Carcinogenic activity of pentabrominated diphenyl ether mixture (DE-71) in rats and mice

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ABSTRACT

Pentabrominated diphenyl ether (PBDE) flame retardants have been phased out in Europe and in the United States, but these lipid soluble chemicals persist in the environment and are found human and animal tissues. PBDEs have limited genotoxic activity. However, in a 2-year cancer study of a PBDE mixture (DE-71) (0, 3, 15, or 50 mg/kg (rats); 0, 3, 30, or 100 mg/kg (mice)) there were treatment-related liver tumors in male and female Wistar Han rats (Crl:WI(Han) after in utero/postnatal/adult exposure, and in male and female B6C3F1 mice, after adult exposure. In addition, there was evidence for a treatment-related carcinogenic effect in the thyroid and pituitary gland tumors in male rats, and in the uterus (stromal polyps/stromal sarcomas) in female rats. The treatment-related liver tumors in female rats were unrelated to the AhR genotype status, and occurred in animals with wild, mutant, or heterozygous Ah receptor. The liver tumors in rats and mice had treatment-related Hras and Ctnnb mutations, respectively. The PBDE carcinogenic activity could be related to oxidative damage, disruption of hormone homeostasis, and molecular and epigenetic changes in target tissue. Further work is needed to compare the PBDE toxic effects in rodents and humans.

1. Introduction

The pentabrominated diphenyl ethers (PBDEs) are used as flame retardants, and are found in human [1] and animal tissues (e.g. eagles [2], starlings [3], whales [4], fish [5]). The most prevalent PBDE congener in tissues is PBDE-47 (2,2′,4,4′-tetrabromodiphenyl ether) [6–8]. Environmental accumulation of PBDEs [9–11] occurs in densely populated areas as well as in remote locations [12]. Concentrations of PBDE in the atmosphere vary with temperature and humidity [13]. While PBDE use has been phased out in Europe [14] and in the United States [15], exposure to these chemicals continues [1], although some studies indicate that PBDE tissue levels are decreasing [16,17]. It is expected that PBDEs will continue to be released into the environment from disposal of TVs and computer parts [18]. Recent studies suggest that marine bacteria can synthesize PBDEs using flavin-dependent brominases [19]. Another source of PBDEs could be photo-degradation of bromophenols [20]. Oral, dermal, and inhalation exposure are all possible routes of PBDE exposure [21].

PBDEs accumulate in tissues, in part, because these are fat soluble chemicals [22–24]. The whole-body half-life (representing primarily elimination from fat) increases with the number of bromine atoms (e.g. whole body half-life for commonly occurring PBDEs: PBDE-153 (six bromine atoms; 11.7 yrs.) > PBDE-99 (five bromine atoms; 5.4 yrs.) > PBDE-47 (four bromine atoms; 3 yrs.) [25]). The PBDE log Kow value, a measure of lipid solubility, also increases with increasing number of bromine atoms (e.g. log Kow 6.81, 7.32, 7.9 for PBDE-47, 99, 153, respectively) [26].

While PBDEs have limited genotoxic activity [26,27], PBDE exposure can disrupt liver and thyroid function. The toxicity of PBDE and its hydroxylated metabolites (e.g. 3-OH-BDE47, 6-OH-BDE47 [28]) may be related in part to the similarity of their structure to the thyroid hormone structure, and competition with T4 for the thyroid hormone receptor resulting in alteration of thyroid hormone transport and metabolism [29]. There is particular concern about early life PBDE exposure and the potential for disruption of thyroid hormone levels [30], because these chemicals occur in household dust [31–33], the hand-to-mouth behavior of young children [34], and the exposure to the infant from mother’s milk [35,36]. PBDEs in food, water, air, soil, sewage...
sludge, and at electronic waste sites allow for oral, dermal, and/or inhalation exposure [37,15]. PBDE exposure is linked to development toxicity in children [38].

This PBDE mixture (DE-71) oral gavage study examined the occurrence of treatment-related non-neoplastic and neoplastic lesions after an in utero/postnatal/adult exposure in rats, and after adult exposure in mice. The occurrence of treatment-related carcinogenic effects was studied in relationship to rat Ah receptor genotype, because activation of this receptor is involved in toxicity from other persistent organic pollutants [39,40]. We discuss how PBDE key characteristics could contribute to its toxic and carcinogenic activity.

2. Methods

2.1. Chemical and gavage formulation

The PBDE mixture (DE-71, lot 1550OK07A, Great Lakes Chemical Corp., El Dorado, AR) was identified using infrared (IR), proton and carbon-13 nuclear magnetic resonance (NMR) spectroscopy and gas chromatography (GC) with mass spectrometry (MS). The purity and composition were assessed using GC with flame ionization detection (FID). 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 dibenzoxodioxins and furans were also identified (approximately 7 × 10⁻³% by weight).

DE-71 formulations were prepared in corn oil to deliver by gavage to rats 0, 3, 15, or 50 mg/kg in a volume of 5 mL/kg body weight and to mice 0, 3, 30, 100 mg/kg at a volume of 10 mL/kg body weight. During the study, dose formulations were analyzed approximately every 2 months and were found to be within 10% of target concentration. Oral gavage dose formulation analysis and stability studies of DE-71 in corn oil was conducted using GC-FID. Stability of corn oil formulations were confirmed for at least for 46 days for formulations stored in amber glass containers sealed with Teflon lined lids at ambient temperature.

2.2. Experimental design

Wistar Han [Crl:WI(Han)] rat dams (referred to as Wistar Han rats) were obtained from Charles River Laboratories (Raleigh, NC) and B6C3F1/N mice from Taconic Farms, Inc. (Germantown, NY). Animals were stratified by weight then randomly assigned to dose groups to ensure equal initial mean weights across dose groups. Fifty B6C3F1/N mice from Taconic Farms, Inc. (Germantown, NY). Animals were stratified by weight then randomly assigned to dose groups to ensure equal initial mean weights across dose groups. Fifty B6C3F1/N mice (age 5–6 weeks of age) per dose group were dosed 5 days per week for 2-years. Twenty-five Wistar Han rat dams (12–13 weeks of age) per dose group were dosed (0, 3, 15 or 50 mg/kg/day) from gestation day (GD) 6 through postnatal day (PND) 21. At PND4 all litters were culled by sex per litter continued in the study for up to two years. Therefore, the control group without correction for multiple testing.

The PBDE mixture (DE-71) oral gavage study examined the occurrence of treatment-related non-neoplastic and neoplastic lesions after an in utero/postnatal/adult exposure in rats, and after adult exposure in mice. The occurrence of treatment-related carcinogenic effects was studied in relationship to rat Ah receptor genotype, because activation of this receptor is involved in toxicity from other persistent organic pollutants [39,40]. We discuss how PBDE key characteristics could contribute to its toxic and carcinogenic activity.
potential litter effects were controlled for in the statistical analyses of the rat study. For body weights, Johnkheere’s test was applied to determine if a dose-related trend was present (p < 0.01). Mixed effects analysis of variance was then applied to body weights, with Williams’ test (if a trend was present) or Dunnett’s test (if a trend was not present) used to compare each dosed group to the control group. Mixed effects logistic regression with litter ID as the random effect and a poly-3-weight adjustment for survival, was used to assess the effects of DE-71 on neoplastic and nonneoplastic lesion incidences.

2.5. Ah receptor (AhR) genotyping

Formalin fixed (FFPE) liver sections from female rats were taken from 60 control liver and 58 female rat liver tumor samples from the 50 mg/kg group (total of 118 female rat liver samples analyzed for AhR genotype). Formalin fixed kidney samples (from the same of the same animals used for liver sample collection) were also analyzed for AhR genotype (64 control and 58 female rats at 50 mg/kg). Genomic DNA (gDNA) was isolated from liver and kidney using the QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA) per manufacturer’s procedure.

The AhR genotypes [39,40] were determined using QPCR and Sanger sequencing methods. A nested PCR product was used for QPCR. The nested PCR product was prepared to amplify the exon 10 region of interest that contained the AhR SNP to determine the genotype (wild, mutant, or heterozygous) for the single nucleotide polymorphism – SNP (mutation) [39,40]. Real time PCR was conducted with a TaqMan® platform in which [VIC/FAM] fluorescence was used to determine the genotype for each sample (adapted from Ref. [53]). The nested PCR product of each sample was analyzed with TaqMan® Genotyping Master Mix (Life Technologies) according to manufacturer’s procedure with the following cycling conditions (95 °C for 10 min, followed by 40 cycles of 95 °C for 20 s, and 66 °C for 1 min) on the ViiA™7 Real Time PCR System (Life Technologies). Upon completion of the PCR run, an endpoint Allelic Discrimination read was performed to determine the genotype for each sample (60 °C for 30 s). The ViiA™7 Real Time PCR System (Life Technologies) software assigned genotypes based on the [VIC/FAM] fluorescence of each sample. A genotype call was deemed ‘Undetermined’ if the Ct value was > 35 or reported by the software. Further details on the AhR genotyping methods are found in the Data in Brief Article [54].

2.6. Tumor mutation analysis

Genetic mutation studies were conducted on PBDE-induced liver tumors from male and female rats and mice to characterize the molecular nature of the tumors. FFPE mouse and rat hepatocellular tumors arising either spontaneously or from chronic PBDE exposure as well as age-matched normal liver tissue were used for mutation analyses. DE-71 exposed and control liver tissue were obtained from rats and mice in this study. The hepatocellular tumor tissues chosen for molecular biology analysis were based on their overall size and viability (minimal to no necrosis or hemorrhage observed microscopically) in order to maximize the amount and quality of DNA obtained from FFPE sections. Further details on the mutation analysis methods are found in the Data in Brief article [54].

2.7. Quantitation of PBDE-47, 99, and 153 tissue levels

PBDE-47, 99, and 153 levels were determined in liver, fat, and plasma of male and female rats (stored at ~80 °C until analyses) and in liver and fat of male (except for 30 mg/kg) and female mice using validated analytical methods. Lipid levels in liver and adipose in male and female rats was determined following extraction with chloroform:methanol (1:1, v/v), hydrolysis with acid, reaction with vanillin reagent followed by detection at 490 nm to allow reporting tissue levels as µg/g lipid in the tissue. There were 4–15 tissue samples available per dose level/sex/species. The methods for the PBDE tissue analysis are found in the Data in Brief Article [54].

3. Results

3.1. Body weights and survival

There were no treatment-related effects on littering parameters after in utero/postnatal PBDE exposure in rats (Table 1). Final mean body weights of 0, 3, 15, and 50 mg/kg male rats and 3 and 15 mg/kg female rats were similar to controls (Table 2). Final mean body weights of 50 mg/kg female rats were reduced. Mean body weights of 30 and 100 mg/kg male and female mice were reduced relative to controls, and this reduction in body weight was attributed to the development of treatment-related liver tumors. Early deaths in rats and mice were

| Table 1 | Littering Parameters for Wistar Han rats after in utero/postnatal exposure. |
|---------|-----------------------------|
| Dose (mg/kg) | 0 | 3 | 15 | 50 |
| Time-Mated Females (GD 6) | 62 | 52 | 52 | 62 |
| Females Pregnant (%) | 54 (87%) | 42 (81%) | 43 (83%) | 51 (82%) |
| Dams with Evidence of Pregnancy Not Delivering (%) | 2 (4%) | 1 (2%) | 4 (9%) | 2 (4%) |
| Dams with Litters on PND 0 (%) | 52 (96%) | 41 (98%) | 39 (91%) | 49 (96%) |
| Dams, Moribund or Natural Deaths | 0 | 0 | 0 | 0 |
| Litters Post-Standardization (PND 4) | 36 | 29 | 28 | 37 |
| Post-Weaning Allocation |
| F1 Males – Core (litters) | 50 (29) | 50 (25) | 50 (25) | 50 (29) |
| F1 Females – Core (litters) | 50 (30) | 50 (25) | 50 (25) | 50 (28) |
| There were no significant differences among the groups for any of the litter parameters. |

Table 2 | Survival and final mean body weight for male and female rats and mice. |
|---------|-----------------------------|
| Dose (mg/kg) | 0 | 3 | 15 | 50 |
| Male rats |
| Survival | 36 | 35 | 38 | 27* |
| Mean body wt. (g) | 673 ± 14 | 669 ± 12 | 695 ± 14 | 678 ± 18 |
| % control | 99% | 103% | 101% |
| Female rats |
| Survival | 38 | 40 | 34 | 38 |
| Mean body wt. (g) | 390 ± 9 | 374 ± 11 | 358 ± 10 | 314 ± 12 |
| % control | 96% | 92% | 81% |
| Male mice |
| Survival | 30 | 33 | 31 | 18 month termination |
| Mean body wt. (g) | 45.7 ± 1.5 | 46.4 ± 1.1 | 38.2 ± 1.1† | 84% |
| % control | 102% | 103% |
| Female mice |
| Survival | 33 | 35 | 38 | 18 month termination |
| Mean body wt. (g) | 51.7 ± 1.4 | 53.4 ± 1.4 | 48.5 ± 1.2 | 94% |
| % control | 103% | 103% |

* Survival is significantly reduced compared to the control group, by Cox’s life table pairwise comparison, p < 0.05.
† Body weight is significantly reduced compared to the control group, by Dunnett’s test, p < 0.05.
* Number of animals surviving to end of the study of 50 per group starting the study.
b Mean ± standard error of the mean.
Table 3  
Treatment-related carcinogenic effects in male and female Wistar Han rats and B6C3F1 mice.

| Dose (mg/kg) | 0  | 3  | 15 | 50 |
|----------------|----|----|----|----|
| **Male rats liver, Number examined**| 49 | 50 | 50 | 50 |
| Hepatocellular adenoma | 3* | 2 | 4 | 8 |
| (6%) (4%) (8%) (16%) | | | | |
| Hepatocellular carcinoma | 0 | 0 | 0 | 2 |
| (0%) (0%) (0%) (0%) | | | | |
| Hepatocellular adenoma or carcinoma | 3**| 2 | 4 | 9* |
| (6%) (4%) (8%) (13%) | | | | |
| Hepatocellular adenoma, hepatocellular adenoma | 0 | 0 | 0 | 2 |
| (0%) (0%) (0%) (0%) | | | | |
| or hepatocellular carcinoma | 3**| 2 | 4 | 11* |
| (6%) (4%) (8%) (22%) | | | | |
| **Male rats thyroid, Number examined**| 45 | 45 | 48 | 46 |
| Thyroid gland: | | | | |
| follicular cell adenoma | 1* | 3 | 2 | 6* |
| (2%) (7%) (4%) (13%) | | | | |
| Thyroid gland: | | | | |
| follicular cell carcinoma | 0 | 2 | 1 | 0 |
| (0%) (4%) (2%) (0%) | | | | |
| Thyroid gland | 1 | 5 | 3 | 6* |
| (0%) (4%) (6%) (13%) | | | | |
| follicular cell adenoma or carcinomain | 0 | 0 | 0 | 2 |
| (0%) (0%) (0%) (0%) | | | | |
| **Male rats pituitary, Number examined** | 49 | 49 | 50 | 50 |
| Pituitary gland: pars distalis or unspecified site | 19** | 12 | 22 | 35** |
| adenoma | (39%) | (24%) | (44%) | (70%) |
| **Female rats liver, Number examined** | 50 | 49 | 50 | 47 |
| Hepatocellular adenoma | 3**| 2 | 8 | 16** |
| (6%) (4%) (16%) (34%) | | | | |
| Hepatocellular carcinoma | 0** | 0 | 1 | 6** |
| (2%) (2%) (13%) | | | | |
| Hepatocellular adenoma or carcinoma | 3**| 2 | 8 | 17** |
| (6%) (4%) (16%) (36%) | | | | |
| Cholangiocarcinoma | 0* | 0 | 0 | 2 |
| (0%) (0%) (0%) (0%) | | | | |
| Hepatocellular adenoma | 0** | 0 | 0 | 8** |
| (2%) (2%) (17%) | | | | |
| Hepatocellular adenoma, hepatocellular adenoma | 3**| 2 | 8 | 21** |
| or hepatocellular carcinomab | (6%) (4%) (16%) (45%) | | | |
| **Female rats uterine, Number examined** | 50 | 50 | 50 | 49 |
| Uterus, metaplasia, squamous | 0 | 2 | 5* | 6* |
| (4%) (10%) (12%) | | | | |
| Cervix, squamous hyperplasia | 2** | 3 | 4 | 8* |
| (4%) (6%) (8%) (16%) | | | | |
| Uterus polyp, stromal | 4 | 12* | 11* | 16 ** |
| (8%) (24%) (22%) (18%) | | | | |
| Uterus, stromal sarcoma | 0 | 0 | 1 | 0 |
| (0%) (2%) | | | | |
| Uterus stromal polyp or stromal sarcomac | 4 | 12* | 12* | 9 |
| (8%) (24%) (24%) (18%) | | | | |
| Vaginal polyp | 0* | 0 | 0 | 2 |
| (0%) (0%) (0%) (0%) | | | | |

| Dose (mg/kg) | 0  | 3  | 15 | 50 |
|----------------|----|----|----|----|
| **Male mice liver, Number examined** | 50 | 50 | 50 | 50 |
| Hepatocellular adenoma | 23** | 35* | 49** | 40** |
| (46%) (70%) (98%) (80%) | | | | |
| Hepatocellular carcinoma | 18** | 15 | 30* | 45** |
| (36%) (30%) (60%) (90%) | | | | |
| Hepatoblastoma | 1** | 1 | 16** | 5* |
| (2%) (2%) (32%) (10%) | | | | |
| Hepatocellular adenoma, adenoma, carcinoma, or hepatoblastoma | 31** | 40 | 49** | 47** |
| (62%) (80%) (98%) (94%) | | | | |
| **Female mice liver, Number examined** | 50 | 49 | 50 | 49 |
| Hepatocellular adenoma | 5** | 7 | 32** | 46** |
| (10%) (14%) (64%) (94%) | | | | |
| Hepatocellular carcinoma | 4** | 2 | 6 | 27** |
| (8%) (4%) (12%) (55%) | | | | |

1P ≤ 0.05, 2P ≤ 0.01.

**Historical Data:**
- **Male rats – liver tumors**
  - Historical controls, gavage corn oil: 3/99 (3.1% ± 4.3%), range.0%-6%.
  - Historical controls, all routes: 4/299 (1.4% ± 2.5%), range.0%-6%.

- **Male rats – Thyroid tumors**
  - Historical controls, gavage corn oil: 4/95 (4.1% ± 2.7%), range.2%-6%.
  - Historical controls, all routes: 5/295 (1.7% ± 2.4%), range.0%-6%.

- **Male rats – Pituitary tumors**
  - Historical controls, gavage corn oil: 4/100 (4.0% ± 2.8%), range.2%-6%.
  - Historical controls, all routes: 6/300 (2.0% ± 2.2%), range.0%-6%.

- **Female rats – liver tumors**
  - Historical controls, gavage corn oil: 4/100 (4.0% ± 2.8%), range.2%-6%.
  - Historical controls, all routes: 6/300 (2.0% ± 2.2%), range.0%-6%.

- **Female rats - uterus**
  - Historical controls, all routes. 5/5/194 (15.1% ± 6.3%), range.8%-22%.

- **Male mice – liver tumors**
  - Historical controls, gavage corn oil: 221/300 (73.7% ± 6.1%), range.62%-78%.
  - Historical controls, all routes: 545/700 (77.3% ± 8.3%), range.62%-90%.

- **Female mice – liver tumors**
  - Historical controls, gavage corn oil: 85/300 (28.3% ± 10.2%), range.16%-40%.
  - Historical controls, all routes: 320/698 (45.9% ± 21.9%), range.16%-82%.

attributed to the carcinogenic effects of the PBDE exposure (Table 2). All high dose mice were sacrificed by 18 months because at this time the mice that had died early were all diagnosed with multiple hepatocellular tumors, and the remaining mice in this group were moribund.

### 3.2. Treatment-related lesions

#### 3.2.1. Rat liver lesions

There were treatment-related benign and malignant liver tumors in male and female rats and mice (Table 3). Treatment-related non-neoplastic lesions of the liver also occurred in male and female rats and mice (Table 4) including centrilobular hepatocellular hypertrophy and fatty change. The hypertrophy was characterized by enlarged hepatocytes with granular eosinophilic cytoplasm and enlarged nuclei. The hepatocytes with fatty change were characterized by vacuolization within the cytoplasm that displaced the nucleus peripherally.

In rats, the incidences of hepatocellular neoplasms (hepatocellular adenomas and hepatocellular carcinomas) were significantly increased in both male and female rats exposed to 50 mg/kg. Additionally, the incidence of hepatocholangiomas was significantly increased in females exposed to 50 mg/kg. Hepatocellular adenomas typically consisted of well-circumscribed masses that caused compression of the surrounding hepatic parenchyma. These neoplasms were composed of a uniform population of hepatocytes and lacked the normal lobular architecture. Some adenomas displayed a little cellular atypia, but it was less common and less pronounced than that seen in the hepatocellular carcinomas. Hepatocellular carcinomas were also invasive and less well-demarcated than adenomas, and frequently contained areas of necrosis and blood-filled spaces. Their growth pattern was characterized by thickened hepatic trabeculae, composed of at least three cell layers wide compared with single-cell wide hepatic cords found in normal liver.

Hepatocholangiomas are thought to arise from cells that can differentiate into either hepatocytes or biliary cells. Hepatocholangiomas were distinguished from hepatocellular adenomas by the presence of dilated nonneoplastic bile ducts, by the increased number of bile ducts...
within hepatocellular adenomas, and by the fact that hepatocellular ade- 
onomas typically lack bile ducts. The biliary epithelial component of the 
hepatocellular adenomas was cuboidal, in contrast to the typically flattened 
epithelium found in biliary cysts.

Cholangiocarcinoma occurred in two 50 mg/kg females and cho-
langiofibrosis occurred in three other 50 mg/kg female rat.

Cholangiofibrosis is believed to be a precursor lesion to cholangio-
carcinoma [55]. Cholangiocarcinoma consisted of an irregular, rela-
tively large, noncircumscribed lesion that effaced and invaded normal 
hepatic parenchyma. The lesion consisted of fibrous connective tissue 
stroma containing numerous atypical bile ducts, which frequently 
contained mucinous material and cellular debris. The epithelium 
forming the atypical bile ducts was often discontinuous, consisted of 
large atypical cells and intestinal goblet cells, and displayed degener-
ative changes. The distinction between cholangiofibrosis and chola-
giangiocarcinoma was primarily based upon liver invasion and size.

Cholangiocarcinoma and cholangiofibrosis are uncommon in control 
rats, but have been observed in previous NTP studies of rats exposed to 
hepatic carcinogens. Consequently, the observations of these neoplasms 
in the livers of rats exposed to the PBDE mixture were considered re-
lated to exposure.

### 3.2.2. Mouse liver lesions

Hepatocellular adenomas were significantly increased in incidence 
in all dosed groups of male mice, and in the 30 and 100 mg/kg groups 
of female mice. In addition, there were significantly increased inci-
dences of hepatocellular carcinoma in 30 and 100 mg/kg males and 
100 mg/kg females; and increased incidences of hepatoblastomas in 30 
and 100 mg/kg males.

Hepatocellular adenomas were discrete, well-circumscribed lesions 
that compressed surrounding parenchyma. They were composed of ir-
regular plates of hepatocytes, which were most commonly eosinophilic, 
but also basophilic or vacuolated. Central veins and portal areas were 
generally absent. Hepatocellular carcinomas were large lesions, fre-
cently with areas of necrosis, which caused compression of, and in-
vasion into, surrounding parenchyma. Typically, hepatocellular carci-
onomas were characterized by hepatocytes forming trabeculae that were 
least three cells thick, although some of the areas of carcinomas were 
of a solid pattern of growth. Cells within the hepatocellular carcinomas 
ranged from eosinophilic to basophilic in staining, and displayed marked pleomorphism and an increased mitotic rate.

Hepatoblastomas were composed of small cells with scant cyto-
plasm and hyperchromatic, oval nuclei. Cells were often arranged in 
rows around variably sized vascular spaces. Most often, hepatoblas-

tomas arose from within a hepatocellular adenoma or carcinoma, 
and when this occurred only the hepatoblastoma was recorded.

### 3.2.3. Treatment-related findings in other tissues

When combined, the incidence of thyroid follicular cell adenoma 
and follicular cell carcinoma were significantly increased in 50 mg/kg 
male rats. The thyroid follicular cell adenoma was a discrete, com-
pressive mass composed of proliferation of follicular cells forming
complex papillary infoldings and irregular follicular structures. The cells were slightly pleomorphic and larger than normal follicular cells. Follicular cell carcinoma displayed more disorganized growth patterns and cellular pleomorphism and invaded the thyroid gland capsule. Treatment-related thyroid gland follicle hypertrophy was also present in male rats (Table 4), and progression from thyroid gland follicle hypertrophy, to follicular cell adenoma and follicular cell carcinoma occurs in rodents. These two neoplasms (thyroid gland follicular cell adenoma and carcinoma) are frequently combined for statistical purposes. There were no treatment-related thyroid tumors in mice or female rats, although the incidence and severity of thyroid gland follicle hypertrophy was increased in the treated groups (Table 4).

In male rats, there was a statistically significant increase in the incidence of adenomas of the pars distalis of the pituitary gland. Pituitary pars distalis adenomas were typically composed of sheets of chromophobes, although scattered acidophils and basophils could be found in some neoplasms. Variable-sized blood vessels, some angiectatic, as well as hemorrhage, were present in many of the neoplasms. The adenomas were usually well-demarcated masses that caused compression of the surrounding parenchyma, with larger neoplasms causing dorsal compression of the hypothalamic region of the brain. There were no increases in pituitary gland tumors in female rats or in male and female mice.

In female rats, there were significantly increased incidences of uterine stromal polyps in the 3 and 15 mg/kg groups based upon examination of the longitudinal cuts and the cross sections of the uterus. In addition, two animals in the 50 mg/kg group had vaginal polyps. The uterine stromal polyps were solitary exophytic nodules that projected into the uterine lumen. They were covered by normal-appearing endometrial surface epithelium, and supported by a broad stalk of endometrial stroma, blood vessels, and a few entrapped glands. Polyps in the vagina were similar to those found in the uterus. Stromal sarcomas were composed of spindle-shaped cells with indistinct cytoplasmic borders that invaded into the uterine wall. Squamous metaplasia was recorded in the uterus when the normal cuboidal to columnar epithelium lining the uterus or endometrial glands was replaced by stratified squamous epithelium. In the cervix, squamous hyperplasia was characterized by increased layers of the normally present squamous epithelium. There were no increases in uterine tumors in normal female mice.

3.3. Mutations in rat and mouse liver tumors

Mutational analysis of PBDE-induced rat and mouse liver tumors for Hras and/or Ctnnb1 mutations was conducted to better characterize PBDE effects (Table 5 and 6). In this study, the rat hepatocellular tumors resulting from PBDE mixture exposure (DE-71) demonstrated mutations exclusively within Hras codon 60 (20% (7/35)), rather than at the common target Hras codon 61 found in spontaneous mouse and human liver tumors. All the mutations were the same G to A transition (Gly to Asp). In the rat, Ctnnb1 mutations were fewer (11% (4/35)), more diverse, identified between codons 33 to 40, and consisted of transitions and transversions. No Hras or Ctnnb1 mutations were noted in the spontaneous hepatocellular adenomas in rats. There were no significant differences in the incidences of mutations between male and female rats (data not presented) and hence the combined data from both male and female rats are presented in Table 5.

In the mouse hepatocellular carcinomas, the incidences of Hras mutations were low (10% (6/62)) and were located within codon 61 mainly C to A or A to T transversions (Table 6). However, there were no significant differences in the incidences of Hras mutations or the mutation spectra between hepatocellular carcinomas occurring spontaneously or resulting from PBDE exposure. Conversely, statistically significant increased incidences of Ctnnb1 mutations were noted in mouse hepatocellular carcinomas resulting from administration of the PBDE mixture compare to controls. None of the hepatocellular carcinomas arising spontaneously harbored Ctnnb1 mutations. These

| Table 5 | Hras and Ctnnb1 Mutations in liver tumors in male and female rats. |
|---------|---------------------------------------------------------------|
| DE-71   | Mutation frequency  | Hras  | Ctnnb1  | Hras G76D | Ctnnb1 Cdn60 |
|         |                    | (%)   | (%)     | GGT to     | GAT          |
| Control – Non-tumor Liver | 0/10 (0%) | 0/10 (0%) | 0 | 0 |
| Control – Spontaneous Liver Tumors | 0/5 (0%) | 0/5 (0%) | 0 | 0 |
| 3 mg/kg | 1/3 (33%) | 0/3 (0%) | 1 | 0 |
| 15 mg/kg | 1/12 (8%) | 1/12 (8%) | 1 | 1 |
| 30 mg/kg | 5/20 (25%) | 3/20 (15%) | 5 | 3 |
| All DE-71 treated groups (Combined) | 7/35 (20%) | 4/35 (11%) | 7 | 4 |

Male and female Wistar Han rats were exposed to 0, 3, 15, or 50 mg/kg PBDE mixture for 2 years. Silent mutations not included; Compared to mice, the hepatocellular carcinoma (HCC) incidence was lower in the rats and hence, hepatocellular adenomas (HCA) were also included in the mutation analysis. The rat HCA and HCC included in this study included: controls (5 HCA); 3 mg/kg (3 HCA); 15 mg/kg (11 HCA and 1 HCC); 50 mg/kg (14 HCA, 6 HCC (3 HCC had Hras mutations, 1 HCC had Ctnnb1 mutation)).

3.4. Ah receptor genotyping in female rats

Because the toxicity of some other persistent organic pollutants depends on a functional AhR receptor, the status of this receptor was analyzed in female rats. The liver tumors in female rats developed independently of the AhR status of the outbred Wistar Han rats (Table 7; Supplement 3), as determined by PCR SNP analysis of the AhR type (whether wild, heterozygous, or homozygous mutant). Of the 118 liver FFPE samples analyzed for AhR genotype, 26 (22.0%) were homozygous wild type G/G; 51 (43.2%) were heterozygous G/A; and 39 (33.1%) were homozygous mutant A/A; and 2 (1.7%) undetermined. The 122 kidney FFPE samples yielded the following genotype totals: 21 (17.2%) homozygous wild type G/G, 51 (41.8%) heterozygous G/A, 38 (31.1%) homozygous mutant A/A, and 12 (9.8%) undetermined. Of the 21 female rats with liver tumors in the 50 mg/kg PBDE exposure group, 6 occurred in female rats with wild AhR (G/G); 4 in female rats with mutant AhR (A/A); 9 with heterozygous AhR (G/A); and 2 in rats with undetermined AhR status.

3.5. PBDE-47, 99, and 153 tissue concentrations

The tissue levels (taken at the terminal necropsy at 2-years) of PBDE-47, 99, and 153 for a given matrix (liver, adipose, or plasma (rat only)) generally occurred at similar tissue concentrations (μg PBDE/g tissue) in both rats and mice [54]. PBDE-153 accumulated in tissues to a greater extent in tissues than predicted based on its content in the mixture (3% PBDE-153, 36% PBDE-47, and 42%, PBDE-99). The levels of PBDEs in adipose tissue were generally 30–60 times the PBDE level in liver tissue in rats and mice. PBDE-47, 99, or 153 concentrations in adipose or liver in female rats or female mice were greater than the corresponding matrix level in male rats or male mice. The total plasma concentration of PBDE-47, 99, and 153 (combined) in male and female rats at 50 mg/kg was 15.09 and 24.68 μg PBDE/g plasma, respectively (6560 and 10,730 μg PBDE/g lipid, using plasma lipid concentration of
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Table 6

| DE-71 | Hras (%) | Ctnnb1 (%) |
|-------|---------|------------|
| Control – Non-tumor liver | 0/8 (0%) | 0/8 (0%) |
| Control – Spontaneous hepatocellular tumors | 2/17 (12%) | 0/17 (05)** |
| 3 mg/kg | 2/14 (14%) | 3/14 (21%) |
| 30 mg/kg | 3/19 (16%) | 1/19 (5%) |
| 100 mg/kg | 1/29 (3%) | 9/29 (31%)** |
| All DE-71 treated groups | 6/62 (10%) | 13/62 (21%)* |

Male and female mice were dosed with 0, 3, 30, or 100 mg/kg PBDE mixture by oral gavage for 2 years. Silent mutations are not included. Non-tumor Liver – 0 mg/kg (3 males + 5 females); Liver Tumors- 0 mg/kg (14 males + 2 females); 3 mg/kg (12 males + 2 females); 30 mg/kg (13 males + 6 females); 100 mg/kg (15 males + 14 females).

Silent mutations not included. 

*p < 0.05, **p < 0.01 by Fisher's exact test to compare each dose group to the controls## p < 0.01 for trend by the Cochran–Armitage trend test.

Table 7

| AhR genotype at exon 10 | Wild | Mutant | Heterozygous | Undetermined | Total |
|------------------------|------|--------|-------------|-------------|-------|
| G/G                     | 13   | 16     | 27          | 0           | 56    |
| (23%)                  | (29%)| (48%)  | (0%)        |             |
| A/A                     | 5    | 18     | 13          | 1           | 37    |
| (13%)                  | (49%)| (35%)  | (3%)        |             |
| G/A                     | 1    | 2      | 1           | 0           | 4     |
| (25%)                  | (50%)| (25%)  | (0%)        |             |
| 50 mg/kg – no liver tumors | 7     | 3      | 10          | 1           | 21    |
| (33%)                  | (14%)| (48%)  | (5%)        |             |

0.0023 per fraction of plasma weight [56].

The PBDE tissue levels were linearly related to the administered doses, and the incidence of liver tumors increased with increasing PBDE dose. Female rats had a higher incidence of hepatocellular tumors per dose group than male rats, and this may have been related to the higher tissue levels of PBDE-47, 99, or 153 than in male rats.

4. Discussion

In these studies, PBDE mixture (DE-71) induced treatment-related carcinogenic effects in the liver of male and female rats and mice, in the thyroid and pituitary of male rats, and the uterus of female rats. This liver carcinogenic response in both sexes and species is an unusual tissue level for a substance with a long tissue half-life, there is potential for continued exposure, as demonstrated by the high levels of PBDE found in the adipose tissue in these studies. As animals age the ability to mount an antioxidant defense decreases [71,72], and, thus, oxidative damage could become more pronounced in older animals.

PBDE metabolic alterations (e.g. upregulation of cytochrome P450s [73,74]) may also have contributed to PBDE carcinogenic effects. Up-regulation of cytochrome P450 systems after PBDE exposure can activate nuclear hormone receptors including the peroxisome proliferation-activated receptor (PPAR) and/or constitutive activated/androstane receptor (CAR) receptor [75,76], both of which regulate lipid metabolism [77]. Metabolism by cytochrome P450 enzymes can generate free reactive oxygen species [78–80], and oxidative damage is a key characteristic for increased risk for liver cancer [81].

Studies in the literature, report that PBDE exposure may lead to DNA adduct formation. PBDEs undergo metabolism to hydroxylated metabolites [82] and to PBDE-quinoine metabolites that interact with DNA forming DNA adducts [83]. In vitro studies show that various types of DNA adducts may be generated from PBDE quinone (2-(2′-bromophenoxyl)-benzoquinone (2′4′BepQ-BQ)) including deoxyguanosine (dG), 2′-deoxyadenosine (dA), 2′-deoxycytidine (dC), or thymidine [84].

Fatty change and cytoplasmic vacuolization in the liver (an indicator for liver fat accumulation [85]) were found after PBDE exposure. Fat accumulation may be a factor that increases the risk for liver cancer development [81]. Other contributing factors to the PBDE-induced liver toxic and carcinogenic effects were the occurrence of hepatocyte hypertrophy; however, not all chemical exposures that cause this effect go on to be liver carcinogens [86,87].

PBDE exposures have been found to cause alterations in thyroid hormone levels [88]. Hydroxylated PBDE metabolites are more toxic [89] than the parent compound, and have higher binding affinity to thyroid receptors [90]. Changes in thyroid hormone levels may not only affect carcinogenic processes in the thyroid, but also in the liver as these hormones are critical regulators of hepatic lipid metabolism [91]. PBDE-induced changes in thyroid hormone levels can occur in both rodents and humans. For example, a human PBDE serum level of 130 pg/g was associated with a decrease in T₄ serum level [92]. In contrast, in rodents, oral PBDE exposure in the mg/kg range (~PBDE plasma level in μg/g range) is usually needed to lower T₄ serum levels [88].

PBDE exposures have the potential to also alter estrogen levels. Binding of hydroxylated PBDE metabolites to sulfotransferase can may be related to oxidative damage. Other in vitro and in vivo studies reported in the literature found that PBDE exposure caused oxidative damage and increased reactive oxygen species [64–69]. In addition, Br-C bonds once broken release free radicals [70]. Oxidative damage can eventually lead to DNA damage and mutations [71]. With the PBDEs, DNA damage is not repaired, which may contribute to the increased risk of cancer development [81].
interfere with sulfonation of estrogen and its excretion, ultimately leading to increased estrogen levels [92]. Such effects on hormone levels could contribute to PBDE carcinogenic effects in the uterus [94] or pituitary gland [95] as occurred in these studies.

The PBDE mixture (DE-71) used in this study contained very low levels of brominated dioxins and furans, and these brominated dioxins and furans have a toxic equivalence factor of 10% that of TCDD [96]. However, to fully investigate whether PBDE-induced liver tumors in the outbred Wistar rat were related to activation of AhR, we characterized AhR status in female Wistar rats. In this outbred rat, the AhR genotype at exon 10 may have a wild, mutant, or heterozygous sequence ([39,40]; Yao et al., 2012). We found that the occurrence of treatment-related liver tumors in female rats was not correlated with AhR type, and the PBDE-induced female rat liver tumors occurred in animals with wild, mutant, or heterozygous AhR. Thus, the occurrence of PBDE-induced liver tumors did not require a functional Ah receptor, and PBDEs themselves are generally not thought to be activators of the AhR [96].

5. Conclusion

In conclusion, PBDE mixture exposure (DE-71) caused toxic and carcinogenic effects in rats and mice. Several key carcinogenic characteristics reported in the literature may contribute to the PBDE carcinogenic activity observed in this study including oxidative damage, alteration in hormone homeostasis, and molecular and epigenetic changes in target tissue. Further work is needed to compare the sensitivity PBDE toxic effects in rodents and humans.

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Conflict of interest

None of the authors have any conflict of interest to declare.

Transparency document

The Transparency document associated with this article can be found in the online version.

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