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Hepatic Transcriptomic Patterns in the Neonatal Rat after Pentabromodiphenyl ether (PBDE) Exposure

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Abstract

Human exposure to pentabromodiphenyl ether (PBDE) mixture (DE-71) and its PBDE-47 congener can occur both in utero and during lactation. Here we tested the hypothesis that PBDE-induced neonatal hepatic transcriptomic alterations in Wistar Han rat pups can inform on potential toxicity and carcinogenicity after longer-term PBDE exposures. Wistar Han rat dams were exposed to either DE-71 or PBDE-47 daily from gestation day (GD 6) through postnatal day four (PND 4). Total plasma thyroxine (T4) was decreased in PND 4 pups. In liver, transcripts for CYPs and conjugation enzymes, Nrf2, and ABC transporters were upregulated. In general, the hepatic transcriptomic alterations after exposure to DE-71 or PBDE-47 were similar and provided early indicators of oxidative stress and metabolic alterations, key characteristics of toxicity processes. Based on liver transcript patterns, the mean BMDLs (mean benchmark dose, lower confidence limit) of the most sensitive biological processes were lower for PBDE-47 than for the PBDE mixture. Neonatal rat liver transcriptomic data provide early indicators on molecular pathway alterations that may lead to toxicity and/or carcinogenicity if the exposures continue for longer durations. These early toxicogenomic indicators may be used to help prioritize chemicals for a more complete toxicity and cancer risk evaluation.

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Declaration of Conflicting Interests

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Keywords

Pentabromodiphenyl ether; PBDE-47; PBDE mixture (DE-71); liver transcriptomic patterns; liver toxicity; thyroxine (T4)

Introduction

Developing strategies to improve public health includes using methods to identify the potential of environmental exposures to cause toxic and/or carcinogenic effects in a more efficient and relevant manner. This includes understanding toxic effects in vulnerable populations, including the very young whose defense systems are not yet fully developed. The current study provides information on how postnatal day (PND) 4 rat pup toxic endpoints can be used to predict longer-term toxicity and/or carcinogenicity potential.

The pentabromodiphenyl ether (PBDE) chemicals, previously used as flame retardants, were selected for study because exposure to these chemicals is still a recurring problem^{1–3}. The PBDEs are fat-soluble chemicals with a long half-life in the body, and transfer can occur from mother-to-fetus, and to the neonate from breast milk⁴. Exposure to PBDEs can result in a variety of toxicities including disruption of thyroid homeostasis and developmental toxicity^{5,6}. While the use of the PBDEs has been phased out, they are still present in e-waste sites and exposures to PBDEs continue^{7–9}. The PBDE mixture (DE-71) used in the current study caused hepatocellular tumors in rats and mice of both sexes¹⁰, but the PBDE-47 congener, one of the most prevalent congeners in human tissues¹¹, has not been tested for carcinogenic activity in model systems. Thus, a comparative evaluation of the toxicity of the PBDE mixture (DE-71) and the PBDE-47 congener in the rat would allow for the identification of similarities and differences between biological effects of the mixture and the congener. Both the PBDE mixture and PBDE-47 were not mutagenic in the presence or absence of metabolic activation¹⁰.

Exposure to hazardous chemicals early in life can have a profound effect on the development of disease later in life^{12,13}. Liver is one of the first organs to be exposed to xenobiotics and is a main organ system for metabolism and excretion of xenobiotics^{14,15}. In this study we provide data to show how liver transcriptomics data can be used to identify early disease biomarkers at PND 4, a time point where exposure to the pup was primarily maternal-to-fetus exposure.

Materials and Methods

Chemicals

PBDE-47 (2,2',4,4'-tetrabromodiphenyl ether; CAS# 5436–43-1) was obtained from Cerillant Corp. (Round Rock, TX; Lot ER081208–02). The identity was confirmed by mass spectrometry and nuclear magnetic resonance spectroscopy (NMR). The purity for PBDE-47 was 99.6% by gas chromatography (GC) analysis using flame ionization detection (FID). There were no quantifiable polybrominated dibenzodioxins or furans present in the PBDE-47 sample.

A PBDE-mixture (DE-71, technical pentabromodiphenyl; CAS# 32534-81-9) was obtained from Great Lakes Corporation (West Lafayette, IN; Lot 2550OA30A). The identity and purity were determined as described previously¹⁰. The DE-71 composition was: PBDE-99 (41.7%), PBDE-47 (35.7%), PBDE-100 (10.4%), PBDE-154 (3.6%), PBDE-153 (3.3%), and PBDE-85 (2%); low levels of polybrominated dibenzodioxins and furans were also identified (approximately $7 \times 10^{-6}\%$ by weight). A high dose of 50 mg/kg was selected for the PBDE-47 and PBDE mixture (DE-71) study to make doses comparable to those used in the DE-71 carcinogenicity study where treatment-related hepatocellular tumors occurred in male and female Wistar Han rats without an effect on survival throughout most of the 2-year study at 50 mg/kg¹⁰. The lower doses were added for benchmark dose analysis of treatment-related transcriptomic changes.

Animals

Timed-mated, female Wistar Han rats (Crl: WI (Han)) were obtained from Charles River Laboratories (Raleigh, NC) at 11–12 weeks of age at gestation day 2 (GD 2). There were 8 dams per dose group. Up to one male and one female pup were randomly selected from each litter for plasma level, thyroid hormone level determinations, and liver transcriptomics (five males only/dose group). All pups available from the litters were examined for liver histopathologic lesions.

Tap water and NIH 07 diet (Ziegler Brothers, Inc., Gardners, PA) were available *ad libitum*. The care of animals on this study was according to NIH procedures as described in the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals (available online at: <https://olaw.nih.gov>). The study protocols were approved by the institutional animal care and use committee.

Experimental Design

PBDE-47 and PBDE mixture (DE-71) dose formulations were prepared at 0 (vehicle control), 0.02, 3, and 10 mg/mL in corn oil to provide oral gavage doses of 0, 0.1, 15, and 50 mg/kg/day when dams were dosed with a dosing volume of 5 mL/kg/day from GD 6 through PND 4, seven days per week. Controls received corn oil vehicle.

Blood was collected from dams (cardiocentesis) for serum thyroid hormone level determination, and plasma from pups for PBDE plasma level and thyroid hormone level determinations. PND 4 pups were euthanized and trunk blood was collected from individual pups. Serum and plasma were isolated and frozen at 80° C until analyzed for PBDE levels and/or thyroid hormones. Immediately after blood collection, the liver was weighed and a section of the liver (~500 mg) was taken from the left lobe, placed in a cryotube, flash frozen and stored at –80° C for toxicogenomic analysis. A 5 mm section of liver immediately adjacent was collected and fixed in 10% neutral buffered formalin (NBF) for histopathology.

Liver Histopathology

At necropsy, sections of liver were removed and fixed in 10% NBF for microscopic evaluation. Additional samples were collected, cubed, and frozen at –80° C. After fixation in NBF, tissues were trimmed, processed, embedded in paraffin, sectioned at a thickness of 5

microns, stained with hematoxylin and eosin (H&E) and examined microscopically by a board-certified veterinary pathologist with experience in toxicologic pathology.

Quantitation of PBDE-47, 99, and 153 Plasma Levels

Plasma levels of PBDE-47, 99, or 153 were determined after PBDE mixture (DE-71) exposure (0, 0.1, 15, or 50 mg/kg) and PBDE-47 determined after PBDE-47 exposure (0, 0.1, 15, and 50 mg/kg) in PND 4 pups using analytical methods previously described¹⁰. Briefly, 100 μ L aliquot of plasma was extracted with toluene (100 μ L PCB 118 in toluene was used as internal standard) and the supernatant was analyzed by GC coupled to an electron capture detector.

Thyroid Hormone Analyses

At PND4, serum samples from dams and plasma samples from pups were analyzed for total thyroxine (T4) by radioimmunoassay (RIA). Samples were processed according to the manufacturers' protocols (MP Biomedicals LLC, Costa Mesa, CA) and analyzed using an Apex Automatic gamma counter (ICN Micromedex Systems, Inc., Huntsville, AL). All samples were run in duplicate.

RNA Collection

RNA was extracted from frozen liver samples (approximately 3–4 mm cubes) from the PND 4 left lateral hepatic lobe, using the Invitrogen PureLink Mini kit (Invitrogen cat# 12183–018A, Carlsbad, CA) according to the manufacturer's protocol. RNA concentration and quality were measured on a Bioanalyzer (Agilent Technologies, Santa Clara, CA). Samples were aliquoted and stored at –80 °C until they were analyzed for gene expression studies.

Microarray Analysis

PND 4 liver gene expression analysis was conducted using Affymetrix Rat Genome 230 2.0 GeneChip® arrays (Affymetrix, Santa Clara, CA). One hundred ng of total RNA was amplified as directed in the Affymetrix 3' IVT Plus kit protocol. 15 μ g of amplified biotin-aRNAs were fragmented and 12.5 μ g were hybridized to each array for 16 hours at 45 °C in a rotating hybridization oven using the Affymetrix Eukaryotic Target Hybridization Controls and protocol. Array slides were stained with streptavidin/phycoerythrin utilizing a double-antibody staining procedure and then washed for antibody amplification according to the GeneChip Hybridization, Wash and Stain Kit and user manual. Arrays were scanned in an Affymetrix Scanner 3000 and data were obtained using the GeneChip® Command Console and Expression Console Software (AGCC; Version 3.2 and Expression Console; Version 1.2).

Microarray Data Normalization

Probe intensity data from all Rat Genome 230 version 2 Affymetrix GeneChip® arrays consisting of 31,099 probe sets were read into the R software environment (<http://www.R-project.org>) directly from .CEL files using the R/affy package¹⁶. Probe-level data quality was assessed using image reconstruction, box plots of raw signal intensities, and histograms of raw signal intensities. Normalization was carried out using the robust multi-array average

(RMA) method using all probe intensity data sets together¹⁷. Briefly, the RMA method adjusts the background of perfect match (PM) probes, applies a quantile normalization of the corrected PM values, and calculates final expression measures using the Tukey median polish algorithm. RMA scatterplots were used as an additional quality control measure.

Statistical Assessment of Differential Gene Expression

Statistical contrasts were used to find pairwise gene expression differences between the control group and each dose group using the R/Maanova package¹⁸. For each chemical, the model

$$Y_i = \mu + \text{DOSE} + \varepsilon_i \quad (1)$$

was used to fit the log2 transformed gene expression measures Y_i , where μ is the mean for each array, DOSE is a four fold level factor representing the dose effect (control, low dose, medium dose, high dose) and ε_i captures random error for probe set i . The null hypothesis for each test is that the mean of the control samples is equal to the mean of the dose group samples and the alternative hypothesis for each test is that the mean of the control group samples is not equal to the mean of the dose group samples. A total of three different comparisons were tested for each probe set (control vs low dose, control vs middle dose, and control versus high dose). All statistical tests were performed using F_s , a modified F-statistic incorporating shrinkage estimates of variance components¹⁹. P-values were calculated by permuting sample labels 1000 times. In order to reduce the number of false positives, p-values were adjusted for multiple hypothesis testing corresponding to all probe sets on the array using the Benjamin-Hochberg false discovery rate procedure implemented using the `p.adjust()` function in R. This correction controls the expected proportion of errors among the significant results²⁰. Unless otherwise noted, a false discovery rate threshold of 0.05 was used for statistical significance. Log₂ fold changes were calculated by subtracting the control and dose treated (low dose, middle dose, or high dose) relative expression values from model (1) above²¹.

Over-represented gene sets were determined from the gene list obtained above by testing for association with gene pathway relationships (www.ingenuity.com). Enrichment of pathway members among differentially expressed probe sets were assessed using the one-tailed Fisher exact test for 2×2 contingency tables.

Benchmark Dose Analysis

PND 4 liver transcriptomic data were used to calculate the benchmark dose (BMD), and a statistically lower confidence bound BMD (BMDL)²² using BMDEExpress 2.0 (<https://github.com/auerbachs/BMDEExpress-2.0/releases>). We adopted the default settings for BMDEExpress 2.0 except that we used an ANOVA p-value < 0.05 and |fold-change| ≥ 1.5 for defining differentially expressed transcripts. Biological Process Gene Ontology terms were used for gene set definitions. BMDEExpress 2.0 is an updated version of BMDEExpress^{23,24}. Reference doses for the altered GO Biological Process Category pathways (<http://geneontology.org/page/go-enrichment-analysis>) were based on liver microarray data from exposed animals. All BMD calculations were performed within the BMDEExpress

framework. First, a classical one-way ANOVA was used to filter the probe list to find transcripts that were differentially expressed across dose groups with a p-value < 0.05 and |fold-change| ≥ 1.5. Next, BMD statistics were calculated for each probe set. When more than one probe set mapped to the same Entrez ID, the BMD values were averaged across probe sets to obtain a single value for each Entrez ID and the GO analyses were performed on a gene-specific basis. The program returned a range of summary exposure levels (mg/kg/day) representing the central tendencies and variability of BMD (chemical exposure level (mg/kg/day) estimated to result in a 10% extra risk of disease) and BMDL (95% lower bound on BMD) values.

Analysis of Body and Organ Weights and Thyroid Hormone Levels

Thyroid hormone (T4) data, which typically have skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley²⁵ and Dunn²⁶. Jonckheere's test (Jonckheere, 1954) was used to assess the significance of dose-response trends and to determine whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose-response (Dunnett's or Dunn's test). Trend-sensitive tests were used when Jonckheere's test was significant at p<0.01.

Prior to analysis, extreme values identified by the outlier test of Dixon and Massey²⁷ were examined. Implausible values, extreme values from animals that were suspected of being sick due to causes other than treatment, and values that the laboratory indicated as being inadequate due to measurement problems were eliminated from the analysis.

Analysis of Pregnancy Outcomes

Trends in fertility indices across dose groups were tested using Cochran-Armitage trend tests. Pairwise comparisons of each dosed group with the control group were conducted using Fisher's exact test. Litter size across dose groups was compared to the control group using the nonparametric multiple comparison methods of Shirley (1977) and Dunn (1964). Jonckheere's test²⁸ was used to assess the significance of dose-response trends and to determine whether the trend-sensitive test (Shirley's test) was more appropriate for pairwise comparisons than the test that does not assume a monotonic dose-response (Dunn's test). Shirley's test was used when Jonckheere's test was significant at p<0.01.

Results

Pregnancy Outcome

There were no treatment-related PBDE-47 or PBDE-mixture effects on fertility, litter size, or male or female pup body weights²⁹.

PBDE-47, 99, and 153 Plasma Levels

After PBDE-mixture exposure, pup plasma levels of PBDE-47, -99, and -153 were measured, and after PBDE-47 exposure, pup PBDE-47 plasma levels were measured. PBDE congener levels increased with increasing dose of the chemical in pups of each sex (Table 1 and Figure 1). The amount of PBDE 47 and PBDE 99 in the PBDE mixture administered to

the dam was ~10–12 times the amount of PBDE 153, while in pup plasma the amount of PBDE 47 and PBDE 99 was ~2 times the amount of PBDE 153. Thus, after PBDE mixture exposure, PBDE-153 was present in pup plasma at a higher level than predicted based on the level in the administered dose.

The plasma levels of PBDE-47 after exposure to either PBDE-47 or PBDE mixture (containing 36% PBDE-47) were approximately equivalent based on the amount of PBDE-47 given to the animal (for the 50, 15, or 0.1 mg/kg dose of PBDE-mixture, the PBDE-47 dose was approximately 17.85, 2.67, or 0.0357 mg/kg, respectively).

Liver Histopathology

Cytoplasmic vacuolization was diagnosed in male and female pups at 15 and 50 mg/kg after PBDE mixture (DE-71) exposure (Table 2). Affected hepatocytes contained primarily irregularly shaped vacuoles separated by strands of cytoplasm, but there were also fewer discrete round vacuoles consistent with lipid. Vacuolization caused enlargement of many of the hepatocytes. Grading was done using a four grade system, with 1 being minimal, 2 mild, 3 moderate, and 4 severe. The severity grade was based upon the number of hepatocytes with the change, and the amount of cytoplasmic vacuolization within the affected hepatocytes, with none of the animals in this study having more than a mild degree of vacuolization. Severity grading was subjective, but in general, animals with minimal vacuolization had approximately 25% or fewer of the hepatocytes affected, while animals with mild vacuolization had 50% or fewer, but more than 25% of the hepatocytes affected. No treatment-related liver lesions were seen after PBDE-47 administration.

Thyroid Hormone Levels

Total T4 levels were significantly decreased in dams treated with 15 and 50 mg/kg PBDE mixture, and in dams treated with 50 mg/kg PBDE-47 (Table 3). At PND 4, T4 was not detected or was below level of detection in male pups for the 15 or 50 mg/kg exposure to PBDE-47 or male or female pups with 50 mg/kg exposure to PBDE mixture (Table 3).

Microarray Analysis

There were 139 and 161 significantly changed levels of liver transcripts compared to controls ($FDR < 0.05$) at 50 mg/kg PBDE-47 or PBDE mixture, respectively (Table 4; Supplement 1 and 2). In addition, there were 40 and 13 significantly changed levels of liver transcripts compared to controls ($FDR < 0.05$) at 15 mg/kg of PBDE-47 or PBDE mixture exposure, respectively. The majority of these changed transcripts were upregulated. Canonical pathways changes after PBDE exposures included those of liver metabolic functions and Nrf2 antioxidant systems (Table 5 & 6). In addition, various glucuronosyl transferases were upregulated in the 15 mg/kg or 50 mg/kg groups. Membrane transport transcripts were upregulated after exposure to PBDE-47 or PBDE mixture, including ATP binding cassette members and solute carrier transcripts (Table 6).

Benchmark Dose Analysis

Benchmark dose analysis of the liver transcript data was performed for PBDE-47 (Supplement 3) and PBDE mixture (DE-71) (Supplement 4) using GO Biological Process

categories. Selected BMDL levels are reported in Table 7, which show GO categories with 5 genes (and 5% of the genes in the category) that pass the selection criteria in BMDExpress 2.0 (p value, ≤ 0.05 and $|FC| > 1.5$). The lowest BMDLs for PBDE-47 exposure were 0.04 mg/kg and for PBDE mixture exposure somewhat higher (in the range of 3 – 4 mg/kg). The lower BMDL levels with PBDE-47 may have been due in part to the finding that there were more significant liver transcripts at 15 mg/kg than there were with the PBDE mixture.

Discussion

We hypothesized that certain health risks from environmental exposures can be identified in neonates after in utero exposures, including decreased thyroid hormone levels, and adding neonatal time points to toxicity studies is key to evaluating risks to humans (Figure 2). Pentabromodiphenyl ethers (PBDEs) are structurally similar to thyroxine (T4) and can interfere with thyroid homeostasis and lower T4 levels³⁰. Low thyroid hormone levels have been associated with liver disease^{31–33} and progression of liver lesions to liver cancer^{34–37}. With long-term PBDE mixture exposure, hepatic cancers occur in rodent model systems¹⁰. Here we report that PBDE-47 or PBDE mixture (DE-71) exposure to the rat dam leads to decreased thyroxine (T4) levels and hepatic transcript change in PND 4 rat pups. Pups do not have a fully functional thyroid system and rely on the dam for supplying sufficient quantities of this hormone³⁸.

Along with decrease in T4 levels there was upregulation of glucuronosyltransferase (UGT) transcripts, enzymes that facilitate the glucuronidation and excretion of thyroid hormones^{39,40}. The changes in liver transcriptomic patterns also included increase in metabolic cytochrome p450 transcripts and Nrf2 antioxidant pathway transcripts. Many of these hepatic transcript expression changes were similar to those seen at PND 22²⁹ after both PBDE-47 and PBDE mixture exposure, and at thirteen weeks after PBDE mixture exposure⁴¹.

The hepatic transcript changes were indicative of key characteristics of carcinogens⁴², including increases in metabolic enzyme systems which can metabolize PBDEs to oxygenated metabolites capable of toxicity and free radical formation^{43–45}. The cytochrome P450 transcript changes included those usually expressed in the liver (e.g. *Cyp2C8*, *Cyp2C9*, *Cyp1A2*), as well as those less abundant in the liver (e.g. *Cyp2A6*, *Cyp2B6*, *Cyp2C19*, *Cyp3A5*)^{36,37}. Activation of the CAR receptor is associated with upregulation of *Cyp2Bs* and *UGTs*⁴⁶. *Cyp1a* can be induced by both AhR independent and dependent events⁴⁷. There are continuing investigations as to how CAR activation in rodent studies can translate to humans^{48,49}.

Hydroxylated PBDE metabolites have been shown to cause oxidative stress in various test systems^{50–52}. In addition, PBDEs may be converted to quinone metabolites capable of forming DNA adducts⁵³. In this study, the early disease biomarkers of oxidative stress included upregulation of the Nrf2 antioxidant pathway transcripts⁵⁴, an adaptive pathway to combat oxidative stress⁵⁵. Oxidative stress can contribute to liver disease development⁵⁶, and may be one factor in PBDE-induced liver toxicity¹⁰. PBDE and its metabolites are

excreted as glucuronide conjugates^{57–62}, and at PND 4, this glucuronide enzyme activity may not be fully developed^{63–65}.

Upregulation of membrane transport transcripts included changes in transcript levels for the *ABCC3* and *ABCB1* genes (also known as MRP3 and MDR1, respectively) after both PBDE-47 and PBDE mixture exposures. These ABC transporters are members of a family of efflux pumps which transport chemicals and drugs across the hepatocyte membrane, and provide some protection from xenobiotic exposure⁶⁶. Lipid binding to these membrane transporters can affect the conformation of the pump to an abnormal open or closed state leading to disruption of homeostasis^{67,68}. Other investigators have also found that brominated chemicals can affect the expression level of membrane transcripts^{69,70}.

Other treatment-related hepatic transcript changes included upregulation of selected SLC transporters, including upregulation of *SLC35E3* (a sugar transporter⁷¹), *SLC4A4* (a sodium bicarbonate transporter⁷²), *SLC22A1* (an organic cation transporter⁷³), and/or *SLC26A1* (an anion transporter⁷⁴). Changes in membrane protein transcripts provide evidence that PBDE exposures alter critical membrane transport systems that govern cellular content and function.

Down regulation of the lipid efflux pump transcripts *ABCG5* and *ABCG8* was observed at PND 22²⁹ but not at PND 4. At PND 4, there was little evidence for liver lipid accumulation after PBDE exposure, and pups do not have much fat at this time point in development^{75,76}. Thus, at PND 4, the need for lipid efflux pumps is probably not required to maintain homeostasis. Low thyroid hormone levels can lead to accumulation of lipids in the liver^{31–33}, but apparently at PND 4 this is less evident than at PND 22²⁹.

Minimal hepatic centrilobular vacuolization occurred only with the PBDE mixture suggesting that components in the mixture (other than PBDE-47) may be responsible for this lesion. There are reports in the literature that PBDE-99, present in the mixture, may have some transient effects not found with PBDE-47^{77–79}, and this may account for the hepatic lesions with the PBDE mixture exposure. However, after longer term PBDE-47 exposure hepatic toxic lesions occur in rats²⁹.

PND 4 hepatic transcript patterns were used to determine the benchmark dose for PBDE-47 and PBDE mixture. Examination of the results indicated that the PBDE-47 congener had a lower benchmark dose than found with the PBDE mixture. This may be related to the finding that there were more significant hepatic transcript changes after PBDE-47 exposure than after PBDE mixture exposure at the lower exposure levels.

Thyroid hormone levels after PBDE exposure can serve as a translational bridge for PBDE effects, because decreases in T4 levels have been reported in both animals and humans after PBDE exposures^{80,81}. Although not studied here, decreases in T4 levels can also lead to development toxicity including decreased neuron formation and maturation^{82,83}.

A goal of 21st century toxicology studies is to “refine, reduce, and replace” the use of animals in toxicology and risk assessment studies⁸⁴. Here we used fewer animals than in traditional rodent toxicology studies, and showed that PND 4 rat liver transcriptomic

patterns provide early indicators of toxicity and/or processes involved in pathways to cancer⁴². These early disease biomarkers may be used to prioritize chemicals for a more complete cancer and risk evaluation. Because PBDE-47 PND 4 hepatic transcript patterns were similar to those after PBDE mixture exposure, we would predict that this PBDE congener would also be a liver carcinogen with longer-term exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
PBDE levels in plasma in PND 4 rat pups

Figure 2.
Summary of PBDE-induced toxicity

Table 1.

Concentration of PBDE congeners in plasma of PND 4 pups exposed to PBDE-47 or PBDE mixture (DE-71)

Dose (mg/kg)	0	0.1	15	50
PBDE-47	Analyte (ng/mL)			
Male pup				
PBDE-47	0 ± 0 [6] ^{a, **}	35 ± 8 [6] ^{**}	6,976 ± 1,166 [5] ^{**}	19,367 ± 2,300 [6] ^{**}
Female pup				
PBDE-47	3 ± 3 [6] ^{**}	47 ± 10 [6] ^{**}	7,243 ± 907 [6] ^{**}	21,317 ± 2,393 [6] ^{**}
PBDE-mixture				
Male pup				
PBDE-47	0 ± 0 [6] ^{**}	4 ± 2 [6] [*]	2,973 ± 579 [6] ^{**}	8,803 ± 971 [6] ^{**}
PBDE-99	0 ± 0 [6] ^{**}	4 ± 2 [6]	3,297 ± 553 [6] ^{**}	11,073 ± 1,530 [6] ^{**}
PBDE-153	0 ± 0 [6] ^{**}	0 ± 0 [6]	1,361 ± 220 [6] ^{**}	4,617 ± 718 [6] ^{**}
Female pup				
PBDE-47	0 ± 0 [6] ^{**}	2 ± 1 [5] [*]	2,616 ± 885 [7] ^{**}	9,674 ± 1,261 [5] ^{**}
PBDE-99	0 ± 0 [6] ^{**}	3 ± 3 [5]	2,729 ± 884 [7] ^{**}	12,900 ± 2,134 [5] ^{**}
PBDE-153	0 ± 0 [6] ^{**}	0 ± 0 [5]	1,133 ± 330 [7] ^{**}	5,660 ± 784 [5] ^{**}

^aMean ± SEM [number of animals per group, 5–7 animals]^{**}P<0.01 (trend test or pairwise comparison between the control and a dose group)

Values below the limit of detection (LOD) were substituted with ½ the LOD

LOD: PBDE-47, 2 ng/mL; PBDE-99, 10 ng/mL; PBDE-153, 20 ng/mL

Table 2.

Hepatic lesions at PND 4 after PBDE mixture (DE-71) Exposure

	Female pups			
	0 mg/kg	0.1 mg/kg	15 mg/kg	50 mg/kg
No. Examined	14	13	23	23
No significant findings	14	13	17	13
Centrilobular vacuolization	0	0	6 (1.0) ^a	10 (1.3)
	Male pups			
	0 mg/kg	0.1 mg/kg	15 mg/kg	50 mg/kg
No. Examined	16	16	21	30
No significant findings	14	16	12	17
Centrilobular vacuolization	2 (1.0)	0	9 (1.1)	13 (1.2)

^a Average severity grading was done using a four grade system, with 1 being minimal, 2 mild, 3 moderate, and 4 severe. The severity grade was based upon the number of hepatocytes with the change, and the amount of cytoplasmic vacuolization within the affected hepatocytes. Only the affected animals are included in the average severity score for the lesions.

Table 3.

Total thyroxine (T4) levels^a in dams or PND 4 pups exposed to PBDE-47 or PBDE mixture (DE-71)

BDE-47 (mg/kg):	0	0.1	15	50
Dams	3.62 ± 0.22 [7] ^{b, **}	3.94 ± 0.12 [8]	3.59 ± 0.13 [8]	2.89 ± 0.05 [8] ^{**}
Male pup	1.14 ± 0.16 [8]	1.18 ± 0.13 [8]	0 ^d	0
Female pup	1.43 ± 0.15 [8]	1.25 ± 0.13 [8]	0.67 ± 0.12[8] ^{**}	1.14 ± 0.13 [8]
PBDE-mixture				
Dams	4.37 ± 0.13 [8] ^{**}	4.38 ± 0.24 [8]	3.48 ± 0.16 [8] ^{**}	3.04 ± 0.30 [8] ^{**}
Male pup	0.96 ± 0.15 [8]	1.51 ± 0.15 [8]	0.59 ± 0.11 [8]	0
Female pup	1.35 ± 0.22 [8]	1.35 ± 0.19 [8]	0.68 ± 0.22 [7]	0

^aSerum from dams and plasma from pups

^bMean ± standard error [number of animals]

^{**}P<0.01 (trend test or pairwise comparison between the control and a dose group)

^dGroups with 80% of values below the limit of detection were removed from the analysis and indicated with a "0" in the table, where the limit of detection was .76 ug/dL.

Number of hepatic transcripts up- or down-regulated after PBDE-47 or PBDE mixture (DE-71) exposure at PND 4 in male pups

Table 4.

	PBDE-47 (mg/kg)			PBDE-mixture (DE-71) (mg/kg)		
	0.1	15	50	0.1	15	50
Total Significant transcripts	2	40	139	0	13	161
Up-regulated	0	36	106	0	13	139
Down-regulated	2	4	33	0	0	22

Number of transcripts significantly changed at a false discovery rate < 0.05 based on gene mapping using Ingenuity excel files

Table 5.

Top Ingenuity Pathways for PBDE-47 or PBDE mixture (DE-71) at 50 mg/kg at PND 4

Top Canonical pathways	PBDE-47		PBDE mixture (DE-71)	
	P value	# significant transcripts/total number in pathway	P value	# significant transcripts/total number in pathway
Nicotine degradation III	3.83E-16	12/56 (21.4%)	1.04E-20	15/56 (26.6%)
Melatonin Degradation I	2.71E-15	12/65 (18.5%)	1.26E-19	15/65 (23.1%)
Nicotine Degradation II	2.71E-15	12/65 (18.5%)	1.26E-19	15/65 (23.1%)
Xenobiotic Metabolism Signaling	1.45E-13	18/299 (6.0%)	4.32E-17	22/29 (7.4%)

Data taken from Ingenuity analysis

Table 6. Selected PND 4 hepatic transcripts following PBDE-47 or PBDE mixture (DE-71) exposure in PND 4 male pups

Symbol Affymetrix No.	PND 4 Expression fold change						
	PBDE-47	PBDE-47	PBDE-47	PBDE Mixture	PBDE Mixture	PBDE Mixture	PBDE Mixture
Dose mg/kg	0.1	15	50	0.1	15	50	50
Metabolic Enzymes							
CYP1A1 1370269_at	-1.07	5.66	10.98	2.68	59.69	91.30	
Cyp1A2 1387243_at	1.31	2.26	2.09	2.06	13.88	53.83	
CYP2A6 1369136_at	-1.18	5.33	7.17	1.72	6.99	16.94	
CYP2B6 1371076_at	1.16	3.98	4.40	1.36	3.41	4.03	
CYP2C8 1370241_at	-1.28	5.73	9.26	1.30	5.53	11.15	
CYP2C19 1370580_a_at	-1.10	3.34	3.49	1.50	4.34	4.89	
CYP3A5 1387118_at	-1.17	1.62	1.71	1.13	1.60	1.78	
POR 1387109_at	-1.25	1.48	1.76	1.11	1.69	2.49	
Nrf2							
AKR7A2 1367843_at	-1.04	1.17	1.44	1.10	1.18	1.47	
DNAJA3 1378392_at	-1.02	1.22	1.28	1.14	1.32	1.54	
EPHX1 1387669_a_at	-1.04	1.78	2.28	1.18	1.80	2.77	
GSTAI 1368180_s_at	-1.01	1.80	2.27	1.34	1.95	3.38	
GSTA3 1371089_at	1.29	1.45	1.76	1.19	1.43	1.78	
GSTM5 1386985_at	1.06	1.27	1.70	1.21	1.40	2.44	

Symbol Affymetrix No.	PND 4 Expression fold change					
	PBDE-47	PBDE-47	PBDE-47	PBDE Mixture	PBDE Mixture	PBDE Mixture
<i>GSTT2/GSTT2B</i> 1368409_at	-1.03	1.31	1.45	1.20	1.23	1.74
<i>PRDX1</i> 1367613_at	-1.01	1.09	1.26	1.06	1.11	1.21
<i>NQO1</i> 1387599_a_at	-1.34	1.05	1.55	1.76	3.16	7.19
Conjugating Enzymes						
<i>UGT2A1</i> 1369850_at	-1.04	1.46	1.63	1.01	1.50	1.98
<i>UGT2B10</i> 1380278_at	1.09	2.66	2.94	1.19	2.65	3.88
<i>UGT2B17</i> 1370698_at	-1.12	1.78	1.74	1.24	1.93	2.05
<i>Ugt2b17</i> 1387955_at	-1.30	1.67	1.64	1.33	1.81	2.06
<i>UGT2B11</i> 1381852_at	1.06	1.30	1.56	1.03	1.69	2.47
<i>UGT2B7</i> 1368397_at	-1.02	1.27	1.24	1.04	1.35	1.41
Membrane Function						
<i>ABCC3</i> 1369698_at	-1.02	2.36	2.83	1.35	2.32	3.76
<i>ABCB1</i> 1370464_at	-1.07	1.41	1.87	1.20	2.28	3.10
<i>ABCG5</i> 1369455_at	1.19	-1.04	-1.06	1.10	1.16	1.29
<i>ABCG8</i> 1369440_at	1.15	-1.02	1.11	1.22	-1.04	-1.02
<i>SLC35E3</i> 1389391_at	-1.10	1.09	1.38	1.08	1.07	1.60
<i>SLC4A4</i> 1372479_at	-1.43	-1.38	-1.40	1.18	1.06	1.77
<i>SLC22A1</i> 1368191_a_at	-1.03	1.53	1.68	1.12	1.26	1.56
<i>SLC26A1</i> 1368600_at	1.16	1.32	1.60	1.05	1.28	1.43

Symbol Affymetrix No.	PND 4 Expression fold change					
	PBDE-47	PBDE-47	PBDE-47	PBDE Mixture	PBDE Mixture	PBDE Mixture
<i>MMGT1</i> 1395325_s_at	-1.05	1.744	2.28	1.23	1.91	2.37
<i>MMGT1</i> 1376168_at	-1.12	1.66	2.09	1.16	1.89	2.14
<i>TMM62</i> 1377854_at	-1.11	1.00	1.23	1.17	1.27	1.61
<i>VMP1</i> 1370807_at	-1.1	-1.02	1.00	1.06	1.31	1.54
<i>TMM10B</i> 1368977_a_at	-1.05	1.21	1.34	1.22	1.04	1.51

Red bolded numbers are significant (FDR <0.05)

Table 7.

Selected mean BMDL levels for PBDE-47 or PBDE mixture based on GO categories using hepatic transcriptomic data in PND 4 male pups

GO Accession Number Column C	GO Name Column N	# Genes in Category Column D	# significant genes Column F	P Value Column K (two Tail fisher test)	Gene Names Column N	Mean BMDL (mg/kg) Column W	BMDL SD (column Z)
PBDE-47							
GO:001607	alpha-amino acid biosynthetic process	56	5	3.73E-04	Thns12;Ass1;Got1;Asl;Oat	0.04	0.04
GO:000652	cellular amino acid biosynthetic process	60	5	5.12E-04	Thns12;Ass1;Got1;Asl;Oat	0.03	0.04
GO:000606	alpha-amino acid catabolic process	76	5	0.002	Thns12;Scly;Dao;Got1;Oat	0.04	0.03
GO:0006063	cellular amino acid catabolic process	86	5	0.003	Thns12;Scly;Dao;Got1;Oat	0.04	0.03
GO:000805	xenobiotic metabolic process	72	11	4.72E-09	Cyp2c22;Aox1;Cyp1a2;Gstm7;Gstm1;RGDI559459;Gstm4;Ugt2b1;Cyp2c6v1;Cyp1a1;Cyp2a3	3.6	6.9
PBDE mixture							
GO:0046686	response to cadmium ion	67	6	0.0016896	Cyp1a2;Cyp3a23/3a1;Gsn;Abcb1a;Cyp2a3;Gsr	3.33803445	7.92746436
GO:004738	exogenous drug catabolic process	27	6	2.75E-05	Cyp2b21;Cyp2c22;Cyp1a2;Cyp2c6v1;Cyp2a1;Cyp2a3	3.4843736	8.27368215
GO:0007164	ammonium ion metabolic process	129	6	0.031596	Gpld1;Sat2;Akr1c14;Apoa4;Por;Ddc	4.26653035	5.21719128
GO:0070988	demethylation	47	5	0.0016234	Cyp1a2;Cyp3a23/3a1;Cyp3a9;Cyp1a1;Por	5.45549956	7.99410794
GO:0010595	positive regulation of endothelial cell migration	85	5	0.020008	Gpld1;Nus1;Amod1;Fgfbp1;Nfe2l2	5.59333146	6.64258719

GO Accession Number Column C	GO Name Column N	# Genes in Category Column D	# significant genes Column F	P Value Column K (two Tail fisher test)	Gene Names Column N	Mean BMDL (mg/kg) Column W	BMDL SD (column Z)
GO:0019748	secondary metabolic process	46	10	2.32E-08	Cyp1a2;Gsta3;Akr1c14;Cyp1a1;Cyp2a1;Cyp2a3;Cyp1b1;Abcc2;Ddc;Nfe2l2	6.33544674	7.82698162
GO:0009404	toxin metabolic process	22	6	4.15E-06	Cyp1a2;Gsta3;Cyp1a1;Cyp1b1;Ddc;Nfe2l2	6.45137688	8.99400848
GO:0072593	reactive oxygen species metabolic process	80	7	0.0013105	Nqo1;Cyp1a2;Txnrd1;Cyp1a1;Cyp1b1;Apoa4;Por	6.70501753	7.88449485
GO:001655	cellular response to ketone	134	7	0.015629	Ugt2b17;Ugt2b1;Abcb1a;Efna5;Ahr;Cyp1b1;Abcc2	7.12729101	9.06913161
GO:0006720	isoprenoid metabolic process	78	5	0.015112	Cyp1a2;Nus1;Cyp2c6v1;Cyp1a1;Cyp1b1	7.21628912	9.76807372
GO:0006805	xenobiotic metabolic process	72	13	1.03E-08	Cyp2b21;Cyp2c22;Sult1b1;Cyp1a2;Gstm1;RGD1559459;Gsta4;Ugt2b1;Cyp2c6v1;Cyp1a1;Cyp2a1;Cyp2a3;Cyp1b1	7.32327662	9.36025023

^aRepresents categories with 5 genes (and 5% of the genes in the category) that pass the selection criteria in BMDExpress 2.0 (p value 0.05 and FC > 1.5)