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PBDE-47 and PBDE mixture (DE-71) toxicities and liver transcriptomic changes at PND 22 after in utero/postnatal exposure in the rat

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Abstract

Pentabromodiphenyl ethers (PBDE) are found in human tissue, in household dust, and in the environment, and a particular concern is the potential for the induction of cancer pathways from these fat-soluble persistent organic pollutants. Only one PBDE cancer study has been conducted and that was for a PBDE mixture (DE-71). Because it is not feasible to test all PBDE congeners in the environment for cancer potential, it is important to develop a set of biological endpoints that can be used in short-term toxicity studies to predict disease outcome after long-term exposures. In this study, PBDE-47 was selected as the test PBDE congener to evaluate and compare toxicity to that of the carcinogenic PBDE mixture. The toxicities of PBDE-47 and the PBDE mixture were evaluated at PND 22 in Wistar Han rat (CrI: WI (Han)) pups after in utero/postnatal exposure (0, 0.1, 15, or 50 mg/kg; dams, GD6-21; pups, PND 12-PND 21; oral gavage daily dosing). By PND 22, PBDE-47 caused centrilobular hypertrophy and fatty change in liver, and reduced serum thyroxine (T₄) levels; similar effects were also observed after PBDE mixture exposure. Transcriptomic changes in the liver included induction of cytochrome p450 transcripts and up-regulation of Nrf2 antioxidant pathway transcripts and ABC membrane transport transcripts. Decreases in other transport transcripts (*ABCG5* & *8*) provided a plausible mechanism for lipid

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accumulation, characterized by a treatment-related liver fatty change after PBDE-47 and PBDE mixture exposure. The benchmark dose calculation based on liver transcriptomic data was generally lower for PBDE-47 than for the PBDE mixture. The up-regulation of the Nrf2 antioxidant pathway and changes in metabolic transcripts after PBDE-47 and PBDE mixture exposure suggest that PBDE-47, like the PBDE mixture (NTP 2016, TR 589), could be a liver toxin/carcinogen after long-term exposure.

Keywords

Pentabrominated diphenyl ethers; PBDE-47; PBDE mixture (DE-71); Liver transcriptomic patterns; Liver toxicity

Introduction

The pentabrominated diphenyl ethers (PBDE) were extensively used as additive flame retardants until they were phased out beginning in 2004. Mixtures of PBDE congeners were added to furniture containing polyurethane foam and other consumer goods at levels up to 2–7% (Agency for Toxic Substances and Disease Registry 2017). Because PBDEs were not covalently bound to base polymers in these products, the PBDEs could leach into the environment (Wu et al. 2011). The PBDE are hydrophobic, lipophilic, have a low vapor pressure and a high log K_{ow} , common characteristics of biopersistent organic chemicals (US Environmental Protection Agency 2008a, b, c). PBDEs have been shown to bioaccumulate in the environment (McGrath et al. 2017; Wang and Kelly 2017) and are found in human tissue (Sjodin et al. 2014). Although the PBDE congeners are no longer used as flame retardants, the potential for exposure remains a concern because of their widespread presence in the environment and the long half-life of PBDEs in humans. PBDEs are found in household dust, foods, and air (Chevrier et al. 2016; Herbstman and Mall 2014; Sethi et al. 2017; US Environmental Protection Agency 2010a) and can cross the placenta (Dassanayake et al. 2009) and partition into breast milk (Fang et al. 2015; Schecter et al. 2006). PBDE exposures are of concern, because PBDE exposures in humans have been linked to thyroid toxicity (Makey et al. 2016) and neurotoxicity (Lam et al. 2017).

DE-71 is an additive flame retardant composed of a mixture of primarily low molecular weight PBDE congeners. This PBDE mixture (DE-71) was found to be a liver carcinogen in both sexes of rats and mice in a 2-year study (National Toxicology Program 2016); however, the potential carcinogenicity of the individual PBDE congeners in this mixture has not been evaluated. Because it is not feasible to test all the PBDE congeners in long-term rodent carcinogenicity studies, we sought to identify common biologic changes following in utero/postnatal exposure of rats to the congener PBDE-47, or to the PBDE mixture that could be used to predict long-term toxicity and carcinogenicity. Use of early biologic changes that can be used to predict disease outcomes (Strimbu and Tavel 2010) could be used in the risk assessment process and in setting guidelines for exposure (Consumer Product Safety Commission 2017). PBDE-47 was used as the test congener for these studies, because it is a major component of the DE-71 mixture (37%), and is the most prevalent PBDE congener in human tissues and in the environment (Sjodin et al. 2014).

In this study, liver histopathology, serum thyroid hormone and lipid levels, and liver transcript patterns were evaluated in dams and postnatal day (PND) 22 pups following in utero/gestational exposure to PBDE-47, the PBDE mixture, or two known liver carcinogens, phenobarbital and PCB-126. PBDE-induced thyroid hormone level changes have been reported in both humans and rodents and could affect normal fetal and infant development (Hallgren and Darnerud 2002; He et al. 2011; Richardson et al. 2008; Staskal et al. 2006a, b; Suvorov et al. 2009; Talsness et al. 2008). Thyroid hormone changes can predispose to neurotoxicity (Giordano and Costa 2012), reproductive toxicity (Eskenazi et al. 2017; Harley et al. 2017; Vuong et al. 2017; Zhang et al. 2017a) and cancer (Sinha et al. 2014). We evaluated liver transcriptomic patterns to characterize and compare the toxicity of PBDE-47 to that of the PBDE mixture to help predict long-term toxicity and the likelihood for development of carcinogenic processes. The liver transcript data were used for preliminary benchmark dose risk analysis as has been done for other chemicals that cause liver toxicity and cancer (Dunnick et al. 2017). This information on PBDE congener toxicity can be used in developing guidelines for flame retardant content in consumer products (Consumer Product Safety Commission 2017).

Methods

Chemicals

PBDE-47 (2,2',4,4'-tetrabromodiphenyl ether; CAS# 5436-43-1) was obtained from Cerillant Corp. (Round Rock, TX). The purity for PBDE-47 was 99.5% by gas chromatography analysis using flame ionization detection GC/FID and 99.3% by high-pressure liquid chromatography with photo-diode array (PDA) detection (HPLC/PDA). There were no quantifiable polybrominated dibenzodioxins or furans present in the PBDE-47 sample. PBDE-47 dose formulations were prepared in corn oil to provide oral gavage doses of 0, 0.1, 15, and 50 mg/kg. A high dose of 50 mg/kg was selected to make doses comparable to those used in the DE71 carcinogenicity study, where treatment-related liver tumors occurred in male and female Wistar Han rats without an effect on survival throughout most of the 2-year study at 50 mg/kg. The lower doses were added for benchmark dose analysis for treatment-related transcript changes.

A PBDE mixture (DE-71, technical pentabromodiphenyl; CAS# 32534-81-9) was obtained from Great Lakes Corporation (West Lafayette, IN; Lot 2550OA30A). 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 PBDE85 (2%); low levels of polybrominated dibenzodioxins and furans were also identified (approximately 7×10^{-6} % by weight). DE-71 dose formulations were prepared in corn oil to provide oral gavage doses of 0, 0.1, 15, and 50 mg/kg (Dunnick and Nyska 2009). The high dose of 50 mg/kg was selected, because at this dose in the DE-71 carcinogenicity study, there were treatment-related liver tumors in male and female Wistar Han rats without an effect on survival throughout most of the 2-year study. Lower doses were added for benchmark dose analysis for treatment-related gene transcript changes.

Phenobarbital free acid (CAS No. 50-06-6) was obtained from Spectrum Chemical Manufacturing Corp. (New Brunswick, NJ). The purity of 99% was confirmed by HPLC

with UV detection. Dose formulations of phenobarbital were prepared in corn oil to provide oral gavage doses of 0, 5, and 45 mg/kg. When phenobarbital was administered at 40 mg/kg to pregnant Sprague–Dawley (CD) rats from day 12 to day 19 of gestation, there was suppression of body weight gain and delay in onset of puberty but no effect on survival of pups (Gupta et al. 1980). Phenobarbital caused cancer in Wistar Han rats when given in the drinking water at 500 mg/L water (~ 500 ppm or ~ 0.5% in water or 30 mg/kg body weight) from 7 weeks of age to up to 152 weeks of age (IARC 2001).

PCB-126 (3,3',4,4',5-pentachlorobiphenyl; CAS# 57465-28-8) was obtained from AccuStandard, Inc. (New Haven, CT). The purity of PCB-126 was 99.5% pure. A purity profile using a GC/high-resolution/mass spectrometry (GC/HR/MS) system detected four impurities. The impurities had a relative area of 0.49%. Two of the impurities were identified as tetrachlorobiphenyls and one of the impurities was identified as a pentachlorobiphenyl. The remaining impurity could not be identified. This impurity did not contain any of the characteristics of dioxins, furans, or PCBs and was most likely a mixture of compounds. Analysis of the GC/HR/MS data for any possible PCBs, furans, or dioxins did not identify any possible impurities other than those found in the purity profile. PCB-126 dose formulations were prepared in corn oil to provide oral gavage doses of 0, 10, 50, and 250 ng/kg. PCB-126 has a toxic equivalency factor (TEF) of 0.1 (National Toxicology Program 2006b; US Environmental Protection Agency 2010b). The selected PCB-126 doses were 10× comparable TCDD doses. PCB-126 exposure over this dose range caused no significant effects on littering and was carcinogenic in the NTP Sprague Dawley rat bioassay (National Toxicology Program 2006a).

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). The NIH 07 diet (Ziegler Brothers, Inc., Gardners, PA) was given to dams and pups throughout the pregnancy and gestation periods and until weaning. Tap water and NIH-07 diet were available ad libitum. The care of animals on this study was according to NIH procedures as described in the US 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

Dams were dosed by gavage with either PBDE-47, PBDE mixture, PCB-126, or phenobarbital in corn oil (dosing volume 5 mL/kg) from GD 6 through postnatal day (PND) 21, 7 days/week. Controls received corn oil vehicle. Pups were dosed by gavage with the same chemical at the same dose as their dam starting on PND 12 and continuing through PND 21, 7 days/week until weaning on PND 21. On PND, 4 litters were standardized, so each litter contained 6 pups (3 males and 3 females). Dams having litters with fewer than 5 pups/litter were euthanized (CO₂) and blood harvested for serum collection.

On PND 22 all dams and pups were euthanized by CO₂ asphyxiation. Blood was collected from dams (cardiocentesis) for serum thyroid hormone and lipid analyses and plasma for

PBDE tissue-level determinations. One male and one female pup, randomly selected from 6 to 9 different litters of each dose level and all 4 chemicals, were designated as “core” animals. Core pups were weighed and then euthanized (CO₂) on PND 22. Pup blood was collected (cardiocentesis), and plasma isolated and frozen at 80 °C until analyzed for PBDE tissue levels, thyroid hormones, and lipids. In pups, these endpoints were all measured in plasma, because there was insufficient blood volume to allow for both serum and plasma collection. Immediately after blood collection, livers were weighed, and then, one section (~500 mg) from the left lobe was collected in a cryotube and flash frozen and stored at – 80 °C for toxicogenomic analysis. A 5 mm section of liver immediately adjacent was collected and fixed in 10% NBF for histopathology. The remaining PND 22 animals (2/sex/litter) were euthanized and then liver, serum, or plasma collected as described above.

Liver histopathology

At necropsy, sections of liver were removed and fixed in 10% neutral buffered formalin for microscopic evaluation. Additional samples were collected, cubed, and frozen at – 80 °C. After fixation in formalin, 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. Oil Red O staining for the presence of lipid was subsequently done on frozen tissue from selected animals exposed to PBDE-47 or the PBDE mixture (DE-71).

For hepatocellular hypertrophy, minimal severity was recorded when approximately 10–25% of hepatocytes in most of the hepatic lobules were enlarged; mild severity was recorded when approximately 25–50% of the hepatocytes in each lobule were enlarged; moderate severity was characterized by approximate involvement of 50–75% of the hepatic lobule, and with marked severity, the entire lobule was characterized by hypertrophic hepatocytes.

Minimal fatty change usually affected only a small number of hepatocytes (< 10%) and the vacuoles were not numerous enough to fill the cytoplasm or displace the nucleus. Mild fatty change was recorded when there were more affected hepatocytes in each lobule (10–25%) or the change was present in the majority of the hepatic lobules. Oil Red O staining was used to confirm the presence of lipid, but not to quantify the amount of lipid within the liver sections. The amount of lipid within a section of liver was only assessed subjectively.

Quantitation of PBDE-47, 99, and 153 plasma levels

PND 22 pup and dam plasma levels of PBDE-47, 99, or 153 were determined after PBDE mixture (DE71) exposure (0, 0.1, 15, or 50 mg/kg). PND 22 plasma levels of PBDE-47 were determined after PBDE-47 exposure (0, 0.1, 15, and 50 mg/kg). The analytical system used was as previously reported (National Toxicology Program 2016), where for detection of the PBDEs, an Agilent 6890 gas chromatograph (Agilent, Santa Clara, CA) was coupled to an electron capture detector. An RTX®-5 column (30 m × 0.25 mm, 1.0 µm film thickness) (Restek, Bellefonte, PA) was used with a helium carrier gas at a flow rate of 3 mL/min. The oven temperature was held at 210 °C for 2 min and then increased to 330 °C at 8 °C/min and held for 3 min. Injector and detector temperatures were 300 °C and 320 °C, respectively.

Thyroid hormone analyses

Serum samples from dams and plasma samples from pups were analyzed for total triiodothyronine (T3) and 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). Serum samples were analyzed for thyroid stimulating hormone (TSH) using a MILLIPEX MAP Rat Pituitary magnetic bead panel according to the protocols provided by the manufacturer (EMB Millipore Corp., Chicago, IL). All samples were run in duplicate.

Lipid analyses

Serum samples from dams and plasma samples from pups were analyzed for cholesterol and triglycerides using an Olympus AU400e clinical analyzer (Beckman Coulter Inc. Irving, TX).

RNA collection

RNA was extracted from frozen liver samples (approximately 3–4 mm cubes) from the PND 22 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 22 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 µg of amplified biotin-aRNAs were fragmented and 12.5 µg were hybridized to each array for 16 h 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 Gene-Chip 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 was read into the R software environment (<http://www.R-project.org>) directly from CEL files using the R/affy package (Gautier et al. 2004). Probe-level data quality was assessed using image reconstruction, box plots of raw signal intensity, and histograms of raw signal intensities and hierarchical clustering of samples. Normalization was carried out using the robust multi-array average (RMA) method using all probe intensity data sets together (Irizarry et al. 2003). Briefly, the RMA method adjusts the background of perfect match

(PM) probes, applies a quantile normalization of the corrected PM values, and calculates the 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

Analysis of variance (ANOVA) was used to find pairwise gene expression differences between the control group and each dose group using the R/maanova package (Wu et al. 2003). For each chemical, the model

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

was used to fit the log-transformed gene expression measures Y_i , where μ is the mean for each array, DOSE is the dose effect (control, high dose), and ε_i captures random error. All statistical tests were performed using F_s , a modified F-statistic incorporating shrinkage estimates of variance components (Cui et al. 2005). p values were calculated by permuting sample labels 1000 times. These p values were corrected for multiple hypothesis testing using the Benjamin–Hochberg false discovery rate (FDR) procedure implemented using the `p.adjust()` function in *R*.

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

Benchmark dose analysis

PND 22 liver transcriptomic data were used to calculate the benchmark dose (BMD) and a statistically lower confidence bound BMD (BMDL) (Crump 1995) 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 $|\text{fold-change}| \geq 1.5$ 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 (Thomas et al. 2012; Yang et al. 2007). Reference doses for which GO Biological Process Category pathways (<http://geneontology.org/page/go-enrichment-analysis>) were altered 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 altered across dose groups with a p value < 0.05 and $|\text{fold-change}| \geq 1.5$ for statistical significance. 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

Organ and body weight data, which have approximately normal distributions, were analyzed using the parametric multiple comparison procedures of Williams (1971, 1972) and Dunnett (1955). Thyroid hormone data, which typically have skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley (1977) and Dunn (1964). Jonckheere's test (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 Massey (1957) 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 test (1954) 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

Littering parameters and body and liver weights

There were no statistically significant toxic effects of PBDE-47, PBDE mixture (DE-71), PCB-126 or phenobarbital on pup body weights at birth or on littering parameters (Supplement 1). The exception was a significantly higher litter size in the high dose group of PBDE mixture (10.90 ± 0.57) compared to controls (8.22 ± 0.85). Liver weight and liver/body weight ratios were increased in the top two exposure levels with PBDE-47, PBDE mixture, and phenobarbital, and liver body weight ratio was increased in the top exposure level in female pups after PCB-126 exposure (Supplement 1).

PBDE blood levels

Concentrations of the PBDE congeners, PBDE-47, PBDE-99, and PBDE-153, were measured in the plasma of PND 22 pups and in serum of dams after exposure to the PBDE mixture (DE-71) (Table 1). The levels of PBDEs at 15 and 50 mg/kg were proportional to exposure levels and were in general low or not detected in the control and 0.1 mg/kg dose groups. The concentrations (mg/ml) of PBDE-47 were measured in the plasma of PND 22 pups and in serum of dams after exposure to PBDE-47 (Table 2). Plasma levels of the PBDE congeners were similar in male and female pups. Levels of PBDE congeners were higher in

pups than in the dams which were attributed to lower metabolic and excretion capabilities of the pups compared to those in adult animals.

Serum thyroid hormone levels and cholesterol levels

Dose-related decreases in thyroxine (T_4) and triiodothyronine (T_3) levels occurred after PBDE-47 and PBDE mixture exposure in male and female pups at PND 22 (Table 3). T_4 levels in dams exposed to PBDE-47 and PBDE mixture were also significantly decreased at PND 22 (Table 4). The decreases in T_4 were generally greater in pups than in the dams treated with either PBDE-47 or DE-71. For example, T_4 levels at 50 mg/kg DE-71 were 74% of controls for dams and 47–48% of controls for male and female pups. In dams, T_3 levels trended lower after PBDE-47 exposure. There were significant increasing dose-related trends in TSH levels of male and female pups after PBDE mixture exposure, although no dose group was significantly different from the controls. At PND 22, serum cholesterol levels trended upward in male and female pups exposed to PBDE mixture. The levels of triglycerides were not significantly affected by exposure to PBDE-47 or PBDE mixture.

Liver histopathology

PBDE-47 In the liver of PND 22 pups (Table 5), significantly increased incidences of centrilobular hepatocellular hypertrophy and fatty change were recorded in 15 mg/kg and 50 mg/kg males and females (Fig. 1). Hepatocellular hypertrophy was characterized by large hepatocytes, with finely vacuolated or granular appearing cytoplasm (Fig. 1a, b). This change was primarily observed in the centrilobular location and with increasing severity affected more of each individual hepatic lobule. Fatty change was a mix of microvesicular fatty change, characterized by many small vacuoles that gave the cytoplasm a grainy appearance, and macrovesicular fatty change, in which the hepatocytes contained one or more, slightly larger, obvious, discrete vacuoles (Fig. 2). Fatty change was typically of minimal severity. The vacuoles did not fill the cytoplasm or displace the nucleus. Oil Red O staining confirmed the increase in lipid in pups exposed to PBDE-47 compared to control pups (Fig. 2a, b).

PBDE mixture In the liver of PND 22 pups (Table 5), increased incidences and severities of centrilobular hepatocellular hypertrophy and fatty change were recorded in 15 mg/kg and 60 mg/kg males and females (Fig. 1). These lesions were similar in character to that observed in the PND 22 livers seen in the PBDE-47 study, but there was more lipid accumulation in the livers of pups exposed to the PBDE mixture compared to those exposed to PBDE-47 (Fig. 1). Oil Red O staining confirmed the increase in lipid in pups exposed to the PBDE mixture compared to control pups (Fig. 2). In addition, increased mitoses were recorded in males and females at 0.1 mg/kg and above. Mitotic figures were fairly common in the control animals due to their young age. Animals that had minimal increased mitotic figures contained more mitotic figures (typically 5 or more fields of 3–5 or more mitoses at 20X, compared to 1–3 in control animals) (Fig. 2c, d).

Phenobarbital In the livers of PND 22 pups (Table 5), there were increased incidences of fatty change and centrilobular hypertrophy in male and female pups dosed with 45 mg/kg phenobarbital. Most of the occurrences were of minimal or mild severity, although there

were a few occurrences that were moderate in severity. Fatty change and centrilobular hypertrophy were similar to that observed in the PBDE-47 and PBDE mixture studies. The number of hepatocytes with a single, large vacuole increased with increasing severity.

PCB-126 There were no treatment-related liver lesions observed at PND 22 after exposure to PCB-126.

Transcriptomic analysis

There was a treatment-related up-regulation of liver enzyme transcripts after PBDE-47, PBDE mixture, and phenobarbital exposure (at an FDR of < 0.05) (Table 6; Fig. 3). This included up-regulation of the nicotine degradation pathways, xenobiotic metabolism, and melatonin degradation pathways (Tables 7, 8). The LPS/IL-1 mediated inhibition of RXR function pathway was down-regulated after these exposures. PCB-126 was the only exposure that up-regulated *Cyp1b1*, while *Cyp1a1* was up-regulated after all four exposures. In general, down-regulated liver transcripts (Supplement 2) had a smaller absolute fold decrease than found with the up-regulated liver transcripts.

The down-regulated liver transcripts with the highest absolute fold-change at 50 mg/kg PBDE-47 were: *ABCG8* (ATP-binding cassette subfamily G member 8 (– 7.7 fold) 1369440_at); *Pcp4l1* (Purkinje cell protein 4-like 1 (– 6.6 fold) 1390227_at); *ABCG5* (ATP-binding cassette subfamily G member 5 (– 6.3 fold) 1369455_at); *Pcp4l1* (Purkinje cell protein 4-like 1 (– 5.0 fold) 1390912_at); and *SCD* (stearoyl-CoA desaturase (– 4.2 fold) 1370355_at). Of these transcripts, *SCD* and *Pcp4l1* were also down-regulated after phenobarbital exposure (– 8.1 fold) but not after PBDE mixture or PCB-126 exposure. There were no down-regulated gene transcripts with PCB-126 at any of the exposure levels.

Several PBDE-47 liver transcripts at 0.1 mg/kg had a significant fold-change $> |1.5 \text{ fold}|$ including: *Rdx* (radixin (2.1 fold) 1375542_at); *Foxn3* (forkhead box N3 (–2.0 fold) 1384728_at); *Thrb* (thyroid hormone receptor beta (–1.7 fold) 1378457_at); *Tmf1* (TATA element modulatory factor 1 (–1.8 fold) 1387350_at); *Ccr1* (C-C motif chemokine receptor 1 (–1.63) 1370083_at). Phenobarbital had the most transcripts at the significant low-dose (5 mg/kg) transcripts, most notably *Cyp2cb* (9.7 fold). At the low exposure level of PCB-126, *Cyp1a1* was up-regulated (3.9 fold). There were no significant PBDE mixture liver transcripts with an absolute fold-change $> |1.5 \text{ fold}|$ at an FDR < 0.05 that were up-regulated at the 0.1 mg/kg exposure level.

BMDL analysis

Using the liver transcriptomic data, the benchmark dose analysis by GO categories provided more than 500 GO categories with a p value < 0.05 for PBDE-47 or PBDE mixture (Supplements 3 and 4). The GO category with 5 genes (and $> 5\%$ of the genes in the category) that passed the selection criteria in BMDExpress 2.0 ($p < 0.05$ and $|FC| > 1.5$) with the lowest mean BMDL was for PBDE-47, 0.05 mg/kg [“response to thyroid hormone” (GO:0097066)] and for PBDE mixture, 0.92 mg/kg [“response to cadmium ion” (GO:0046686)] (Table 9). The mean BMDL pattern for PBDE-47 was “left-shifted” (or smaller) than for the PBDE mixture (Fig. 4). Using uncertainty factors of 10 for species variation and

10 for intraspecies variation provides an estimated oral minimum risk level for PBDE-47 toxicity of ~ 0.0005 mg/kg compared to 0.009 mg/kg for the PBDE mixture. Therefore, the PBDE-47 exposure was considered have more toxic potential than the PBDE mixture.

Discussion

In utero/postnatal exposure to the PBDE mixture (DE-71) and PBDE-47 congener caused hepatotoxicity in PND 22 pups which increased with increasing PBDE exposure. The neonate is particularly vulnerable to toxic chemicals in the environment (Landrigan and Goldman 2011). The pups had higher PBDE congener tissue levels than the dam which was attributed to undeveloped metabolic and excretion capabilities (Coughtrie et al. 1988; Emond et al. 2013). Toxic endpoints included liver transcript changes and decreases in thyroid hormone levels, and these treatment-related changes were similar after exposure to either the PBDE-47 congener or the PBDE mixture. This suggests that PBDE-47 may also be a liver toxin and/or carcinogen after longer term exposure, as reported for the PBDE mixture (DE-71). Because liver disease and cancer is a worldwide concern (Fitzmaurice et al. 2017; World Health Organization Interagency for Research on Cancer 2012), it is important to identify possible environmental exposures that may contribute to the development of liver disease, including exposure to persistent organic pollutants (e.g., pentabromodiphenyl ethers).

At PND 22, PBDE-47-induced liver transcript patterns similar to those induced by the PBDE mixture or phenobarbital, both known liver carcinogens. These transcript patterns are characteristic of multiple toxicities including fatty liver, oxidative damage, alterations in metabolism, and changes in thyroid hormone levels. Some of these changes occur with up-regulation of the nicotine degradation, melatonin, and PXR/RXR/CAR pathways (Tolson and Wang 2010). These liver transcript changes could contribute to the occurrence of thyroid disease, liver disease, and/or cancer.

The observed up-regulation of glucuronosyl transferase and sulfotransferase transcripts could help explain the PBDE-induced reduction in T₄ levels. These findings are supported by other studies which showed that PBDE exposure reduced T₄ levels (Szabo et al. 2009). However, our findings are characterized the PBDE effect in the neonate. PBDE exposures in humans are also associated with alterations in thyroid homeostasis, but at a much lower PBDE exposure level than needed to cause this effect in rodents (Makey et al. 2016) suggesting that humans may be particularly vulnerable to PBDE toxicity.

PBDE exposure caused up-regulation of the Nrf2 antioxidant and melatonin pathways both of which can occur after oxidative damage (Esteban-Zubero et al. 2016; Karin and Dhar 2016; Reiter et al. 2009; Zhang et al. 2017b). Continued oxidative stress can promote protumorigenic processes such as up-regulation of P62 (Karin and Dhar 2016; Umemura et al. 2016) and liver disease (Cichoz-Lach and Michalak 2014). Oxidative stress may be involved in the development of liver fatty change (Antonucci et al. 2017; Thoolen et al. 2010). Up-regulation of the Nrf2 pathway also occurs after exposure to other rat and mouse liver carcinogens that cause oxidative damage (Dunnick et al. 2017).

Liver transcript biomarkers of exposure included up-regulation of liver cytochrome p450 levels. Up-regulation of cytochrome p450 levels have previously been reported after phenobarbital (Lamba et al. 2003, 2005) or PBDE mixture exposure (Dunnick et al. 2012; Dunnick and Nyska 2009). Cytochrome p450 systems can detoxify xenobiotics, initiate the formation of free radicals, and activate xenobiotics to carcinogens (Bondy and Naderi 1994; Khan and Ali Khan 2015; Zangar et al. 2004). These liver enzyme transcripts are induced after exposure to other rodent hepatocarcinogens including furan (Dong et al. 2016), DMPT (Dunnick et al. 2017), conazoles (Bhat et al. 2013), and nitrotoluenes (Deng et al. 2011). Thus, the up-regulation of PND 22 cytochrome transcripts is early indicators of liver toxicity and/or carcinogenicity processes, and help to identify changes in biologic pathways more rapidly than the more widely used 13-week toxicity study, a goal for the twenty-first century science studies (National Academy of Sciences 2017).

Other mechanistic insights were obtained by from the liver transcript patterns. *Cyp1a1* transcript was up-regulated after exposure to PBDE mixture, PBDE-47, phenobarbital, or PCB-126 (a chemical added to this study as a known dioxin-like chemical) (National Toxicology Program 2006b). *Cyp1a1* can be induced by both AhR independent and dependent events (Sadar et al. 1996; Tolson and Wang 2010). However, *Cyp1b1* was up-regulated only by PCB-126 (Li et al. 2017). *Cyp1b1* induction requires aryl hydrocarbon receptor-interacting protein (AIP), whereas *Cyp1a1* and *Cyp1a2* activation can be caused by multiple types of chemical exposures (Nukaya et al. 2009). Thus, the PBDE patterns were more similar to that of phenobarbital than to PCB-126.

Down-regulation of *Abcg5* and *Abcg8* transcripts occurred after both PBDE and phenobarbital exposures. These ATP-binding cassette (ABC) proteins translocate lipids, and work to excrete sterols from the cell and to maintain sterol balance (Lee et al. 2016). The down-regulation of these efflux pumps could be one mechanism for the observed PBDE-induced liver fatty change, a change associated with ongoing liver disease in other studies (Kneeman et al. 2012; Thoolen et al. 2010).

Other ATP-binding cassette (ABC) transcripts which code for xenobiotic efflux pumps on the plasma membrane (Locher 2016) were changed after PBDE exposure including up-regulation of *Abcb1*, *Abcc2*, and *Abcc3*. These changes could work to decrease toxic chemical accumulation (Hodges et al. 2011) by excreting xenobiotics (Wang et al. 2016). *Tmem27* was up-regulated after PBDE exposure, but a functional role for this protein in the liver has not been elucidated (Lambert et al. 2010). The *Gmp6a* transcript which codes for a neuronal membrane glycoprotein (an NGF-gated Ca²⁺ channel) (Monteleone et al. 2014) was increased only after phenobarbital exposure.

While the PND 22 PBDE liver toxic response was occurring, the animal was mounting disease protective responses which included as mentioned above up-regulation of antioxidant and membrane transport pathways. Up-regulation of *Ces2c*, aldehyde dehydrogenases (*Aldh1a1* and *Aldh1a7*), and lipoprotein lipase transcripts may also be classified as protective responses. *Ces2c* codes for an enzyme that hydrolyzes esters and detoxifies xenobiotics (Staudinger et al. 2010). Aldehyde dehydrogenases catalyze the oxidation of alcohols to aldehydes and oxidation of aldehydes to less toxic carboxylic acids

(Koppaka et al. 2012; Singh et al. 2013). Lipoprotein lipase (LPL) is the rate-limiting enzyme for hydrolysis of lipoprotein triglyceride (Walton et al. 2015). While not measured in these studies, the protective pathways can decrease as animals' age. For example, there are lower levels of *Ces2c* and other enzymes in older animals (Fu et al. 2012).

PBDEs induced *Apoa2* (apolipoprotein A-II) and *App* (amyloid beta precursor protein) liver transcripts at PND 22. Apolipoprotein A-II is the second most abundant protein of high-density lipoprotein particles and may play a role in lipoprotein triglyceride catabolism and regulation of LPL activity (Julve et al. 2010). Others report that up-regulation of peripheral amyloid proteins can contribute to Alzheimer disease (Sutcliffe et al. 2011); however, further work is required to evaluate how these changes could contribute to disease.

The PBDE benchmark dose (BMD) and BMDL was determined using PND 22 liver transcriptomic data mapped to gene ontology (GO) categories. The PBDE-47 GO category with the lowest BMDL was response to thyroid hormone. Using 10 for interspecies and 10 intraspecies variation, the mean PBDE-47 BMDL was 0.0005 mg/kg. In addition to alterations in thyroid hormone homeostasis, PBDE exposures can lead to other toxicities including development impairments (Casey et al. 2017; Cooper and Pearce 2017) and liver disease (Brown et al. 2017; Diehl and Day 2017; Frau et al. 2015).

Conclusions

PBDE-47 liver toxicity and transcript changes were similar to those of the PBDE mixture (a known rodent carcinogen), and included changes characteristic of oxidative stress, alterations in metabolic processes, decreases in T_4 levels, and liver fatty change (Fig. 5). These biological responses are early indicators of disease, and can be used to identify other chemical toxins using short-term toxicity studies, as recommended by recent scientific work groups (National Academy of Sciences 2017).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Pathology of liver lesions after PBDE-47 or PBDE- mixture exposure. **a** Liver from a PND 22 female control rat pup. There is a lack of fat vacuoles within the liver. **b** Liver from a PND 22 female rat pup exposed to 50 mg/kg PBDE-47. Mild fatty change is characterized by clear, discrete vacuoles within scattered hepatocytes. **c** Liver from a PND 22 male rat pup exposed to DE-71 with centrilobular hypertrophy and widespread fatty change in the hepatocytes. **The fatty change is more severe than that seen in the section of the liver from the animal exposed to PBDE-47.** **d** Higher magnification of the liver from the same animal as that in **c**. Clear discrete vacuoles, suggestive of fat, are present within hepatocytes

Fig. 2.

Oil Red O Stain for lipids in liver. **a** Liver from a PND 22 control male mouse from the PBDE-47 study group. The liver is stained with Oil Red O, which stains lipids bright red. There are a few very small droplets of lipid within some hepatocytes. **b** Liver from a PND 22 male mouse exposed to PBDE-47, stained with Oil Red O. Compared to **a**, there are more lipid droplets. The changes in this liver indicate an increased lipid content, consistent with fatty change. **c** Liver from a PND 22 control male mouse from the PBDE mixture (DE-71) study group. The liver is stained with Oil Red O, which stains lipids bright red. Similar to the control from the PBDE-47 study group, there are a few very small droplets of lipid within some hepatocytes. **d** Liver from a PND 22 male mouse exposed to the PBDE mixture (DE-71), stained with Oil Red O. Compared to **c**, there are many more lipid droplets, and the individual droplets are larger. When compared to **c**, there also appears to be increased lipid in this animal compared to the liver of a pup exposed to PBDE-47.

Fig. 3.
Dendrogram of significant liver transcripts. **a** Liver transcripts after PBDE-47 exposure at 0, 15, or 50 mg/kg. **b** Liver transcript changes after PBDE mixture (DE-71) exposure at 0, 15, or 50 mg/kg

Fig. 4.
Mean BMDL pattern for PBDE mixture

Fig. 5.
Overview of mechanistic findings from liver transcript analysis

Table 1

Concentrations of PBDE congeners in plasma of PND 22 pups and dams exposed to PBDE mixture (DE-71)

Male pups	PBDE mixture (DE-71) Dose Levels (mg/kg)			
Analyte (µg/ml)	0	0.1	15	50
PBDE-47	0 ^{**}	0	1.19 ± 0.16 ^{**}	3.57 ± 0.32 ^{**}
PBDE-99	0 ^{**}	0	0.66 ± 0.10 ^{**}	2.44 ± 0.29 ^{**}
PBDE-153	0 ^{**}	0	0.68 ± 0.10 ^{**}	2.88 ± 0.21 ^{**}
Female pups				
PBDE-47	0 ^{**}	0	0.98 ± 0.09 ^{**}	2.28 ± 0.13 ^{**}
PBDE-99	0 ^{**}	0.01	0.52 ± 0.06 ^{**}	1.30 ± 0.13 ^{**}
PBDE-153	0 ^{**}	0	0.55 ± 0.06 ^{**}	1.77 ± 0.06 ^{**}
Dams				
PBDE-47	0 ^{**}	0	0.17 ± 0.025 ^{**}	0.53 ± 0.056 ^{**}
PBDE-99	0.01 ^{**}	0	0.16 ± 0.02 ^{**}	0.44 ± 0.05 ^{**}
PBDE-153	0 ^{**}	0	0.098 ± 0.02 ^{**}	0.265 ± 0.04 ^{**}

PBDE congeners were measured in plasma of PND 22 pups and in serum of dams at PND 22 after DE-71 exposure (Table 1) or after PBDE-47 exposure (Table 2)

Means ± SE, $n = 6$

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

^{**} $p < 0.01$ (pairwise comparison in dose group column; trend test p value in the control (0 mg/kg) column)

Table 2

Concentrations of PBDE-47 in plasma of PND 22 pups and dams exposed to PBDE-47

	PBDE-47 (mg/kg)			
Male pups	0	0.1	15	50
PBDE-47 (µg/ml)	0.01 ± 0.01 **	0.044 ± 0.01	3.85 ± 0.57 **	15.05 ± 4.06 **
Female pups				
PBDE-47 (µg/ml)	0.007 ± 0.01 **	0.042 ± 0.01	3.39 ± 0.34 **	10.66 ± 1.25 **
Dams				
PBDE-47 (µg/ml)	0 **	0	0.56 ± 0.05 **	2.03 ± 0.43 **

PBDE congeners were measured in plasma of PND 22 pups and in serum of dams at PND 22 after DE-71 exposure (Table 1) or after PBDE-47 exposure (Table 2)

Means ± SE, $n = 6$

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

** $p < 0.01$ (pairwise comparison in dose group column; trend test p value in the control (0 mg/kg) column)

Table 3

Male and female pups: PND 22 thyroid hormone and lipid levels after PBDE-47 or PBDE mixture (DE-71) exposure

mg/kg	Male pups			Female pups				
	0	0.1	15	50	0	0.1	15	50
T ₄ (ug/dL)								
PBDE-47	5.08 ± 0.21**	5.05 ± 0.26	3.20 ± 0.08**	3.00 ± 0.10**	4.98 ± 0.16**	4.74 ± 0.33	3.04 ± 0.12**	2.98 ± 0.22**
	-	99%	63%	59%	-	95%	61%	60%
PBDE mixture	5.67 ± 0.36**	5.67 ± 0.31	3.66 ± 0.19**	2.69 ± 0.23**	5.80 ± 0.43**	5.37 ± 0.18	3.62 ± 0.28**	2.72 ± 0.15**
(DE-71)	-	100%	65%	48%	-	92%	62%	47%
T ₃ (ng/dL)								
PBDE-47	151.2 ± 7.81**	148.6 ± 7.88	146.2 ± 26.37*	126.7 ± 5.86*	148.2 ± 5.35**	130.2 ± 5.56*	106.7 ± 5.17**	116.3 ± 5.22**
	-	98%	97%	84%	-	88%	72%	78%
PBDE mixture	150.3 ± 7.88**	133.8 ± 5.60	111.2 ± 7.92**	122.7 ± 6.56*	129.9 ± 6.01*	132.3 ± 8.08	107.2 ± 5.34*	115.2 ± 5.64
(DE-71)	-	89%	74%	82%	-	102%	83%	89%
TSH (pg/ml)								
PBDE-47	732.7 ± 115.7	753.6 ± 123.2	734.2 ± 139.6	1135 ± 237.5	1423 ± 210.5	1062 ± 132.4	1293 ± 256.8	1244 ± 120.0
	-	103%	100%	155%	-	75%	91%	87%
PBDE mixture	673.8 ± 158.7*	672.5 ± 132.7	766.2 ± 79.16	872.4 ± 83.15	1191 ± 209.6*	857.3 ± 81.11	1054 ± 101.9	1642 ± 235.5
(DE-71)	-	100%	114%	130%	-	72%	88%	138%
Cholesterol (mg/dL)								
PBDE-47	117.9 ± 6.81	119.6 ± 6.69	121.9 ± 6.23	120.1 ± 6.40	117.6 ± 7.52	117.9 ± 4.70	119.5 ± 7.14	130.1 ± 6.40
	-	102%	103%	110%	-	100%	102%	109%
DE-71	115.7 ± 6.55*	110.7 ± 4.12	119.1 ± 4.93	148.3 ± 9.39	114.0 ± 5.62*	118.3 ± 6.67	119.5 ± 5.17	154.7 ± 9.17**
-	-	96%	103%	128%	-	104%	104%	136%
Triglycerides (mg/dL)								
PBDE-47	193.0 ± 28.00	175.4 ± 20.90	153.0 ± 20.36	153.9 ± 17.27	193.3 ± 27.32	166.6 ± 22.78	137.6 ± 11.76	138.8 ± 18.21
	-	91%	79%	80%	-	86%	71%	72%
PBDE mixture	146.4 ± 23.60	165.3 ± 22.94	108.1 ± 19.09	136.7 ± 13.14	155.1 ± 16.14	156.0 ± 24.59	113.7 ± 19.80	131.2 ± 20.93
(DE-71)	-	113%	74%	93%	-	101%	73%	85%

Mean ± standard error

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$N = 8$ pups per dose level
Values in bold represents control
* $p < 0.05$;
** $p < 0.01$ (pairwise comparison in dose group column; trend test p value in the control column)

Table 4

Dams: PND22 serum thyroid hormone and lipid levels

mg/kg	0	0.1	15	50
T₄ (ug/dL)				
PBDE-47	4.48 ± 0.16 ^{**}	4.30 ± 0.14	3.92 ± 0.15 [*]	3.43 ± 0.13 ^{**}
	-	96%^a	88%	77%
PBDE mixture (DE-71)				
	3.80 ± 0.11 ^{**}	3.58 ± 0.12	3.38 ± 0.14 [*]	2.82 ± 0.15 ^{**}
	-	94%	89%	74%
T₃ (ng/dL)				
PBDE-47	67.46 ± 3.95 [*]	69.48 ± 4.56	63.79 ± 3.28	56.28 ± 1.54
	-	103%	95%	83%
PBDE mixture (DE-71)				
	65.17 ± 3.85	65.26 ± 2.53	64.29 ± 3.27	60.38 ± 2.76
	-	100%	99%	93%
TSH (pg/ml)				
PBDE-47	1681 ± 540.8	1639 ± 203.2	1564 ± 606.9	1620 ± 404.8
	-	97%	93%	96%
PBDE mixture (DE-71)				
	1030 ± 120.5	1739 ± 277.5	1218 ± 219.2	1155 ± 166.3
	-	169%	118%	112%
Cholesterol (mg/dL)				
PBDE-47	64.22 ± 3.18	68.50 ± 2.99	67.38 ± 2.60	68.67 ± 2.60
	-	107%	105%	107%
PBDE mixture (DE-71)				
	63.11 ± 3.21	67.67 ± 2.49	63.67 ± 3.01	70.80 ± 2.69
	-	107%	101%	112%
Triglycerides (mg/dL)				
PBDE-47	66.222 ± 5.68	76.51 ± 9.50	69.63 ± 3.65	65.44 ± 4.81
	-	115%	105%	99%
PBDE mixture (DE-71)				
	87.78 ± 5.16	74.22 ± 4.87	87.44 ± 5.09	82.90 ± 3.79
	-	84%	100%	94%

Mean ± standard error

N = 8 dams per dose level

S_g Serum used for dam analysis and lipid for pup analysis
 p pairwise comparison in dose group column; trend test p value in the control column)

**

$p < 0.05$;

*

Values in bold represent control

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Table 5
Treatment-related liver lesions at PND 22 after PBDE-47, PBDE mixture (DE-71), and phenobarbital exposure

	Male pups				Female pups			
PBDE-47 (mg/kg)	0	0.1	1.5	50	0	0.1	1.5	50
Number examined	27	24	24	26	26	24	23	26
Fatty change ^a	1 ^{**} (1.0)	0	8 ^{**} (1.0)	21 ^{**} (1.1)	0 ^{**}	0	4 [*] (1.0)	14 ^{**} (1.2)
Centrilobular hypertrophy	0 ^{**}	0	24 ^{**} (1.8)	26 ^{**} (3.2)	0 ^{**}	0	22 ^{**} (1.6)	26 ^{**} (3.0)
PBDE mixture (DE-71) (mg/kg)	0	0.1	1.5	50	0	0.1	1.5	50
Number examined	26	21	26	30	22	31	26	28
Fatty change	0 ^{**}	1 (1.0)	16 ^{**} (1.5)	30 ^{**} (2.7)	0 ^{**}	0	16 ^{**} (1.4)	28 ^{**} (2.9)
Centrilobular hypertrophy	0 ^{**}	0	26 ^{**} (1.3)	30 ^{**} (3.5)	0 ^{**}	1 (1.0)	26 ^{**} (1.2)	26 ^{**} (3.5)
Increased mitoses	0 ^{**}	6 ^{**} (1.0)	12 ^{**} (1.0)	13 ^{**} (1.0)	0 ^{**}	5 (1.0)	13 ^{**} (1.0)	12 ^{**} (1.0)
Phenobarbital (mg/kg)	0	5	45	-	0	5	45	-
Number examined	20	19	19	-	21	20	21	-
Fatty change	0 ^{**}	1 (1.0)	14 ^{**} (1.7)	-	0 ^{**}	0	16 ^{**} (1.8)	-
Centrilobular hypertrophy	0 ^{**}	4 [*] (1.0)	18 ^{**} (1.8)	-	0 ^{**}	2 (1.0)	20 ^{**} (1.7)	-

No treatment-related liver lesions with PCB 126 on PND22

^{*} Control and two doses with phenobarbital

^{*} $p < 0.05$;

^{**} $p < 0.01$ by the Cochran-Armitage trend test if in the control column or by Fisher's exact test if in a dose column

^a Incidence (Severity grade, where 1 = minimal; 2 = mild; 3 = moderate; 4 = marked)

Table 6
Liver genes up- or down-regulated after PBDE-47, PBDE mixture (DE-71) phenobarbital, or PCB 126 exposure

	PBDE-47			PBDE mixture (DE-71)			Phenobarbital			PCB-126		
	0.1 mg/kg	15 mg/kg	50 mg/kg	0.1 mg/kg	15 mg/kg	50 mg/kg	5 mg/kg	45 mg/kg	10 ng/kg	50 ng/kg	250 ng/kg	
Total	17	284	628	1	104	244	132	612	1	1	10	
Up	4	200	376	1	93	170	74	297	1	1	10	
Down	9	64	217	0	7	61	53	275	0	0	10	

Genes significant at a false discovery rate (FDR) < 0.05

Table 7

Selected liver gene transcript expression for PBDE-47, PBDE mixture (DE-71), phenobarbital, or PCB-126

	PBDE-47 (mg/kg)			PBDE mixture (DE-71) (mg/kg)			Phenobarbital (mg/kg)			PCB-126 (ng/kg)		
	0.1	15	50	0.1	15	50	5	45	10	50	250	
Liver enzymes—xenobiotic metabolizing enzymes (nicotine degradation pathway in ingenuity)												
<i>Cyp2C8</i> cytochrome P450 family 2 subfamily C member 8 1370241_at	-1.11	24.91	31.68	3.00	46.27	55.80	9.66	73.50	1.18	1.70	1.03	
<i>Cyp2A6</i> cytochrome P450 family 2 subfamily A member 6 1369136_at	1.04	14.55	23.38	1.06	14.92	14.31	2.93	22.81	1.21	1.82	1.16	
<i>Cyp2B6</i> cytochrome P450 family 2 subfamily B member 6 1371076_at	1.16	4.25	4.52	1.55	4.88	4.70	2.19	3.91	1.01	-1.40	-1.28	
<i>Cyp2C9</i> cytochrome P450 family 2 subfamily C member 9 1387328_at	1.73	3.65	3.94	1.50	3.60	3.54	2.87	4.48	-1.05	1.22	1.23	
<i>Ces2c</i> (includes others) carboxylesterase 2C 1368905_at	-1.02	10.86	14.86	1.69	9.93	7.89	3.18	13.99	1.06	-1.03	1.02	
<i>Sult1e1</i> sulfotransferase family 1E member 1 1368733_a	1.20	5.95	9.19	2.01	6.32	45.34	1.34	2.92	-1.03	1.04	1.19	
<i>Cyp1a1</i> cytochrome P450 family 1 subfamily A member 1 1370269_at	-2.07	1.91	4.28	5.13	60.47	77.64	1.60	2.16	3.89	7.66	18.89	
<i>Cyp1b1</i> cytochrome P450 family 1 subfamily B member 1 1368990_at	-1.14	-1.20	-1.13	-1.04	2.51	5.99	1.02	-1.07	-1.01	1.06	23.12	
<i>Pltp</i> phospholipid transfer protein 1391435_at	-1.24	-1.72	-2.34	-1.00	-1.26	-1.13	-1.12	-1.18	-1.06	-1.06	-1.04	
Membrane transport/lipid metabolism												
<i>ABCB1</i> 1370464_at	1.10	2.10	2.20	1.22	1.98	1.60	1.62	3.18	1.00	-1.15	-1.20	
<i>ABCC2</i> ATP-binding cassette subfamily C member 2 1368497_at	1.10	1.70	2.10	1.26	1.94	2.20	1.35	2.19	-1.10	1.10	1.13	
<i>ABCC3</i> ATP-binding cassette subfamily C member 3 1369698_at	1.01	2.99	3.42	1.40	3.40	3.37	1.50	2.39	-1.10	-1.23	1.44	
<i>ABCG5</i> ATP-binding cassette subfamily G member 5 (—6.3) 1369455_at	-1.07	-3.34	-6.30	-1.17	-2.20	-3.05	-1.61	-2.67	1.07	1.17	1.10	
<i>ABCG8</i> ATP-binding cassette subfamily G member 8 1369440_at	-1.26	-5.15	-7.72	-1.28	-2.14	-3.50	-2.02	-3.09	1.10	-1.06	1.30	
<i>TMEM27</i> transmembrane protein 27 1387013_at	1.01	11.24	19.38	1.64	10.70	14.91	2.93	22.81	1.12	1.81	1.16	
<i>GPM6A</i> glycoprotein M6A 1373773_at	-1.25	-3.30	-5.24	1.35	-1.74	1.18	3.82	9.68	-1.66	-2.31	-1.10	
<i>Aldh1a1</i> aldehyde dehydrogenase 1 family member A1 1387022_at	1.25	2.95	3.72	1.24	2.42	2.65	1.49	2.42	-1.09	-1.08	1.04	
<i>Aldh1a7</i> aldehyde dehydrogenase family 1, subfamily A7 1368718_at	1.00	2.91	6.16	1.57	3.01	4.76	1.69	4.34	-1.1	1.02	1.38	
<i>APOA2</i> apolipoprotein A2 1369727_at	1.78	3.68	5.14	1.24	3.15	5.50	-1.36	1.73	-1.13	1.13	1.35	
<i>APP</i> amyloid beta precursor protein 1380533_at	-1.04	1.98	3.16	-1.02	4.78	2.62	2.15	6.48	1.40	1.06	1.56	
<i>LPL</i> lipoprotein lipase 1386965_at	-1.30	3.88	4.18	-1.13	2.34	1.80	-1.30	2.67	1.20	-1.21	-1.22	
Nrf2 pathway												
<i>GPX2</i> glutathione peroxidase 2 1374070_at	1.05	4.32	6.38	1.03	2.52	4.92	1.28	1.17	-1.22	-1.20	-1.37	
<i>AKR7A3</i> aldo-keto reductase family 7 member A3 1368121_at	1.37	2.10	3.00	1.54	4.90	6.92	2.14	3.18	1.44	1.16	1.99	
<i>EPHX1</i> epoxide hydrolase 1 1387669_a_at	1.01	2.40	2.94	1.50	3.46	3.35	1.96	2.57	1.18	1.00	1.61	

	PBDE-47 (mg/kg)				PBDE mixture (DE-71) (mg/kg)				Phenobarbital (mg/kg)				PCB-126 (mg/kg)			
	0.1	15	50	0.1	15	50	0.1	15	50	5	45	10	50	10	50	250
<i>GSTA1</i> glutathione S-transferase alpha 1 1368180_s_at	1.01	2.05	2.45	1.46	2.99	3.61	1.90	2.46	2.46	1.90	2.46	1.90	2.46	1.90	2.46	2.46
<i>GSTA3</i> glutathione S-transferase alpha 3 1371089_at	1.20	1.45	1.90	1.26	1.36	1.91	1.04	1.25	1.08	1.04	1.25	1.08	1.04	1.04	1.08	1.16
Thyroid hormone conjugating enzymes																
<i>SULT1B1</i> sulfotransferase family 1B member 1 1387314_at	1.14	3.74	5.17	1.19	2.83	4.22	1.88	4.69	2.24	1.88	4.69	2.24	1.67	1.16	1.16	1.16
<i>UGT2B28</i> UDP glucuronosyltransferase family 2 member B28 1387825_at 4.013	-1.12	3.57	4.01	1.79	1.99	6.15	1.32	1.89	1.01	1.32	1.89	1.01	1.25	1.18	1.18	1.18
UGT2B17 UDP glucuronosyltransferase family 2 member B17 1370698_at	1.01	2.05	2.00	1.33	2.15	2.34	1.95	2.61	1.05	1.95	2.61	1.05	1.13	1.07	1.07	1.07
Other																
<i>SNX10</i> sorting nexin 10 1383585_s_at	2.04	3.09	5.06	1.25	4.06	8.08	1.34	4.28	1.22	1.34	4.28	1.22	1.52	2.05	2.05	2.05
<i>CEACAM4</i> carcinoembryonic antigen related cell adhesion molecule 4 1370371_a_at	-1.29	-1.38	-1.24	1.39	6.33	25.18	1.16	1.02	1.29	1.16	1.02	1.29	1.31	11.26	11.26	11.26
<i>LOC100360095</i> (includes others) urinary protein 2 1370349_a_at	-2.12	3.61	2.50	3.24	8.35	45.56	5.13	1.00	1.17	5.13	1.00	1.17	1.34	16.70	16.70	16.70
Unidentified 1396155_at	1.04	12.53	19.10	2.00	11.62	29.90	1.55	15.31	1.02	1.55	15.31	1.02	1.06	1.11	1.11	1.11
<i>ZDHHC2</i> zinc finger DHHC-type containing 2 1370828_at	-1.14	3.69	6.17	1.15	2.80	4.67	1.43	4.90	1.16	1.43	4.90	1.16	1.20	1.17	1.17	1.17

Bolded numbers are significant (FDR < 0.05)

Table 8

Top ingenuity pathways for PBDE-47 and PBDE mixture (DE-71) at 50 mg/kg

Top canonical pathways	PBDE-47 <i>p</i> value	# Significant transcripts/ total number in pathway	PBDE mixture (DE-71) <i>p</i> value	# Significant transcripts/ total number in pathway
Nicotine degradation II	4.28E-14	44.7% 17/38	6.89E-14	28.3% 13/46
Nicotine degradation III	1.60E-13	45.7% 16/35	8.80E-15	32.5% 13/40
Melatonin degradation I	1.27E-12	41.0% 16/39	2.07E-17	34.9% 15/43
Superpathway of melatonin degradation	4.98E-12	38.1% 16/42	9.74E-17	31.9% 15/47
LPS/IL-1 mediated inhibition of RXR function	1.57E-10	17.9% 27/151	1.56E-10	10% 18/180
CAR/RXR activation	2.83E-14	60.9% 14/23	1.73E-16	50.0% 12/24
NRF2-mediated oxidative stress response	1.59E-02	8.3% 13/157	1.08E-05	6.9% 12/175
Top cell functions/diseases	<i>p</i> value	Number of transcripts changed	<i>p</i> value	Number of transcripts changed
Lipid metabolism	2.40E-02 to 1.70E-12	136	1.47E-02 to 3.80E-12	59
Endocrine system development and function	1.65E-02 to 1.41E-08	57	8.99E-03 to 4.12E-09	22
Liver steatosis	5.14E-02 to 1.26E-04	26	4.64E-01 to 2.35E-03	12

Results for PBDE-47 and DE-71 at 50 mg/kg or distribution across doses for response of selected genes in these pathways or cellular functions in the 0.1, 15, or 50 mg/kg groups

Table 9

Selected mean BMDL levels for PBDE-47 and PBDE mixture (DE-71) based on GO categories using liver transcriptomic data

GO term	GO term name	# Genes in GO category	# Significant genes	p value	Gene names	Mean BMDL (mg/kg)
PBDE-47						
GO:0097066	Response to thyroid hormone	36	5 (13.8%)	0.006	Hes1; Ctss; Inhbb; Rdx; Abcb1a	0.05
GO:0046686	Response to cadmium ion	63	5 (8%)	0.05	Akr1c3; Cyp3a23/3a1; Abcb1a; Cyp2a3; Gsr	0.19
GO:1990748	Cellular detoxification	73	6 (8%)	0.03	Sesn2; Gstl1; Gpx2; Mgst2; Gsr; Prdx3	0.78
GO:0006855	Drug transmembrane transport	18	5 (27%)	0.00	Abcb1a; Slco1b2; Abcc3; Abcg5; Abcc2	2.04
GO:0006575	Cellular modified amino acid metabolic process	125	11 (8.8%)	0.002	Vnn1; Gmmt; Sult1b1; Gstl1; Ptds s2; Mgst2; Gsta4; Ass1; Pemt; Gsr; Egl3	2.30
GO:0042632	Cholesterol homeostasis	54	8 (14.8%)	0.002	Lpl; Lrp5; ApoA2; Abcg5; Abcg8; Fabp4; Scarb1	2.84
GO:0055092	Sterol homeostasis	54	8 (14.8%)	0.002	Lpl; Lrp5; ApoA2; Abcg5; Abcg8; Fabp4; Scarb1	2.84
GO:0046470	Phosphatidylcholine metabolic process	30	5 (16.6%)	0.002	Gpld1; Fabp5; ApoA2; Pemt; Chka	2.84
PBDE mixture (DE-71)						
GO:0046686	Response to cadmium ion	63	6 (9%)	0.04	Cyp1a2; Cyp3a23/3a1; Abcb1a; Sod2; Cyp2a3; Gsr	0.92
GO:0006778	Porphyryn-containing compound metabolic process	34	6 (18%)	0.00	Tmem14c; Cyp1a2; Bdh2; Cyp1a1; Cyp2a3; Alas1	1.01
GO:0006855	Drug transmembrane transport	18	7 (39%)	0.00	Slc17a3; Abcb1a; Slco1b2; Abcc3; Abcg5; Abcc2; Slc22a1	3.18
GO:0019369	Arachidonic acid metabolic process	39	7 (18%)	0.002	Cyp2b21; Mgl1; Cyp2c6v1; Cyp2a1; Cyp2a3; Cyp1b1; Cyp4a8	3.29
GO:0006914	Autophagy	107	9 (8.4%)	0.05	Rab7a; Rab1b; Fam134b; Stbd1; Gabarapl1; Sqstm1; Lgals8; Anxa7; Cttd	3.82
GO:0035902	Response to immobilization stress	39	5 (13%)	0.02	Cyp1a2; Mdm2; Cyp1a1; Sod2; Ren	3.95
GO:0035902	Response to immobilization stress	39	5 (13%)	0.02	Cyp1a2; Mdm2; Cyp1a1; Sod2; Ren	3.95
GO:0001523	Retinoid metabolic process	42	5 (12%)	0.03	Aldh1a1; Cyp1a1; Adh4; Cyp1b1; Adh1	3.98

^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)