11 and two of its metabolites, 4-OH-PCB 11 and the corresponding sulfate metabolite, increase axonal and dendritic growth in cultures of cortical and hippocampal pyramidal neurons.¹⁷ Reporter gene assays and other functional cellular bioassays reveal that PCB 11, 4-OH-PCB 11, and the corresponding sulfate metabolites are endocrine-disrupting chemicals.^{32, 33} Because PCB sulfates can competitively bind to serum proteins, these metabolites may interfere with the transport of vitamins, and hormones.³⁴ Analogous to other, lower chlorinated PCBs, hydroquinone, catechol or quinone metabolites of PCB 11 are expected to cause oxidative stress and to directly or indirectly damage DNA.
35–43

Although humans are exposed to PCB 11, its metabolism in humans has not been characterized to date. The present study used a combination of *in silico* predictions and liquid chromatography/mass spectrometry (LC-MS)-based non-target analyses to gain insights into PCB 11 metabolites potentially formed in humans. Metabolism studies were performed with HepG2 cells, a hepatocellular carcinoma cell line that retains the expression of key metabolic enzymes and has been used to characterize the metabolism of environmental contaminants in humans.⁴⁴ Our results demonstrate the PCB 11 is metabolized in HepG2 cells to methoxy, sulfo- and glucuronide conjugates of mono-, di- and trihydroxy PCB 11 metabolites, including potentially toxic PCB 11 catechol metabolites. These initial findings will guide more in-depth studies on the metabolism and toxicity of PCB 11 and its metabolites in humans.

EXPERIMENTAL SECTION

In silico metabolite predictions with ADMET Predictor and MetaDrug.

A structure of PCB 11 was drawn in Mol file format using ChemBioDraw Ultra and uploaded to ADMET Predictor software (Simulations Plus, Lancaster, CA, USA) and MetaDrug (Thompson Reuters, New York, NY, USA), as we reported previously.⁴⁵ The structures of possible PCB 11 metabolites were predicted with the default settings of the Metabolism Module of ADMET Predictor or MetaDrug to aid in the interpretation of the metabolism studies in HepG2 cells. In addition, PCB 11 was classified as a substrate of the cytochrome P450 isoforms included in ADMET Predictor (i.e., CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C19, CYP2C9, CYP2D6, CYP2E1, and CYP3A4) and MetaDrug (i.e., CYP1A2, CYP2B6, CYP2D6, and CYP3A4).

Chemicals.

The highly pure 3,3'-dichloro-biphenyl (PCB 11) (99.97%) used for the metabolism studies was synthesized from the corresponding benzidine.^{46, 47} For a description of other chemicals and materials, see the Supporting Information.

Exposure of HepG2 cells to PCB 11 for metabolite analysis.

HepG2 cells (6×10^6 /well) in 3 mL complete minimum essential medium (MEM) were seeded into 6-well plates (for additional information regarding the HepG2 cells and their maintenance, see the Supporting Information). After 48 h of attachment, cells were exposed for 24 h in triplicates to 10 μ M PCB 11 (0.1 % DMSO) in exposure medium (no FBS, 3 mL per well). This concentration was selected because, compared to lower PCB 11 concentrations, this concentration resulted in higher metabolite levels, thus facilitating the detection of PCB 11 metabolites. The control group was treated for 24 h with the exposure medium with 0.1 % DMSO. After 24 h incubation, the media were transferred into glass vials, and the cells were washed once with PBS (1 mL). The cells were harvested into PBS (1 mL) with a rubber policeman and added into a separate glass vial, and the wells were washed once with PBS (1 mL). The vials were stored at -20°C until analysis. The plates were wrapped in aluminum foil and stored at 4°C until analysis. Plates containing exposure medium and 10 μ M PCB 11, but no cells, were incubated for 24 h, and media were collected to assess if PCB 11 undergoes abiotic transformation under the experimental conditions. No PCB 11 transformation products were observed in these control experiments. For a summary of parallel cytotoxicity studies, see the Supporting Information.

Extraction of PCB 11 metabolites from the medium.

The exposure medium (roughly 4 mL) was spiked with 4'-chloro-3'-fluoro-4-sulfoxy-biphenyl (3-F-4'-PCB 3 sulfate, 200 ng) and 4'-chloro-3'-fluoro-4-hydroxy-biphenyl (3-F-4'-OH-PCB 3, 200 ng) as surrogate recovery standards, and acidified with 10 % formic acid (v/v, 400 μ L). Acetonitrile (4 mL) was added to each sample, and phase separation was assisted by adding magnesium sulfate (1.2 g) and sodium chloride (0.3 g). The vials were inverted for 5 min and centrifuged at 1,811 g for 5 min. The organic layer was passed through an anhydrous magnesium sulfate (2 g) column to remove dissolved water and salts. The aqueous phase was re-extracted using hexane (3 mL). The combined extracts were evaporated to dryness under a gentle stream of nitrogen and reconstituted in acetonitrile/water (200 μ L; 15:85, vol/vol). Deconjugation experiments were performed using a modified approach that is described in the Supporting Information.

Consistent with other studies,⁴⁸ cell pellets, extracted analogously, contained lower levels of PCB 11 metabolites than medium, with several metabolite classes being below the limit of detection of our analytical method. Therefore, cell pellets were not analyzed further in this study.

Liquid Chromatography-High Resolution Mass Spectrometry.

Initial screening analyses were carried out with an Ultra-Performance Liquid Chromatography (UPLC) (Waters Acquity UPLC, Milford, MS, USA) coupled with Quadrupole Time-of-flight Mass Spectrometer (QToF-MS) (Waters Q-ToF Premier, Milford, MA, USA) at the High-Resolution Mass Spectrometry Facility of the University of Iowa (Iowa City, IA, USA). Data and operating conditions from the LC-QTOF-MS analyses are summarized in the Supporting Information (Table S1). Subsequently, untargeted MS and targeted MS/MS experiments were performed with an ultra-high performance liquid chromatograph (Ultimate 3000 UHPLC+ Focused, Thermo Fisher, Waltham, MA, USA)

coupled with a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (LC-Orbitrap MS) (Thermo Fisher) at the Center of Mass Spectrometry and Proteomics at the University of Minnesota (Minneapolis, MN, USA) (Tables 1 and S2, respectively).

Two different LC columns (Waters Acquity UPLC BEH C18 (2.1 × 100 mm, 1.7 μM) for Table 1 and Waters XBridge BEH C18 (2.1 × 100 mm, 3.5 μM) for Table S2) were used for the chromatographic separation of the PCB 11 metabolites on the LC-Orbitrap MS, with a flow rate of 0.3 mL/min. The following gradient (% (B) acetonitrile) was used: 0-1 min, 15 %; 1-3 min, 15-60 %, 3-11.5 min, 60-90 %; 11.5-12 min, 90-98 %, 12-12.5 min, 98%; 12.5-13 min 98-15 %, 13-14 min, 15 %. Water with 10 mM ammonium acetate (pH = 7.0) was used as solvent (A). The Orbitrap MS system was equipped with heated-electrospray ionization (HESI-II) probe source and operated in negative mode at the following conditions: Spray voltage, 3.0 kV; sheath gas flow rate, 50 arb; capillary temperature, 320 °C; auxiliary gas heater, 400 °C; S-lens, 55 V. Full-scan accurate mass spectra were obtained in the scan range from m/z 70 to m/z 1050 with a resolution of 70000 FWHM. Targeted MS/MS spectra were obtained in the data-dependent mode in which only precursor ions with intensities larger than 8×10^4 were fragmented with a ramped normalized collision energy of 30, 45, and 75 eV. Because epoxide and quinone metabolites of aromatic compounds can be detected by positive mode LC-MS analysis,⁴⁹ selected extracts were analyzed in the positive mode by both LC-QTOF MS (Waters Acquity UPLC BEH C18) and LC-Orbitrap MS (Waters XBridge BEH C18 column); however, no PCB 11 metabolites were detected in these screening experiments.

Full scan raw data for three biological replicates for both exposure and control samples were converted to mzXML format using Proteowizard software and uploaded to XCMS Online for an untargeted metabolomics analysis to identify PCB 11 metabolites (for a summary of these data, see Table S2). The parameters for XCMS Online processing are detailed in the Supporting Information (Table S3). PCB 11 metabolites identified by XCMS Online were confirmed based on their accurate mass and isotopic mass patterns with Thermo Xcalibur software and further identified based on their MS/MS data.

Metabolomics analysis.

Metabolomics data was acquired together with PCB 11 metabolites on three biological replicates of control and treated HepG2. The raw data files acquired in both negative and positive modes were extracted using *apLCMS*⁵⁰ and *xMSanalyzer*⁵¹ to obtain a peak table with mass to charge ratio (m/z), retention times, and intensities for each mass spectral feature. A feature was included when it was detected in at least two out of the three replicates in the same group. The missing values were imputed with one-half of the lowest signal detected for that feature across all samples. The data were $\log_2$ transformed and normalized by the sum of the peak areas of all the ions. Metabolic features differed between exposed and control groups were selected using the *limma* test⁵² for generating raw p-values and adjusted p-values for false discovery rate (FDR) with the Benjamini and Hochberg method.⁵³ Volcano plots, representing the $-\log_{10}$ p-values versus $\log_2$ fold change, were used to visualize the feature distribution (raw p-values 0.05; q-values 0.05 for FDR correction). Pathway enrichment analysis using *Mummichog* (version 2.0.6)⁵⁴ was

performed on the raw p-values. *Mummichog* provides a biological interpretation of the data without a priori identification of the metabolites in the untargeted feature table. We used less stringent criteria of *raw P* < 0.05 to obtain optimal coverage of metabolites (100-500 metabolites) for permutation testing. This approach protects against type 1 statistical error by permutation testing in pathway enrichment analysis that is additional to *limma* test.⁵⁵ Student's t-test (two-tailed) was used to test the statistical significance of the normalized raw intensities of selected metabolites between groups. All the metabolites were putatively annotated using *xMSannotator*⁵⁶ with confidence scores based upon the Human Metabolome DataBase (HMDB)⁵⁷ and Kyoto Encyclopedia of Genes and Genomes (KEGG).⁵⁸ A high confidence score of 3 was assigned for those metabolites with the detection of both primary molecular ions (e.g., [M+H]⁺ for positive mode and [M-H]⁻ for negative mode) and clustered adducts. A medium confidence score of 2 was assigned for those metabolites with the detection of only the primary molecular ions. Conservatively, lower confidence annotations with scores of 1 for low confidence and 0 for no confidence were not included for further data interpretation.

Quality assurance/quality control.

Extracted ion chromatograms are based on calculated extract masses with a mass window of 10 ppm. PCB 11 metabolites are only reported if they were detected in all triplicate samples with an abundance > 10-times the background levels to ensure the reproducibility of the untargeted analysis (Table S3). The following criteria were used to identify PCB 11 metabolites: All differences between measured and calculated accurate mass were < 5 ppm. The isotopic mass patterns of the molecular ions were consistent with the pattern predicted for dichlorinated PCB 11 metabolites within a 20 % error (i.e., A : A+2 : A+4 = 9 : 6 : 1, where A represents the isotopic ion with two ³⁵Cl, A+2 represents the isotopic ion with one ³⁵Cl and one ³⁷Cl, and A+4 represents isotopic ion with two ³⁷Cl). Whenever possible, MS/MS spectra were acquired for at least one isomer of each PCB 11 metabolite class to confirm key structural elements. 3-F-4'-OH-PCB 3 and 3-F-4'-PCB 3 sulfate were used to semi-quantitatively monitor the performance of the extraction and instrumental analysis. 3-F-4'-PCB 3 sulfate was also spiked into the blank solvent (buffer) as surrogate standards to assure the efficiency of the deconjugation and derivatization. Control samples analyzed in parallel included blank incubations exposed to 0.1 % DMSO only and cell-free incubations (only MEM medium, 10 μ M PCB 11, and 0.1 % DMSO (v/v)). No metabolites were detected in any control sample.

RESULTS AND DISCUSSION

Prediction of PCB 11 metabolites using ADMET Predictor and MetaDrug.

Two commercial software packages, ADMET Predictor and MetaDrug, were used to predict the PCB 11 metabolites formed in humans. Both software packages provide complimentary estimates that can be used to guide PCB metabolism studies and identify possible P450 enzymes involved in the biotransformation of a specific PCB congener; however, both packages have limitations in predicting the metabolism of PCBs in humans, as we reported previously for the metabolism of higher chlorinated PCBs.⁴⁵

ADMET Predictor estimated the formation of one hydroxylated PCB 11 metabolite, 4-OH-PCB 11, by CYP1A2 and CYP2C9 and, possibly, CYP2C19 and CYP3A4. Consistent with this prediction, studies with highly purified rat P450 enzymes demonstrated that PCB 11 is primarily metabolized by CYP1A2 in the 4-position.²³ Studies of a pentachlorobiphenyl (PCB 101) with human recombinant P450 enzymes did not suggest a major role of CYP2C and CYP2D enzymes in the metabolism of PCBs; however, these earlier studies investigated a PCB congener with two *ortho* chlorine substituents.⁵⁹ Because rat cytochrome P450 enzymes have drastically different specificities for non-*ortho* vs. di-*ortho* chlorinated PCB congeners,²³ in-depth metabolism studies are needed to confirm the role of these isoforms in the metabolism of PCB congeners, such as PCB 11, without *ortho* chlorine substituents in humans.

MetaDrug predicted the formation of three of the four possible OH-PCB 11 metabolites, including 4-OH-PCB 11, and several dihydroxylated and sulfated PCB 11 metabolites, including 5,6-di-OH-PCB 11 (3',5-dichloro-2,3-dihydroxybiphenyl) and 4,5-di-OH-PCB 11. MetaDrug also suggested the formation of 2,3-, 3,4-, 4,5- and 5,6-arene oxides of PCB 11 and metabolites derived from these arene oxides, including dihydrodiols and glutathione adducts, and two mono-methoxylated PCB 11 derivatives. Our studies with human liver microsomes and recombinant P450 enzymes using PCBs 91, 95, 132, and 136 indicate the congener-specific formation of PCB arene oxides that rearrange to the corresponding OH-PCBs, typically by mechanisms involving a 1,2 chlorine shift.^{45, 60} Rat P450 enzymes also metabolize PCB 11 via an arene oxide to a dihydrodiol.²⁴ It is therefore not entirely unexpected that MetaDrug predicts the formation of arene oxide intermediates in the metabolism of PCB 11. ADMET Predictor does not consider arene oxide intermediates in its predictions, highlighting a limitation of this software package.

Formation of the PCB 11 metabolites in the medium of PCB 11 exposed HepG2 cells.

We used an untargeted approach to characterize the PCB 11 metabolites formed in HepG2 cells as a model system. This human hepatoma cell line is readily available, easy and inexpensive to maintain,⁶¹ and widely used as a model for drug metabolism and toxicity studies.^{62, 63} The expression of cytochrome P450 enzymes in HepG2 cells is lower compared to primary hepatocytes,^{44, 64–66} which represents a limitation of this cell line for metabolism studies. We employed a PCB 11 concentration that was used in an earlier toxicity study.³² Based on earlier metabolism studies, this concentration was expected to yield metabolite levels that could be detected with our analytical methods. No appreciable effect of PCB 11 on cell viability was observed under the experimental conditions employed in this study, as determined using the resazurin and cell count methods (Fig. S1). Analyses of the cell culture medium collected after 24 h exposure to PCB 11 revealed the formation of complex mixtures of oxygenated PCB 11 metabolites, including mono- and di-hydroxylated PCB 11 metabolites, and the corresponding sulfates and glucuronides (Classes 1 and 2). We also observed several novel methoxylated metabolites (Classes 3 and 4).

Class 1 – mono-hydroxylated PCB 11 metabolites and their conjugates.

We identified three different types of mono-hydroxylated PCB 11 derivatives in the medium from PCB 11-exposed HepG2 cell. Only one mono-hydroxylated PCB 11 isomer (OH-PCB

11, Class 1.1, Fig. S2) was identified after 24 h incubations. This metabolite corresponds to 4-OH-PCB 11 based on a comparison of its retention time and MS data with an authentic standard (Fig. S2). It is possible that other OH-PCBs were present but could not be detected due to their low concentration, their transient formation that progressed to downstream metabolites, or because of co-elution issues. Also, two sulfate (PCB 11 sulfate, Class 1.2, Fig. S3) and two glucuronide conjugates of monohydroxylated PCB 11 metabolites (PCB 11 glucuronide, Class 1.3, Fig. S4) were detected. The major sulfate metabolite corresponded to 4-PCB 11 sulfate, as confirmed with an authentic standard (Fig. S3). Aliquots of a representative extract were deconjugated with sulfatase/glucuronidase and analyzed as the methylated derivatives by both GC-ECD (Fig. S5) and GC-MS (Figs. S6–S7). We observed a peak corresponding to 4-OH-PCB (analyzed as methylated derivatives) in the extract without sulfatase/glucuronidase treatment. The 4-OH-PCB 11 peak increased after sulfatase/glucuronidase treatment (Fig. S6), which is consistent with the detection of sulfate and glucuronide metabolites in the LC-Orbitrap MS analysis.

Two isomers of PCB 11 glucuronides were detected with an accurate mass of m/z 413.01948 ($[M-H]^-$) (Fig. S4). Based on a loss of 176 Da ($C_6H_8O_6$) in the MS/MS analysis, these metabolites contain a cyclic form of glucuronic acid. In addition, two isomers with shorter retention times (i.e., higher hydrophilicity) were detected with an accurate mass of m/z 431.03005 ($[M-H]^-$). Based on the accurate mass and the loss of H_2O from the molecular ion in the MS spectrum, we propose that these two glucuronide isomers correspond to a ring-opened (linear) form of glucuronic acid. Protein-glucose adducts with a similar structure have been reported previously.^{67, 68}

The Class 1 metabolites of PCB 11 formed by HepG2 cells are consistent with the predictions using ADMET Predictor and MetaDrug. Moreover, several of the metabolites detected in this study, in particular, 4-OH-PCB 11 and the corresponding PCB 11 sulfate, have been detected in animal tissues^{20, 21, 69} and human serum.¹⁸ These earlier studies only reported the presence of *para*-substituted metabolites, whereas this study detected the formation of several isomers of the Class 1 metabolites of PCB 11 in a human model. The preferential formation of *para* hydroxylated PCB metabolites by human cytochrome P450 enzymes, in particular CYP2A6, has been reported for some PCB congeners,^{45, 70} whereas other PCB congeners are hydroxylated in the *meta* position with or without a 1,2-chlorine shift.^{45, 60, 71} It is also noteworthy that we did not detect PCB 11 dihydrodiols, metabolites that are formed by rat cytochrome P450 enzymes,^{23, 24} or PCB 11 glutathione adducts.

Class 2 – dihydroxylated PCB 11 metabolites and their conjugates.

One dihydroxylated PCB 11 (di-OH-PCB 11; Class 2.1) and four sulfate (OH-PCB 11 sulfate, Class 2.2) and two glucuronide conjugates (OH-PCB 11 glucuronide, Class 2.3) of di-OH-PCB 11 were formed by HepG2 cells exposed to PCB 11 (Figs. S8–S9). The di-OH-PCB 11 co-eluted with an OH-PCB 11 sulfate isomer (Fig. S8–A). Because the molecular ion of the di-OH-PCB 11 metabolite and a major fragment ion of the OH-PCB 11 sulfate have the same m/z of 252.98231, we could not unambiguously identify this metabolite as a di-OH-PCB 11 based on our initial LC-Orbitrap MS analysis. We subsequently confirmed the presence of the di-OH-PCB 11 on a different Acquity BEH C-18 column, which could

separate the two co-eluting metabolites (Fig. S8–A). It is noteworthy that we only detected one di-OH-PCB 11 isomer. Experiments with rat microsomes discovered three di-OH-congeners with PCB 3 (4-chlorobiphenyl), most likely because of the lack of phase 2 metabolism.⁷² In the present study with living cells other di-OH-PCB 11 metabolites were below the detection limit because they are either minor metabolites or are rapidly converted to sulfated (Class 2.2), glucuronidated (Class 2.3) and methoxylated (Classes 3 and 4) metabolites. Also, it can be expected that similar to other lower chlorinated PCB congeners, PCB 11 catechols can be further oxidized to reactive and potential highly toxic PCB 11 quinones.^{35, 40, 72–76}

The presence of a di-OH-PCB 11 and the corresponding conjugated (Class 2.2 and 2.3) metabolites was confirmed after deconjugation, followed by derivatization and gas chromatographic analysis (Figs. S5–S7). A comparison of the retention time of the di-OH-PCB 11 metabolite to the retention time of authentic standards allowed us to identify this metabolite as 4,5-di-OH-PCB 11. This observation is consistent with previous studies demonstrating that *para* hydroxylated PCBs are further oxidized to PCB catechols by human liver microsomes.^{60, 70, 71, 77} The formation of 4,5-di-OH-PCB 11 in HepG2 cells is noteworthy, because this metabolite, like structurally related PCB catechols, most likely can redox cycle and form reactive quinone metabolites.²² The presence of four Class 2.2 metabolites suggests that di-OH-PCBs other than 4,5-di-OH-PCB 11 were formed by HepG2 cells. As predicted by MetaDrug, these metabolites may also be catechols or contain a hydroxy group in both phenyl rings. Limited evidence suggests the formation of similar PCB metabolites in certain animal species.⁷⁸ Likely, the levels of these metabolites were not detected in the GC analyses due to fast phase 2 biotransformation.

Class 3 – methoxylated-hydroxylated PCB 11 metabolites and their conjugates.

HepG2 cell culture medium contained three types of methoxylated PCB 11 metabolites derived from di-OH-PCB 11 metabolites, including methoxylated-hydroxylated PCB 11 metabolites (MeO-OH-PCB 11, Class 3.1) and the corresponding sulfate (MeO-PCB 11 sulfate, Class 3.2) and glucuronide conjugates (MeO-PCB 11 glucuronide, Class 3.3) (Fig. 1). Two isomers were detected for all Class 3 metabolites with a confidence level of 2 based on the Schymanski framework (Table 1).⁷⁹ Figure 3 shows the MS and MS/MS data of one representative isomer from each metabolite Class; for the MS and MSMS data of other Class 3 metabolites shown in Table 1, see Fig. S10.

The formation of the hydroxylated-methoxylated PCB metabolites (Class 3.1) for other PCB congeners has been observed in several disposition studies in rats,⁸⁰ rabbits,⁸¹ and guinea pig.⁸² However, the formation of the corresponding sulfate and glucuronide of Class 3.1 metabolites has not been reported previously. Based on *in vivo* studies, the formation of hydroxylated-methoxylated PCBs is thought to occur by the methylation of PCB catechols.⁸³ The two isomers of hydroxylated-methoxylated PCB 11 metabolites are likely isomers of 4,5-di-OH-PCB 11 that undergo further sulfation and glucuronidation to other Class 3 metabolites; however, additional work is needed to confirm the stereochemistry of these PCB 11 metabolites.

Class 4 – methoxylated-dihydroxylated PCB 11 metabolites and their conjugates.

Three types of methoxylated PCB 11 metabolites derived from trihydroxylated PCB 11 derivatives were observed in medium from PCB 11 exposed HepG2 cells, including methoxylated-dihydroxylated PCB 11 metabolites (MeO-di-OH-PCB 11, Class 4.1), and the corresponding sulfate (MeO-OH-PCB 11 sulfate, Class 4.2) and glucuronide conjugates (MeO-OH-PCB 11 glucuronide, Class 4.3) (Fig. 1). We detected four methoxylated-dihydroxylated PCB 11 metabolites, four sulfate conjugates, and two glucuronide conjugates. Fig. 2 shows the MS and MS/MS data of one representative isomer from each metabolite Class; for the MS and MS/MS data of other Class 4 metabolites shown in Table 1, see Fig. S11. No trihydroxylated PCB 11 metabolite was observed. Three Class 4.1, two Class 4.2, and one Class 4.3 metabolite were identified with a confidence level of 2 according to the Schymanski framework (Table 1).⁷⁹ The remaining Class 4 metabolites were identified with a confidence level of 3 because we were unable to obtain MS/MS data of these minor metabolites. The formation of these metabolites has not been reported previously, and further research is needed to elucidate their structure and toxicities.

Proposed metabolic pathways of PCB 11 in HepG2 cells.

This study is a comprehensive investigation of the metabolic pathway of PCB 11 in HepG2 cells. We detected a total of 30 PCB 11 metabolites belonging to 4 metabolite Classes. Based on the *in silico* predictions and our identification of four Classes of PCB 11 metabolites, we propose the metabolism scheme for PCB 11 shown in Fig. 3. Briefly, PCB 11 is oxidized to monohydroxylated PCBs (Class 1.1), in particular, 4-OH-PCB 11, by hepatic cytochrome P450 enzymes. The formation of OH-PCBs may occur either by direct insertion of an O atom into a C-H bond or via an arene oxide. It is noteworthy that we did not detect dihydrodiols or glutathione conjugates of PCB 11, metabolites that are formed via arene oxide intermediates. OH-PCBs are readily metabolized by SULTs and UGTs to the corresponding sulfates (Class 1.2) and glucuronides (Class 1.3), which is consistent with metabolism studies in rodents and other *in vitro* models.^{22, 84} Alternatively, OH-PCBs can be further oxidized to di-OH-PCB 11 metabolites, with 4,5-di-OH-PCB 11 being a major metabolite (Class 2.1).

Class 1 and Class 2.1 metabolites are further oxidized or conjugated to hydroxylated PCB 11 sulfate (Class 2.2) and glucuronide metabolites (Class 2.3), respectively. Hydroxylated PCB sulfates have been detected in rats^{85–87} and polar bears,³⁰ whereas no hydroxylated PCB 11 glucuronides have been reported to-date. There is also evidence that these metabolites are formed in mice; however, it is unknown if these metabolites were PCB sulfates or glucuronides.⁸⁸ In addition to the formation of Class 1 and 2 metabolites, we also observed the formation of methoxylated metabolites of di- and tri-hydroxylated metabolites of PCB 11 by HepG2 cells (Classes 3 and 4). We posit that these metabolites are formed by methylation of PCB 11 catechols 4,5-di-OH-PCB 11, with catechol-*O*-methyltransferase (COMT). Overall, the proposed metabolism pathway is consistent with observations in humans. Specifically, the formation of 4-OH-PCB 11 and the corresponding sulfate is consistent with a human biomonitoring study^{18, 19} and an *in vitro* study demonstrating that 4-OH-PCB 11 is a substrate for human sulfotransferases.²⁵ Other PCB congeners appear to be preferentially metabolized to *para*-hydroxylated and *para/meta*-dihydroxylated

metabolites by human cytochrome P450 enzymes.^{70, 89} However, additional metabolism studies are needed with experimental models that better approximate the hepatic metabolism and clearance in humans than HepG2 cells, such as primary hepatocytes, HepaRG cells, hepatospheres, and others.^{44, 90}

Toxicological implications of PCB 11 metabolism.

The formation of redox-active or reactive PCB metabolites is linked to a range of adverse health outcomes, such as cancer, associated with exposure to PCBs.^{22, 84} For example, metabolites of PCB 3 formed in rats have been associated with relevant cancer endpoints. 3',4'-di-OH-PCB 3 (4'-chloro-3,4-dihydroxybiphenyl), a metabolite of PCB 3 formed in rats, increases sister chromatid exchanges (SCE) and causes chromosome loss and chromosome breaks in V79 cells.^{39, 91} *In vivo* studies also demonstrate that metabolic activation of PCB 3 plays a role in the initiating activity/genotoxicity of PCB 3.^{74, 92, 93} The metabolic pathway shown in Fig. 3 demonstrates that PCB 11 is similarly metabolized in a model of human drug metabolism and forms metabolites, such as 4,5-di-OH-PCB 11, that are redox-active or can form reactive quinone or arene oxide intermediates. Our study demonstrates that sulfation, glucuronidation, and methylation reactions can eliminate these potentially toxic metabolites. Indeed, UGTs and COMTs protect primary rat hepatocytes from the toxicity of PCB catechols.⁹⁴ However, sulfated and glucuronidated metabolites may be transported to other target sites, where they undergo deconjugation to toxic metabolites.^{22, 84} Moreover, the methylation of a hydroxy group increases the lipophilicity of the corresponding metabolite (Table S4), which may increase their retention *in vivo* and, thus, their toxicity. More research is therefore needed to investigate the toxicity of the PCB 11 metabolites identified in this study, assess their disposition *in vivo*, and study their occurrence in humans.

Changes in endogenous metabolism in media from PCB 11-exposed HepG2 cells.

The analysis of the data revealed distinct differences in features that were not associated with PCB 11 metabolites but reflected the disruption of endogenous metabolism following PCB 11 exposure. A total of 864 out of 6141 features differed between the control and exposed HepG2 cells in positive metabolomics data, and 368 out of 3476 features differed in negative data. Further analysis of the positive and negative ESI data (Fig. 4) with false discovery rate (FDR) correction in multiple comparisons identified 55 and 28 significant metabolites, respectively, in the medium from PCB 11 exposed compared to vehicle exposed HepG2 cells ($p < 0.05$; $FDR < 0.05$; Fig. 4a). A pathway enrichment analysis of positive and negative ESI data identified 4 and 9 significantly affected pathways, respectively ($p < 0.05$; Fig. 4b), in particular, the vitamin B₆ (pyridoxine) pathway (Fig. 4c).

There is evidence that PCB exposure alters this pathway in other model systems. For example, levels of pyridoxal phosphate (PLP), the biologically active form of vitamin B₆, were significantly reduced in the rat liver following exposure to Kanechlor, a PCB mixture.⁹⁵ Although we did not identify PLP with a high level of confidence, we observed a consistent decrease in other forms of vitamin B₆ (i.e., pyridoxal, pyridoxine, or pyridoxate). Our results suggest a dysregulation in vitamin B₆ (pyridoxine) metabolism in PCB 11

exposed HepG2 cells, likely due to a deficiency in PLP, thus identifying a potential target for future studies of the hepatotoxicity of PCB 11.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

Thanks to Mr. Vic Parcell from the High-Resolution Mass Spectrometry Facility, University of Iowa, and Dr. Stephen Harvey from the Center of Mass Spectrometry and Proteomics at the University of Minnesota for help with the chemical analysis, and Drs. Xueshu Li, Xianran He, and Wenjin Xu from the University of Iowa for the synthesis of several PCB 11 metabolite standards.

FUNDING SOURCES

This work was supported by grants ES027169 [HJL], ES019776 [XL], ES014901 [HJL], ES013661 [HJL, GL], and ES005605 [HJL] from the National Institute of Environmental Health Sciences, National Institutes of Health. The content is solely the responsibility of the authors. It does not necessarily represent the official views of the National Institute of Environmental Health Sciences/National Institutes of Health. The findings and conclusions in this presentation have not been formally disseminated by the Centers for Disease Control and Prevention/the Agency for Toxic Substances and Disease Registry and should not be construed to represent any agency determination or policy.

REFERENCES

1. PCBs-overview. <http://www.pops.int/Implementation/IndustrialPOPs/PCB/Overview/tabid/273/Default.aspx> (Accessed on Jun 26, 2020),
2. Anezaki K; Nakano T Concentration levels and congener profiles of polychlorinated biphenyls, pentachlorobenzene, and hexachlorobenzene in commercial pigments. *Environ. Sci. Pollut. Res.* 2014, 21, 998–1009.
3. Herkert NJ; Jahnke JC; Hornbuckle KC Emissions of tetrachlorobiphenyls (PCBs 47, 51, and 68) from polymer resin on kitchen cabinets as a non-aroclor source to residential air. *Environ. Sci. Technol.* 2018, 52, 5154–5160. [PubMed: 29667399]
4. Hu DF; Hornbuckle KC Inadvertent polychlorinated biphenyls in commercial paint pigments. *Environ. Sci. Technol.* 2010, 44, 2822–2827. [PubMed: 19957996]
5. Pizzini S; Sbicego C; Corami F; Grotti M; Magi E; Bonato T; Cozzi G; Barbante C; Piazza R 3,3'-dichlorobiphenyl (non-Aroclor PCB-11) as a marker of non-legacy PCB contamination in marine species: comparison between Antarctic and Mediterranean bivalves. *Chemosphere* 2017, 175, 28–35. [PubMed: 28211332]
6. Praipipat P; Rodenburg LA; Cavallo GJ Source apportionment of polychlorinated biphenyls in the sediments of the selaware river. *Environ. Sci. Technol.* 2013, 47, 4277–4283. [PubMed: 23586856]
7. Basu I; Arnold KA; Vanier M; Hites RA Partial pressures of PCB-11 in air from several great lakes sites. *Environ. Sci. Technol.* 2009, 43, 6488–6492. [PubMed: 19764206]
8. Rodenburg LA; Ralston DK Historical sources of polychlorinated biphenyls to the sediment of the New York/New Jersey Harbor. *Chemosphere* 2017, 169, 450–459. [PubMed: 27889511]
9. Hu DF; Martinez A; Hornbuckle KC Sedimentary records of non-Aroclor and Aroclor PCB mixtures in the Great Lakes. *J. Great Lakes Res.* 2011, 37, 359–364. [PubMed: 23538476]
10. Shang HT; Li YM; Wang T; Wang P; Zhang HD; Zhang QH; Jiang GB The presence of polychlorinated biphenyls in yellow pigment products in China with emphasis on 3,3'-dichlorobiphenyl (PCB 11). *Chemosphere* 2014, 98, 44–50. [PubMed: 24231041]
11. Hu DF; Martinez A; Hornbuckle KC Discovery of non-Aroclor PCB (3,3'-dichlorobiphenyl) in Chicago air. *Environ. Sci. Technol.* 2008, 42, 7873–7877. [PubMed: 19031874]

12. Rodenburg LA; Guo J; Du SY; Cavallo GJ Evidence for unique and ubiquitous environmental sources of 3,3'-dichlorobiphenyl (PCB 11). *Environ. Sci. Technol* 2010, 44, 2816–2821. [PubMed: 20384375]
13. Hites RA Atmospheric concentrations of PCB-11 near the Great Lakes have not decreased since 2004. *Environ. Sci. Technol. Lett* 2018, 5, 131–135.
14. Rodenburg LA; Krumins V; Curran JC Microbial dechlorination of polychlorinated biphenyls, dibenzo-p-dioxins, and -furans at the Portland harbor superfund site, Oregon, USA. *Environ. Sci. Technol* 2015, 49, 7227–7235. [PubMed: 26010118]
15. Marek RF; Thorne PS; Wang K; DeWall J; Hornbuckle KC PCBs and OH-PCBs in serum from children and mothers in urban and rural U.S. communities. *Environ. Sci. Technol* 2013, 47, 9555–9556. [PubMed: 24143032]
16. Koh WX; Hornbuckle KC; Thorne PS Human serum from urban and rural adolescents and their mothers shows exposure to polychlorinated biphenyls not found in commercial mixtures. *Environ. Sci. Technol* 2015, 49, 8105–8112. [PubMed: 26053216]
17. Sethi S; Keil KP; Chen H; Hayakawa K; Li XS; Lin YP; Lehmler HJ; Puschner B; Lein PJ Detection of 3,3'-dichlorobiphenyl in human maternal plasma and its effects on axonal and dendritic growth in primary rat neurons. *Toxicol. Sci* 2017, 158, 401–411. [PubMed: 28510766]
18. Grimm FA; Lehmler HJ; Koh WX; DeWall J; Teesch LM; Hornbuckle KC; Thorne PS; Robertson LW; Duffel MW Identification of a sulfate metabolite of PCB 11 in human serum. *Environ. Int* 2017, 98, 120–128. [PubMed: 27816204]
19. Zhu YM; Mapuskar KA; Marek RF; Xu WJ; Lehmler HJ; Robertson LW; Hornbuckle KC; Spitz DR; Aykin-Burns N A new player in environmentally induced oxidative stress: polychlorinated biphenyl congener, 3,3-dichlorobiphenyl (PCB11). *Toxicol. Sci* 2013, 136, 39–50. [PubMed: 23997111]
20. Hu X; Adamcakova-Dodd A; Thorne PS The fate of inhaled C-14-labeled PCB11 and its metabolites in vivo. *Environ. Int.* 2014, 63, 92–100. [PubMed: 24275706]
21. Hu X; Lehmer HJ; Adamcakova-Dodd A; Thorne PS Elimination of inhaled 3,3'-dichlorobiphenyl and the formation of the 4-hydroxylated metabolite. *Environ. Sci. Technol* 2013, 47, 4743–4751. [PubMed: 23582014]
22. Grimm FA; Hu DF; Kania-Korwel I; Lehmler HJ; Ludewig G; Hornbuckle KC; Duffel MW; Bergman A; Robertson LW Metabolism and metabolites of polychlorinated biphenyls. *Crit. Rev. Toxicol* 2015, 45, 245–272. [PubMed: 25629923]
23. Kaminsky LS; Kennedy MW; Adams SM; Guengerich FP Metabolism of dichlorobiphenyls by highly purified isozymes of rat-liver cytochrome-P-450. *Biochemistry-U S* 1981, 30, 577–588.
24. Kennedy MW; Carpentier NK; Dymerski PP; Kaminsky LS Metabolism of dichlorobiphenyls by hepatic-microsomal cytochrome-P-450. *Biochem. Pharmacol* 1981, 30, 577–588. [PubMed: 6791661]
25. Ekuase EJ; Liu YG; Lehmler HJ; Robertson LW; Duffel MW Structure-activity relationships for hydroxylated polychlorinated biphenyls as inhibitors of the sulfation of dehydroepiandrosterone catalyzed by human hydroxysteroid sulfotransferase SULT2A1. *Chem. Res. Toxicol* 2011, 24, 1720–1728. [PubMed: 21913674]
26. Ekuase EJ; Lehmler HJ; Robertson LW; Duffel MW Binding interactions of hydroxylated polychlorinated biphenyls (OHPCBs) with human hydroxysteroid sulfotransferase hSULT2A1. *Chem. Biol. Interact* 2014, 212, 56–64. [PubMed: 24508592]
27. Parker VS; Squirewell EJ; Lehmler HJ; Robertson LW; Duffel MW Hydroxylated and sulfated metabolites of commonly occurring airborne polychlorinated biphenyls inhibit human steroid sulfotransferases SULT1E1 and SULT2A1. *Environ. Toxicol. Pharmacol* 2018, 58, 196–201. [PubMed: 29408762]
28. Li SN; Cao YF; Sun XY; Yang K; Liang YJ; Gao SS; Fu ZW; Liu YZ; Yang K; Fang ZZ Hydroxy metabolites of polychlorinated biphenyls (OH-PCBs) exhibit inhibitory effects on UDP-glucuronosyltransferases (UGTs). *Chemosphere* 2018, 212, 513–522. [PubMed: 30165278]
29. Tampal N; Lehmler HJ; Espandiari P; Mahnberg T; Robertson LW Glucuronidation of hydroxylated polychlorinated biphenyls (PCBs). *Chem. Res. Toxicol* 2002, 15, 1259–1266. [PubMed: 12387623]

30. Liu YN; Richardson ES; Derocher AE; Lunn NJ; Lehmler HJ; Li XS; Zhang YF; Cui JY; Cheng LH; Martin JW Hundreds of unrecognized halogenated contaminants discovered in polar bear serum. *Angew. Chem. Int. Edit* 2018, 57, 16401–16406.
31. Sethi S; Morgan RK; Peng W; Lin YP; Li XS; Luna C; Koch M; Bansal R; Duffel MW; Puschner B; Zoeller RT; Lehmler HJ; Pessah IN; Lein PJ Comparative analyses of the 12 most abundant PCB congeners detected in human maternal serum for activity at the thyroid hormone receptor and ryanodine receptor. *Environ. Sci. Technol* 2019, 53, 3948–3958. [PubMed: 30821444]
32. Pencikova K; Svrzkova L; Strapacova S; Neca J; Bartonkova I; Dvorak Z; Hyzdalova M; Pivnicka J; Palkova L; Lehmler HJ; Li XS; Vondracek J; Machala M In vitro profiling of toxic effects of prominent environmental lower-chlorinated PCB congeners linked with endocrine disruption and tumor promotion. *Environ. Pollut* 2018, 237, 473–486. [PubMed: 29518658]
33. Flor S; He XR; Lehmler HJ; Ludewig G Estrogenicity and androgenicity screening of PCB sulfate monoesters in human breast cancer MCF-7 cells. *Environ. Sci. Pollut. Res* 2016, 23, 2186–2200.
34. Grimm FA; Lehmler HJ; He XR; Robertson LW; Duffel MW Sulfated metabolites of polychlorinated biphenyls are high-affinity ligands for the thyroid hormone transport protein transthyretin. *Environ. Health Perspect* 2013, 121, 657–662. [PubMed: 23584369]
35. Ludewig G; Lehmann L; Esch H; Robertson LW Metabolic activation of PCBs to carcinogens in vivo - A review. *Environ. Toxicol. Pharmacol* 2008, 25, 241–246. [PubMed: 18452002]
36. Robertson LW; Ludewig G Polychlorinated Biphenyl (PCB) carcinogenicity with special emphasis on airborne PCBs. *Gefahrstoffe-Reinhalt Luft* 2011, 71, 25–32.
37. Lehmann L; Flor S; Zettner M; Esch HL; Robertson LW; Ludewig G Mutagenicity of 4-monochlorobiphenyl and its metabolites in vitro and in vivo. *Drug Metab. Rev* 2006, 38, 163–163. [PubMed: 16684655]
38. Jacobus JA; Flor S; Klingelutz AJ; Robertson LW; Ludewig G 2-(4'-chlorophenyl)-1,4-benzoquinone increases the frequency of micronuclei and shortens telomeres. *Environ. Toxicol. Pharmacol* 2008, 25, 267–272. [PubMed: 18438462]
39. Flor S; Ludewig G Polyploidy-induction by dihydroxylated monochlorobiphenyls: Structure-activity-relationships. *Environ. Int* 2010, 36, 962–969. [PubMed: 20471090]
40. Xie W; Wang K; Robertson LW; Ludewig G Investigation of mechanism(s) of DNA damage induced by 4-monochlorobiphenyl (PCB3) metabolites. *Environ. Int* 2010, 36, 950–961. [PubMed: 20129669]
41. Ludewig G; Robertson LW Polychlorinated biphenyls (PCBs) as initiating agents in hepatocellular carcinoma. *Cancer Lett.* 2013, 334, 46–55. [PubMed: 23211541]
42. Li M; Teesch LM; Murry DJ; Pope RM; Li YL; Robertson LW; Ludewig G Cytochrome c adducts with PCB quinoid metabolites. *Environ. Sci. Pollut. Res* 2016, 23, 2148–2159.
43. Ludewig G; Robertson LW; Glauert HP, Carcinogenicity and mechanisms of persistent organic pollutants In *Effects of Persistent and Bioactive Organic Pollutants on Human Health*; Carpenter DO, Ed.; John Wiley & Sons: New York, 2013; pp 57–99.
44. Guo L; Dial S; Shi LM; Branham W; Liu J; Fang JL; Green B; Deng H; Kaput J; Ning BT Similarities and differences in the expression of drug-metabolizing enzymes between human hepatic cell lines and primary human hepatocytes. *Drug Metab. Dispos* 2011, 39, 528–538. [PubMed: 21149542]
45. Uwimana E; Ruiz P; Li XS; Lehmler HJ Human CYP2A6, CYP2B6, AND CYP2E1 atropselectively metabolize polychlorinated biphenyls to hydroxylated metabolites. *Environ. Sci. Technol* 2019, 53, 2114–2123. [PubMed: 30576102]
46. Li X; Holland EB; Feng W; Zheng J; Dong Y; Pessah IN; Duffel MW; Robertson LW; Lehmler HJ Authentication of synthetic environmental contaminants and their (bio)transformation products in toxicology: polychlorinated biphenyls as an example. *Environ. Sci. Pollut. Res* 2018, 25, 16508–16521.
47. Holland EB; Feng W; Zheng J; Dong Y; Li XS; Lehmler HJ; Pessah IN An extended structure-activity relationship of nondioxin-like PCBs evaluates and supports modeling predictions and identifies picomolar potency of PCB 202 towards ryanodine receptors. *Toxicol. Sci* 2017, 155, 170–181. [PubMed: 27655348]

48. Rodriguez EA; Vanle BC; Doorn JA; Lehmler HJ; Robertson LW; Duffel MW Hydroxylated and sulfated metabolites of commonly observed airborne polychlorinated biphenyls display selective uptake and toxicity in N27, SH-SY5Y, and HepG2 cells. *Environ. Toxicol. Pharmacol* 2018, 62, 69–78. [PubMed: 29986280]
49. Huang M; Mesaros C; Hackfeld LC; Hodge RP; Blair IA; Penning TM Potential metabolic activation of representative alkylated polycyclic aromatic hydrocarbons 1-methylphenanthrene and 9-ethylphenanthrene associated with the deepwater horizon oil spill in human hepatoma (HepG2) cells. *Chem. Res. Toxicol* 2017, 30, 2140–2150. [PubMed: 29035516]
50. Yu TW; Park Y; Johnson JM; Jones DP apLCMS-adaptive processing of high-resolution LC/MS data. *Bioinformatics* 2009, 25, 1930–1936. [PubMed: 19414529]
51. Uppal K; Soltow QA; Strobel FH; Pittard WS; Gernert KM; Yu TW; Jones DP xMSanalyzer: automated pipeline for improved feature detection and downstream analysis of large-scale, non-targeted metabolomics data. *BMC Bioinformatics* 2013, 14, 15. [PubMed: 23323971]
52. Ritchie ME; Phipson B; Wu D; Hu YF; Law CW; Shi W; Smyth GK limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* 2015, 43, e47. [PubMed: 25605792]
53. Benjamini Y; Hochberg Y Controlling the false discovery rate - a practical and powerful approach to multiple testing. *J. R. Stat. Soc. B* 1995, 57, 289–300.
54. Li SZ; Park Y; Duraisingham S; Strobel FH; Khan N; Soltow QA; Jones DP; Pulendran B Predicting network activity from high throughput metabolomics. *Plos Comput. Biol* 2013, 9, e1003123. [PubMed: 23861661]
55. Go YM; Walker DI; Soltow QA; Uppal K; Wachtman LM; Strobel FH; Pennell K; Promislow DEL; Jones DP Metabolome-wide association study of phenylalanine in plasma of common marmosets. *Amino Acids* 2015, 47, 589–601. [PubMed: 25526869]
56. Uppal K; Walker DI; Jones DP xMSannotator: An R package for network-based annotation of high-resolution metabolomics data. *Anal. Chem* 2017, 89, 1063–1067. [PubMed: 27977166]
57. Wishart DS; Jewison T; Guo AC; Wilson M; Knox C; Liu YF; Djoumbou Y; Mandal R; Aziat F; Dong E; Bouatra S; Sinelnikov I; Arndt D; Xia JG; Liu P; Yallou F; Bjorn Dahl T; Perez-Pineiro R; Eisner R; Allen F; Neveu V; Greiner R; Scalbert A HMDB 3.0-The human metabolome database in 2013. *Nucleic Acids Res.* 2013, 41, D801–D807. [PubMed: 23161693]
58. Kanehisa M; Furumichi M; Tanabe M; Sato Y; Morishima K KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Res.* 2017, 45, D353–D361. [PubMed: 27899662]
59. McGraw JE; Waller DP Specific human CYP 450 isoform metabolism of a pentachlorobiphenyl (PCB-IUPAC# 101). *Biochem. Bioph. Res. Co* 2006, 344, 129–133.
60. Uwimana E; Li XS; Lehmler HJ Human liver microsomes atropselectively metabolize 2,2',3,4',6-pentachlorobiphenyl (PCB 91) to a 1,2-shift product as the major metabolite. *Environ. Sci. Technol* 2018, 52, 6000–6008. [PubMed: 29659268]
61. Rudzok S; Schlink U; Herbarth O; Bauer M Measuring and modeling of binary mixture effects of pharmaceuticals and nickel on cell viability/cytotoxicity in the human hepatoma derived cell line HepG2. *Toxicol. Appl. Pharm* 2010, 244, 336–343.
62. Wang W; Qian YC; Li JH; Aljuhani N; Siraki AG; Le XC; Li XF Characterization of mechanisms of glutathione conjugation with halobenzoquinones in solution and HepG2 cells. *Environ. Sci. Technol* 2018, 52, 2898–2908. [PubMed: 29420883]
63. Geng NB; Zhang HJ; Zhang BQ; Wu P; Wang FD; Yu ZK; Chen JP Effects of short-chain chlorinated paraffins exposure on the viability and metabolism of human hepatoma hepG2 cells. *Environ. Sci. Technol* 2015, 49, 3076–3083. [PubMed: 25660998]
64. Wilkening S; Stahl F; Bader A Comparison of primary human hepatocytes and hepatoma cell line HEPG2 with regard to their biotransformation properties. *Drug Metab. Dispos* 2003, 31, 1035–1042. [PubMed: 12867492]
65. Hart SN; Li Y; Nakamoto K; Subileau EA; Steen D; Zhong XB A comparison of whole genome gene expression profiles of HepaRG cells and HepG2 cells to primary human hepatocytes and human liver tissues. *Drug Metab. Dispos* 2010, 38, 988–994. [PubMed: 20228232]

66. Yokoyama Y; Sasaki Y; Terasaki N; Kawataki T; Takekawa K; Iwase Y; Shimizu T; Sanoh S; Ohta S Comparison of drug metabolism and its related hepatotoxic effects in HepaRG, cryopreserved human hepatocytes, and HepG2 cell cultures. *Biol. Pharm. Bull* 2018, 41, 722–732. [PubMed: 29445054]
67. Wang Y; Yu HY; Shi XL; Luo ZP; Lin DH; Huang MD Structural mechanism of ring-opening reaction of glucose by human serum albumin. *J. Biol. Chem* 2013, 288, 15980–15987. [PubMed: 23592780]
68. Morais MPP; Mackay JD; Bhamra SK; Buchanan JG; James TD; Fossey JS; van den Elsen JMH Analysis of protein glycation using phenylboronate acrylamide gel electrophoresis. *Proteomics* 2010, 10, 48–58. [PubMed: 19899078]
69. Grimm FA; He XR; Teesch LM; Lehmler HJ; Robertson LW; Duffel MW Tissue distribution, metabolism, and excretion of 3,3'-dichloro-4'-sulfooxy-biphenyl in the rat. *Environ. Sci. Technol.* 2015, 49, 8087–8095. [PubMed: 26046945]
70. Uwimana E; Li XS; Lehmler HJ 2,2',3,5',6-pentachlorobiphenyl (PCB 95) is atropselectively metabolized to para-hydroxylated metabolites by human liver microsomes. *Chem. Res. Toxicol* 2016, 29, 2108–2110. [PubMed: 27989147]
71. Wu XA; Kammerer A; Lehmler HJ Microsomal oxidation of 2,2',3,3',6,6'-hexachlorobiphenyl (PCB 136) results in species-dependent chiral signatures of the hydroxylated metabolites. *Environ. Sci. Technol* 2014, 48, 2436–2444. [PubMed: 24467194]
72. McLean MR; Robertson LW; Gupta RC Detection of PCB adducts by the P-32-Postlabeling technique. *Chem. Res. Toxicol* 1996, 9, 165–171. [PubMed: 8924587]
73. Srinivasan A; Lehmler HJ; Robertson LW; Ludewig G Production of DNA strand breaks in Vitro and reactive oxygen species in Vitro and in HL-60 cells by PCB metabolites. *Toxicol. Sci.* 2001, 60, 92–102. [PubMed: 11222876]
74. Espandiari P; Glauert HP; Lehmler HJ; Lee EY; Srinivasan C; Robertson LW Initiating activity of 4-chlorobiphenyl metabolites in the resistant hepatocyte model. *Toxicol. Sci* 2004, 79, 41–46. [PubMed: 14976334]
75. Pereg D; Robertson LW; Gupta RC DNA adduction by polychlorinated biphenyls: adducts derived from hepatic microsomal activation and from synthetic metabolites. *Chem. Biol. Interact* 2002, 139, 129–144. [PubMed: 11823002]
76. Spencer WA; Lehmler HJ; Robertson LW; Gupta RC Oxidative DNA adducts after Cu²⁺-mediated activation of dihydroxy PCBs: Role of reactive oxygen species. *Free Radic. Biol. Med* 2009, 46, 1346–1352. [PubMed: 19233261]
77. Schnellmann RG; Putnam CW; Sipes IG Metabolism of 2,2',3,3',6,6'-hexachlorobiphenyl and 2,2',4,4',5,5'-hexachlorobiphenyl by human hepatic microsomes. *Biochem. Pharmacol* 1983, 32, 3233–3239. [PubMed: 6416258]
78. Sundström GJ, B. The metabolism of 2,2',3,5',6-pentachlorobiphenyl in rats, mice and quails. *Chemosphere* 1975, 4, 361–370.
79. Schymanski EL; Jeon J; Gulde R; Fenner K; Ruff M; Singer HP; Hollender J Identifying small molecules via high resolution mass spectrometry: communicating confidence. *Environ. Sci. Technol* 2014, 48, 2097–2098. [PubMed: 24476540]
80. Goto M; Sugiura K; Hattori M; Miyagawa T; Okamura M Metabolism of 2,3,5,6-tetrachlorobiphenyl-¹⁴C and 2,3,4,5,6-pentachlorobiphenyl-¹⁴C in the rat. *Chemosphere* 1974, 5, 233–238.
81. Hutzinger O; Jamieson WD; Safe S; Paulmann L; Ammon R Identification of metabolic dechlorination of highly chlorinated biphenyl in rabbit. *Nature* 1974, 252, 698–699. [PubMed: 4215979]
82. Ariyoshi N; Koga N; Yoshimura H; Oguri K Metabolism of 2,4,5,2',4',5'-hexachlorobiphenyl (PCB153) in guinea pig. *Xenobiotica* 1997, 27, 973–983. [PubMed: 9381737]
83. Safe S; Hutzinger O; Jones D The mechanism of chlorobiphenyl metabolism. *J. Agric. Food Chem* 1975, 23, 851–853. [PubMed: 1159182]
84. Dhakal K; Gadupudi GS; Lehmler HJ; Ludewig G; Duffel MW; Robertson LW Sources and toxicities of phenolic polychlorinated biphenyls (OH-PCBs). *Environ. Sci. Pollut. Res* 2018, 25, 16277–16290.

85. Dhakal K; He XR; Lehmler HJ; Teesch LM; Duffel MW; Robertson LW Identification of sulfated metabolites of 4-chlorobiphenyl (PCB3) in the serum and urine of male rats. *Chem. Res. Toxicol* 2012, 25, 2796–2804. [PubMed: 23137097]
86. Dhakal K; Adamcakova-Dodd A; Lehmler HJ; Thorne PS; Robertson LW Sulfate conjugates are urinary markers of inhalation exposure to 4-chlorobiphenyl (PCB3). *Chem. Res. Toxicol* 2013, 26, 853–855. [PubMed: 23713983]
87. Dhakal K; Uwimana E; Adamcakova-Dodd A; Thorne PS; Lehmler HJ; Robertson LW Disposition of phenolic and sulfated metabolites after inhalation exposure to 4-chlorobiphenyl (PCB3) in female rats. *Chem. Res. Toxicol* 2014, 27, 1411–1420. [PubMed: 24988477]
88. Wu XN; Barnhart C; Lein PJ; Lehmler HJ Hepatic metabolism affects the atropselective disposition of 2,2',3,3',6,6'-hexachlorobiphenyl (PCB 136) in mice. *Environ. Sci. Technol* 2015, 49, 616–625. [PubMed: 25420130]
89. Uwimana E; Maiers A; Li XS; Lehmler HJ Microsomal metabolism of prochiral polychlorinated biphenyls results in the enantioselective formation of chiral metabolites. *Environ. Sci. Technol* 2017, 51, 1820–1829. [PubMed: 28038482]
90. Ashraf MN; Asghar MW; Rong Y; Doschak MR; Kiang TKL Advanced In Vitro HepaRG Culture Systems for Xenobiotic Metabolism and Toxicity Characterization. *Eur. J. Drug. Metab. Ph* 2019, 44, 437–458.
91. Zettner MA; Flor S; Ludewig G; Wagner J; Robertson LW; Lehmann L Quinoid metabolites of 4-monochlorobiphenyl induce gene mutations in cultured Chinese hamster V79 cells. *Toxicol. Sci* 2007, 100, 88–98. [PubMed: 17686921]
92. Espandiari P; Glauert HP; Lehmler HJ; Lee EY; Srinivasan C; Robertson LW Polychlorinated biphenyls as initiators in liver carcinogenesis: resistant hepatocyte model. *Toxicol. Appl. Pharm* 2003, 186, 55–62.
93. Lehmann L; Esch HL; Kirby PA; Robertson LW; Ludewig G 4-monochlorobiphenyl (PCB3) induces mutations in the livers of transgenic Fisher 344 rats. *Carcinogenesis* 2007, 28, 471–478. [PubMed: 16950798]
94. Sadeghi-Aliabadi H; Chan K; Lehmler H-J; Robertson LW; O'Brien ML Molecular cytotoxic mechanisms of catecholic polychlorinated biphenyl metabolites in isolated rat hepatocytes. *Chem. Biol. Interact* 2007, 167, 184–192. [PubMed: 17408604]
95. Fujiwara M; Kuriyama K Effect of PCB (polychlorobiphenyls) on L-ascorbic acid, pyridoxal phosphate and riboflavin contents in various organs and on hepatic metabolism of L-ascorbic acid in the rat. *J. Pharmacol* 1977, 27, 621–7. [PubMed: 412990]

Fig. 1.

Metabolism of PCB 11 by HepG2 cells results in the formation of novel methoxylated PCB metabolites. Class 3 metabolites are dihydroxylated PCB 11 metabolites and include (a) MeO-OH-PCB 11 (m/z 266.99796), (b) MeO-PCB 11 sulfate (m/z 346.95477) and (c) MeO-PCB 11 glucuronide (m/z 443.03006) metabolites. Class 4 metabolites are trihydroxylated PCB 11 metabolites and include (d) MeO-di-OH-PCB 11 (m/z 282.99287), (e) MeO-OH-PCB 11 sulfate (m/z 362.94969) and (f) MeO-OH-PCB 11 glucuronide (m/z 459.02496) metabolites. For each metabolite class, up to three isomers were observed (Table 1).

Analyses were performed by LC-Orbitrap MS in the negative mode. The chromatograms are the extracted ion chromatograms (EIC) based on the calculated accurate masses of each metabolite class, with a mass window of 10 ppm. For selected MS and MS/MS spectra, see Fig. 2 and the Supporting Information.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 2.

The accurate mass of the molecular ion $[M-H]^-$, an isotope pattern consistent with a dichlorinated compounds, and the MS/MS data support the formation of six classes of methoxylated PCB 11 metabolites by HepG2 cells exposed to PCB 11. Representative MS spectra (a1-c1) for Class 3.1-3.3 and (d1-f1) for Class 4.1-4.3 metabolites and (a2-f2) the corresponding MS/MS of one isomer for each metabolite Class. The collision energies on the fragmentation cell for the MS/MS spectra from (a2) to (f2) were 75, 45, 45, 45, 30 and 45 eV, respectively. The placement of the functional groups on different phenyl rings is for illustration purposes only and does not indicate their actual position. Analyses were performed in the negative mode on an LC-Orbitrap MS; for additional details, see the Experimental Section. For the MS and MS/MS spectra of other isomers for methoxylated PCB 11 metabolites listed in Table 1, see the Supporting Information.

Fig. 3.

Scheme showing the complex metabolism of PCB 11 in HepG2 cells. Structures shown in blue were detected in this study. Structures shown in black and red were not detected, but their formation is expected based on the available evidence from this and other studies. Catechol metabolites and their quinone derivatives are shown in red. These metabolites are likely reactive and highly toxic. Class numbers with a green label were predicted by Metadrag, whereas class numbers in black were not predicted. The placement of the functional groups on different phenyl rings is for illustration purposes only and does not indicate their actual position. **P450**: Cytochrome P450 enzyme; **Glc**: Glucuronide; **DHDH**: Dihydrodiol dehydrogenase; **SULT**: Sulfotransferase; **EH**: Epoxide hydrolase; **UGT**: Uridine 5'-diphospho-glucuronosyltransferase; **COMT**: Catechol-O-methyltransferase; **ND1** and **ND2**: Metabolite classes were not detected.

Fig. 4.

The untargeted metabolomics analysis of medium samples revealed distinct differences between experiments with PCB 11 or vehicle (DMSO) exposed HepG2 cells. (a) The volcano plot selected 864 and 368 metabolic features above the threshold of $p = 0.05$ and 55 and 28 features above the threshold of $FDR = 0.05$ in the positive and negative ESI data, respectively. (b) A pathway enrichment analysis of positive and negative ESI data using feature lists with raw p-values identified 4 and 9 significantly affected pathways, respectively ($p < 0.05$). Vitamin B₆ (pyridoxine) metabolism was significant in both positive and negative ESI data. The number of features altered by PCB 11 in these pathways is depicted as overlap/total features. (c) Selected metabolites annotated in vitamin B₆ (pyridoxine) metabolism were dysregulated by PCB 11 exposure (bar plots showing relative levels of normalized raw intensities). Putative metabolites that are significantly changed ($p <$

0.05 (*) and $p < 0.01$ (**)), or not changed ($p > 0.05$) are shown in red and green backgrounds. The accurate masses, retention times, adducts, significances, and confidence scores of annotations of the metabolites in vitamin B₆ metabolism are listed in Table S5.

Table 1.

Several different metabolite classes were detected by LC-Orbitrap MS in medium from HepG2 cells exposed to PCB 11.^a

Class No ^b	Metabolites	Retention time, min	Formula	[M-H] ⁻			MS ² (Da)	Confidence level ^c
				Calculated (Da)	Measured (Da)	Differences (ppm)		
1.1	OH-PCB 11	5.86	C ₁₂ H ₇ Cl ₂ O ⁻	236.98740	236.98824	3.54	201.01, 165.04	1
1.2	PCB 11 sulfate	4.09	C ₁₂ H ₇ Cl ₂ O ₄ S ⁻	316.94421	316.94489	2.15	236.99	2
		4.13			316.94476	1.74	236.99	1
1.3	PCB 11 glucuronide	3.25	C ₁₈ H ₁₇ Cl ₂ O ₈ ⁻	431.03005	431.03096	2.11		3
		3.40			431.03085			3
		3.60			413.02054		236.99	2
		3.65		413.01948	413.02057	2.64	236.99, 201.01	2
2.1	di-OH-PCB 11	4.29	C ₁₂ H ₇ Cl ₂ O ₂ ⁻	252.98231	252.98314	3.28	207.02, 189.01, 163.00, 153.04, 143.05	2
2.2	OH-PCB 11 sulfate	3.57	C ₁₂ H ₇ Cl ₂ O ₅ S ⁻	332.93912	332.94006	2.82		3
		3.69			332.94003			3
		4.19			332.94003	2.73	235.02, 217.01, 207.02, 189.01, 179.03, 161.02, 135.00	2
		4.29			332.94006	2.82	252.98, 235.02, 217.01, 207.02, 189.01, 179.03, 161.02, 135.00	2
2.3	OH-PCB 11 glucuronide	3.00	C ₁₈ H ₁₅ Cl ₂ O ₈ ⁻	429.01440	429.01520	1.86		3
3.1	MeO-OH-PCB 11	3.64	C ₁₃ H ₉ Cl ₂ O ₂ ⁻	266.99796	429.01529	2.07	252.98	2
		6.14			266.99863	2.51	251.96, 216.00, 188.00	2
3.2	MeO-PCB 11 sulfate	6.27	C ₁₃ H ₉ Cl ₂ O ₅ S ⁻	346.95477	266.99857	2.28	251.96, 216.00, 188.00	2
		4.02			346.95554	2.22	267.00, 251.98	2
3.3	MeO-PCB 11 glucuronide	4.20	C ₁₉ H ₁₇ Cl ₂ O ₈ ⁻	443.03005	346.95548	2.05	267.00, 251.97	2
		3.63			443.03101	2.17	267.00, 251.98	2
		3.70			443.03104	2.23	267.00, 251.98	2
Class No	Metabolites	Retention time, min	Formula	[M-H] ⁻			MS ² (Da)	Confidence level ^b
				Calculated (Da)	Measured (Da)	Differences (ppm)		

Class No ^b	Metabolites	Retention time, min	Formula	[M-H] ⁻			MS ² (Da)	Confidence level ^c
				Calculated (Da)	Measured (Da)	Differences (ppm)		
4.1	MeO-di-OH-PCB 11	4.39	C ₁₃ H ₉ Cl ₂ O ₃ ⁻	282.99287	282.99362	2.65	267.97, 231.99, 204.00	2
		4.53			282.99353	2.33	267.97, 231.99, 204.00	2
		4.74			282.99377	3.18		3
		6.27			282.99347	2.12	267.97, 238.97, 204.00, 175.00	2
4.2	MeO-OH-PCB 11 sulfate	3.42	C ₁₃ H ₉ Cl ₂ O ₆ S ⁻	362.94969	362.95065	2.64		3
		3.68			362.95068	2.73	282.99, 267.97, 235.00, 231.99, 204.00	2
		3.78			362.95065	2.64	282.99, 267.97	2
		4.49			362.95041	1.98		3
4.3	MeO-OH-PCB 11 glucuronide	3.67	C ₁₉ H ₁₇ Cl ₂ O ₉ ⁻	459.02496	459.02618	2.66	282.99, 267.97, 204.00, 175.00	2
		3.81			459.02628	2.87		3

^aHepG2 cells were exposed for 24 h to PCB 11 (10 µM), metabolites were extracted from the cell culture medium by QuEChERS extraction, and extracts were analyzed by LC-Orbitrap MS. Most of these metabolites were also detected in LC-QToF analyses of the same extracts (Table S1). Some of these metabolites were also present in the cell pellet, as determined by LC-QToF-MS. Because the metabolite levels in the cell pellet were much lower and in several cases below the detection limit of the LC-QToF-MS analyses, extracts of cell pellets were not analyzed by LC-Orbitrap MS. See the Experimental Section for additional details.

^bClass numbers were assigned as follows: Class 1 (1.1, 1.2, 1.3) are mono-hydroxylated PCB 11 metabolites and their conjugates; Class 2 (2.1, 2.2, 2.3) are di-hydroxylated PCB 11 metabolites and their conjugates; Class 3 (3.1, 3.2, 3.3) are methoxylated-hydroxylated PCB 11 metabolites and their conjugates; and Class 4 (4.1, 4.2, 4.3) are methoxylated-di-hydroxylated PCB 11 metabolites and their conjugates.

^cConfidence levels were assigned using the Schymanski framework.⁷⁹ Level 1: metabolites were not only identified based on accurate mass, isotope pattern, MS, and MS², but also with authentic standards. Level 2: metabolites were identified based on accurate mass, isotope pattern, MS, and MS/MS. Level 3: metabolites were identified based on accurate mass, isotope pattern, and MS, but not MS/MS.

Note: We also observed several mono-chlorinated metabolites that are likely metabolites of the minor PCB 2 impurity present in the PCB 11 used in this study,^{46, 47} however, we cannot dismiss the possibility that these metabolites are dechlorination products formed from PCB 11.