**Table 3A**
GO-annotated biological categories for genes up-regulated in the embryo following maternal thalidomide exposure.

| Category | Term | Count | P value | List Total | Pop Hits | Pop Total | Log 2 Fold Change |
|---|---|---|---|---|---|---|---|
| GOTERM_BP_4 | Biological Process (level 4) | | | | | | |
| GO:0015931 | Nucleobase, nucleoside, nucleotide and nucleic acid transport | 15 | 0.001 | 694 | 100 | 13,532 | +2.92 |
| GO:0050658 | RNA transport | 13 | 0.002 | 694 | 87 | 13,532 | +2.91 |
| GO:0050657 | Nucleic acid transport | 13 | 0.002 | 694 | 87 | 13,532 | +2.91 |
| GO:0051236 | Establishment of RNA localization | 13 | 0.002 | 694 | 87 | 13,532 | +2.91 |
| GO:0051028 | mRNA transport | 11 | 0.007 | 694 | 79 | 13,532 | +2.71 |
| GO:0045941 | Positive regulation of transcription | 40 | 0.000 | 694 | 326 | 13,532 | +2.39 |
| GO:0007507 | Heart development | 15 | 0.006 | 694 | 128 | 13,532 | +2.28 |
| GO:0051276 | Chromosome organization and biogenesis | 45 | 0.000 | 694 | 394 | 13,532 | +2.23 |
| GO:0006281 | DNA repair | 28 | 0.001 | 694 | 267 | 13,532 | +2.04 |
| GO:0022618 | Protein–RNA complex assembly | 12 | 0.035 | 694 | 116 | 13,532 | +2.02 |
| GO:0031325 | Positive regulation of cellular metabolic process | 42 | 0.000 | 694 | 416 | 13,532 | +1.97 |
| GO:0009893 | Positive regulation of metabolic process | 44 | 0.000 | 694 | 445 | 13,532 | +1.93 |
| GO:0051169 | Nuclear transport | 14 | 0.035 | 694 | 145 | 13,532 | +1.88 |
| GO:0016481 | Negative regulation of transcription | 28 | 0.003 | 694 | 300 | 13,532 | +1.82 |
| GO:0006461 | Protein complex assembly | 27 | 0.005 | 694 | 295 | 13,532 | +1.78 |
| GO:0045786 | Negative regulation of progression through cell cycle | 19 | 0.022 | 694 | 209 | 13,532 | +1.77 |
| GO:0009892 | Negative regulation of metabolic process | 38 | 0.002 | 694 | 436 | 13,532 | +1.70 |
| GO:0031324 | Negative regulation of cellular metabolic process | 32 | 0.009 | 694 | 387 | 13,532 | +1.61 |
| GO:0000074 | Regulation of progression through cell cycle | 42 | 0.005 | 694 | 526 | 13,532 | +1.56 |
| GO:0051726 | Regulation of cell cycle | 42 | 0.005 | 694 | 529 | 13,532 | +1.55 |
| GO:0007010 | Cytoskeleton organization and biogenesis | 41 | 0.008 | 694 | 526 | 13,532 | +1.52 |
| GO:0016192 | Vesicle-mediated transport | 39 | 0.013 | 694 | 509 | 13,532 | +1.49 |
| GOTERM_CC_4 | Cellular component (level 4) | | | | | | |
| GO:0005830 | Cytosolic ribosome (sensu Eukaryota) | 10 | 0.017 | 743 | 76 | 14,201 | +2.51 |
| GO:0005681 | Spliceosome | 16 | 0.004 | 743 | 134 | 14,201 | +2.28 |
| GO:0000785 | Chromatin | 22 | 0.001 | 743 | 194 | 14,201 | +2.17 |
| G O:0031965 | Nuclear membrane | 15 | 0.012 | 743 | 136 | 14,201 | +2.11 |
| GO:0012506 | Vesicle membrane | 13 | 0.030 | 743 | 125 | 14,201 | +1.99 |
| GO:0005874 | Microtubule | 23 | 0.005 | 743 | 233 | 14,201 | +1.89 |
| GO:0005635 | Nuclear envelope | 18 | 0.015 | 743 | 182 | 14,201 | +1.89 |
| GO:0005768 | Endosome | 18 | 0.028 | 743 | 196 | 14,201 | +1.76 |
| GO:0005694 | Chromosome | 32 | 0.011 | 743 | 385 | 14,201 | +1.59 |
| GO:0030529 | Ribonucleoprotein complex | 41 | 0.047 | 743 | 584 | 14,201 | +1.34 |
| GOTERM_MF_4 | Molecular Function (level 4) | | | | | | |
| GO:0051427 | Hormone receptor binding | 10 | 0.001 | 578 | 57 | 12,599 | +3.82 |
| GO:0051020 | GTPase binding | 11 | 0.003 | 578 | 78 | 12,599 | +3.07 |
| GO:0003712 | Transcription cofactor activity | 41 | 0.000 | 578 | 311 | 12,599 | +2.87 |
| GO:0003779 | Actin binding | 27 | 0.002 | 578 | 302 | 12,599 | +1.95 |
| GO:0008234 | Cysteine-type peptidase activity | 15 | 0.027 | 578 | 172 | 12,599 | +1.90 |
| KEGG_PATHWAY | | | | | | | |
| hsa05220 | Chronic myeloid leukemia | 10 | 0.016 | 225 | 74 | 4,214 | +2.53 |
| hsa05222 | Small cell lung cancer | 11 | 0.016 | 225 | 87 | 4,214 | +2.37 |
| hsa05215 | Prostate cancer | 11 | 0.016 | 225 | 87 | 4,214 | +2.37 |
| hsa04350 | TGF-beta signaling pathway | 11 | 0.020 | 225 | 90 | 4,214 | +2.29 |
| hsa04912 | GnRH signaling pathway | 11 | 0.026 | 225 | 94 | 4,214 | +2.19 |
| hsa04910 | Insulin signaling pathway | 14 | 0.025 | 225 | 134 | 4,214 | +1.96 |

cle mass during early limb myogenesis [32]. Although these facts may implicate IGF signals as a potential mediator of thalidomide embryopathy, the present study did not find significant expression or thalidomide-induced alteration in the global pattern of several key transcripts in this signaling pathway, including IGFBPs 13, 5, 6 and 7, IGF1, IGF1R, and IRS14 (data not shown). It is certainly plausible that thalidomide exposure may locally alter upstream events in IGF-1 signaling without necessarily altering the molecular abundance profiles of the pathway in the developing limb of monkey embryos. On the other hand, our preliminary microarray analysis does find evidence for the up-regulation of GSK3β and AKT1 transcripts that are downstream in the insulin signaling pathway. Effects on TGF-beta and WNT signaling may be critical here. Thalidomide-induced oxidative stress in chick embryos can enhance signaling through BMPs (bone morphogenetic proteins), leading to up-regulation of the WNT antagonist Dickkopf1 (Dkk1) and subsequent cell death [33]. We note here a significant up-regulation of genes in the TGF-beta pathway and similarities with genes in the cytoskeletal cycle and WNT pathways for the murine FAS [12].

Some of the responsive genes found in this study are known to play roles in vascular development pathways. For example, vascular endothelial growth factor (VEGF) was down-regulated and platelet-derived growth factor receptor β (PDGFRβ) was up-regulated during early stages in thalidomide embryopathy. VEGF is a key stimulator of vascular cell migration and proliferation and acts directly on endothelial cells, whereas PDGF attracts connective tissue cells that can also stimulate angiogenesis. The reciprocal effect on these transcript profiles, potentially leading to an overall decrease in VEGF/PDGFRβ activities, might be predicted to interfere with vascular cell recruitment and proliferation in the developing embryo or limb. It is well known that thalidomide reduces the activity or production of VEGF and TNF-α, leading to inhibition of angiogenesis [34]. The present microarray data are consistent with this effect. Furthermore, VEGF stimulates PDGFRβ and induces tyrosine phosphorylation [35]. The reciprocal effect that maternal thalidomide exposure had on these transcripts may suggest a key event in the programming or induction of vascular cells or their progenitors has been disrupted within 6 h after exposure. This notion is supported by the study of D'Amato et al. [36] that

**Table 3B**
GO-annotated biological categories for genes down-regulated in the embryo following maternal thalidomide exposure.

| Category | Term | Count | P value | List Total | Pop Hits | Pop Total | Log2Fold Change |
|---|---|---|---|---|---|---|---|
| GOTERM_BP_4 | Biological Process (level 4) | | | | | | |
| GO:0008284 | Positive regulation of cell proliferation | 24 | 0.000 | 556 | 240 | 13,532 | −2.43 |
| GO:0007517 | Muscle development | 16 | 0.006 | 556 | 177 | 13,532 | −2.20 |
| GO:0009889 | Regulation of biosynthetic process | 18 | 0.005 | 556 | 207 | 13,532 | −2.12 |
| GO:0006417 | Regulation of translation | 14 | 0.027 | · 556 | 174 | 13,532 | −1.96 |
| GO:0032940 | Secretion by cell | 23 | 0.004 | 556 | 287 | 13,532 | −1.95 |
| GO:0001944 | Vasculature development | 15 | 0.026 | 556 | 191 | 13,532 | −1.91 |
| GO:0045045 | Secretory pathway | 18 | 0.020 | 556 | 239 | 13,532 | −1.83 |
| GO:0051246 | Regulation of protein metabolic process | 23 | 0.008 | 556 | 307 | 13,532 | −1.82 |
| GO:0006873 | Cellular ion homeostasis | 16 | 0.031 | 556 | 214 | 13,532 | −1.82 |
| GO:0006954 | Inflammatory response | 22 | 0.012 | 556 | 301 | 13,532 | −1.78 |
| GO:0016192 | Vesicle-mediated transport | 35 | 0.004 | 556 | 509 | 13,532 | −1.67 |
| GO:0042127 | Regulation of cell proliferation | 34 | 0.005 | 556 | 499 | 13,532 | −1.66 |
| GO:0019752 | Carboxylic acid metabolic process | 36 | 0.012 | 556 | 572 | 13,532 | −1.53 |
| GO:0046907 | Intracellular transport | 40 | 0.043 | 556 | 714 | 13,532 | −1.36 |
| GOTERM_CC_4 | Cellular component (level 4) | | | | | | |
| GO:0005625 | Soluble fraction | 21 | 0.004 | 602 | 244 | 14,201 | −2.03 |
| GO:0005768 | Endosome | 15 | 0.039 | 602 | 196 | 14,201 | −1.81 |
| GO:0005789 | Endoplasmic reticulum membrane | 28 | 0.031 | 602 | 435 | 14,201 | −1.52 |
| GO:0044432 | Endoplasmic reticulum part | 30 | 0.047 | 602 | 494 · | 14,201 | −1.43 |
| GO:0005624 | Membrane fraction | 44 | 0.026 | 602 | 749 | 14,201 | −1.39 |
| GO:0005783 | Endoplasmic reticulum | 46 | 0.049 | 602 | 827 | 14,201 | −1.31 |
| GOTERM_MF_4 | Molecular Function (level 4) | | | | | | |
| GO:0030594 | Neurotransmitter receptor activity | 14 | 0.000 | 531 | 99 | 12,599 | −3.36 |
| GO:0051020 | GTPase binding | 11 | 0.002 | 531 | 78 | 12,599 | −3.35 |
| GO:0016747 | Transferase activity, transferring other than amino-acyl groups | 15 | 0.028 | 531 | 188 | 12,599 | −1.89 |
| GO:0004175 | Endopeptidase activity | 31 | 0.012 | 531 | 463 | 12,599 | −1.59 |
| KEGG_PATHWAY | | | | | | | |
| hsa04640 | Hematopoietic cell lineage | 12 | 0.005 | 223 | 85 | 4,214 | −2.67 |
| hsa04612 | Antigen processing and presentation | 10 | 0.024 | 223 | 80 | 4,214 | −2.36 |

Results for the embryo 6 h after a teratogenic dose of thalidomide (20 mg/kg) on day 26 of gestation for 1281 significantly up-regulated genes (Table 3A) and 1081 significantly down-regulated genes (Table 3B) based on the population of arrayed genes. The annotated system used the NIH/NIAID Database for Annotation, Visualization, and Integrated Discovery (DAVID) at level 4. Count refers to the number of altered genes in the ontology (min = 10 and max = 50). P value refers to results from Fisher exact test ($P \leq 0.05$); List Total refers to the number of annotated genes on the array; Pop Hits and Pop Total refers to the number of annotated genes in the database for the category and overall; Log 2 Fold Change is computed as the mean Log 2 (treated/control) for genes in the category.

suggested limb defects caused by thalidomide were secondary to inhibition of blood vessel growth in the developing limb bud. Down-regulation of the vascular development program is consistent with this notion and with the supposition that correct limb bud formation requires a complex interaction of both vasculogenesis and angiogenesis during development [37]. Perhaps these genes might be considered as potential biomarkers of thalidomide-induced teratogenesis in cynomolgus monkeys. A recent study with the teratogenic thalidomide analogue, CPS49, has shown direct evidence for the suppression of endothelial angiogenetic sprouting and failure to establish a normal vascular network as a key event in thalidomide embryopathy [38]. CPS49 mimics the antiangiogenic properties, but not anti-inflammatory properties, of thalidomide.

Finally, the inflammatory response pathway was found to be significantly down-regulated in the early thalidomide embryome. Although down-regulation of the inflammatory response might be anticipated to protect the embryo, studies in laboratory animals have implicated a role for reactive oxygen species (ROS) in thalidomide embryopathy [39]. In that study, thalidomide was found to preferentially increase ROS in embryonic limb cells from a sensitive species (rabbit) but not the insensitive species (rat). Down-regulation of the inflammatory pathways in thalidomide-exposed monkey embryos reinforces this notion.

In conclusion, these findings show that thalidomide exposure perturbs a general program of morphoregulatory processes in the cynomolgus monkey embryo. Bioinformatics analysis has now identified many key pathways implicated in thalidomide

embryopathy in cynomolgus monkeys, and has also revealed some novel processes that can help unravel the mechanism of this important developmental phenotype. Several pathways, including actin cytoskeleton remodeling and downstream insulin signaling-related genes, in addition to vascular development pathways may provide candidate biomarkers for key events underlying the teratogenicity of thalidomide in primates. To clarify the molecular mechanisms further studies must examine protein expression, phosphorylation, and other modifications in the precursor target organ system.

## Conflict of interest statement

None.

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RESEARCH ARTICLE

# Gender-related difference in the toxicity of 2-(2'-hydroxy-3',5'-di-*tert*-butylphenyl)benzotriazole in rats: Relationship to the plasma concentration, *in vitro* hepatic metabolism, and effects on hepatic metabolizing enzyme activity

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**Abstract**
Previously, we showed that the toxic susceptibility of male rats to an ultraviolet absorber, 2-(2'-hydroxy-3',5'-di-*tert*-butylphenyl)benzotriazole (HDBB), was nearly 25 times higher than that of females. The present study aimed to clarify the mechanism of gender-related differences in HDBB toxicity. Male and female rats were given HDBB by gavage at 0.5, 2.5, or 12.5 mg/kg/day for 28 days, and plasma HDBB levels were measured at various time points by using liquid chromatography–tandem mass spectrometry. HDBB was rapidly absorbed and eliminated from the plasma in both sexes, and no sexual variations were found in the plasma levels. In the plasma, HDBB metabolites were not detected at any dose by the liquid chromatography–photodiode array detector. In an *in vitro* metabolic study using hepatic microsomes from male and female rats, HDBB was slightly metabolized, but no sexual differences were found in the residual HDBB ratio after a 60-minute incubation with an NADPH-generation system. Following 28-day HDBB administration, sexually different changes were found in cytochrome P450–dependent microsomal mixed-function oxidase activities in the liver. In males, 7-ethoxyresorufin *O*-deethylase activity decreased and lauric acid 12-hydroxylase activity increased at all doses. Decreases in aminopyrine *N*-demethylase activity and testosterone 2α- and 16α-hydroxylase activity were also found at 2.5 mg/kg and above in males. In females, the only significant change was increased lauric acid 12-hydroxylase activity at 12.5 mg/kg. These findings indicate that HDBB would have hepatic peroxisome proliferative activity, and the difference in susceptibility of male and female rats to this effect might lead to marked gender-related differences in HDBB toxicity.

**Keywords:** *Benzotriazole UV absorber; gender-related difference; hepatic metabolizing enzyme activity; in vitro hepatic metabolism; plasma concentration; rat*

## Introduction

2-(2'-hydroxy-3',5'-di-*tert*-butylphenyl)benzotriazole (CAS No. 3846-71-7; HDBB) is an ultraviolet (UV) absorber used in plastic resin products, such as building materials and automobile components (METI, 2006). Previously, we showed a marked gender-related difference in the toxicity of HDBB in 28-day and 52-week repeated oral dose toxicity studies using rats (Hirata-Koizumi et al., 2007; 2008a). In the 28-day study, toxic effects were observed mainly in the liver, such as hypertrophy and vacuolar degeneration of hepatocytes, focal necrosis, and bile duct proliferation. HDBB also caused anemia,

degeneration and hypertrophy of myocardium in the heart, hypertrophy of tubular epithelium in the kidneys, and diffuse follicular cell hyperplasia in the thyroids. Adverse effects were found even at the lowest dose of 0.5 mg/kg in males, but in females, they were detected only at 12.5 mg/kg and above. In the 52-week study, histopathological findings in the liver included precancerous changes (i.e., altered hepatocellular foci). Based on hepatic changes, the no observed adverse effect level (NOAEL) for repeated dose toxicity of HDBB was concluded to be 0.1 mg/kg/day in males and 2.5 mg/kg/day in females. These findings show that male rats have a nearly 25 times higher susceptibility to HDBB toxicity than female rats.

Gender-related differences in the toxic susceptibility of rats have been documented for many other industrial chemicals (Ema et al., 2008; Muraoka and Itoh, 1980), environmental pollutants (Knuckles et al., 2004), insecticides (Agarwal et al., 1982; Carlson and DuBois, 1970), and pharmaceuticals (Coleman et al., 1990; McGovren et al., 1981; Stern et al., 2007; Wang et al., 2001). Various causes of such sexual differences are indicated mainly for toxicokinetic determinants, such as hepatic metabolism (Gad, 2006) and membrane transporter in various organs, including the kidneys and intestine (Morris et al., 2003). For example, Coleman et al. (1990) reported that higher sensitivity of male rats to hematotoxicity of dapsone, which is a major component of the multidrug regimen for the treatment of leprosy, was due to the greater capacity for N-hydroxylation. Another example was an amino-acid antitumor agent, acivicin, of which the $LD_{50}$ was much higher in male mice than in females. McGovren et al. (1981) showed that the plasma half-time was much longer in female mice and speculated that the sexual variation may be related to differences in renal excretion.

The aim of the present study is to clarify the mechanism for gender-related differences in HDBB toxicity. We determined plasma HDBB levels in male and female rats given HDBB, by liquid chromatography–tandem mass spectrometry (LC/MS), and the metabolites in plasma were analyzed by using a liquid chromatography–photodiode array detector. The enzymatic transformation of HDBB was also investigated with hepatic S9 fractions and microsomes prepared from male and female rats, and with the single-enzyme systems, microsomes containing cDNA-expressed individual rat cytochrome P450 (CYP) enzymes. Further, we investigated the effects of HDBB on typical CYP-dependent microsomal mixed-function oxidase (MFO) activities [i.e., aminopyrine N-demethylation, 7-ethoxycoumarin O-deethylation (ECOD), 7-ethoxyresorufin O-deethylation (EROD), testosterone 6β-, 2α- and 16α-hydroxylation, and

lauric acid 12-hydroxylation], in the liver, which is the main target of HDBB.

## Materials and methods

This study was performed at Drug Safety Research Laboratories (Kagoshima, Japan) and the Pharmacokinetics and Bioanalysis Center (Kainan, Japan) of Shin Nippon Biomedical Laboratories, Ltd. (SNBL) in 2007–2008. The experiment was approved by the Institutional Animal Care and Use Committee of SNBL and was performed in accord with the ethics criteria contained in the bylaws of the Committee.

### Materials

HDBB (Lot no. AY11) was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). The HDBB used in this study was 100% pure and was stored in a light-resistant, tight container at room temperature until use. 2-(3′,5′-di-*tert*-butyl-2′-hydroxyphenyl)-5-chlorobenzotriazole (DBHCB), used as an internal standard for plasma HDBB determination, was also obtained from Tokyo Chemical Industry Co., Ltd. Corn oil, formic acid (special grade), acetonitrile [high-performance liquid chromatography (HPLC) grade] and aminopyrine (for biochemistry) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 7-ethoxycoumarin and 7-ethoxyresorufin were from Sigma-Aldrich Japan K.K. (Tokyo, Japan), and [4-$^{14}$C]-testosterone and [1-$^{14}$C]-lauric acid were from GE Healthcare Bio-Sciences KK (Tokyo, Japan). All other reagents and solvents were of the highest quality commercially available.

For *in vitro* metabolism studies, hepatic S9 fractions and microsomes prepared from male and female Sprague-Dawley (SD) rats were purchased from Xenotech LLC (Lenexa, Kansas, USA) and BD Gentest (Woburn, Massachusetts, USA), respectively. The single-enzyme systems, microsomes prepared from baculovirus-infected insect cells expressing CYP1A1, CYP1A2, CYP2A1, CYP2A2, CYP2B1, CYP2C6, CYP2C11, CYP2C12, CYP2C13, CYP2D1, CYP2D2, CYP2E1, CYP3A1, or CYP3A2, were also obtained from BD Gentest.

### Animals and housing conditions

Crl:CD(SD) rats (4 weeks old) were purchased from Hino Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan). After a 7-day acclimation, they were subjected to treatment at 5 weeks of

age. On the day before the first dosing, rats found to be in good health were selected and assigned to three groups of 4 males and 4 females to measure plasma HDBB levels and to four groups of 5 males and 5 females to determine hepatic CYP activity by stratified randomization (MiTOX System, ver. 2.0; Mitsui Zosen Systems Research Inc., Chiba, Japan), according to body weight to minimize bias in body weight among groups.

All animals were maintained in an air-conditioned room at 21.8–22.6°C with a relative humidity of 43–52%, a 12-hour light-dark cycle, and ventilation with 15 air changes/hour. Animals were housed individually in stainless cages suspended over a cage board. A basal diet (CE-2; CLEA Japan, Inc., Tokyo, Japan) and water, which meets the drinking water standard under the Water Works Law of Japan, were provided *ad libitum*.

### Measurement of plasma HDBB concentration

Male and female rats (4/sex/group) were given HDBB by gavage once-daily for 28 days. The dosage levels of HDBB were determined to be 0.5, 2.5, or 12.5 mg/kg/day, based on the results of our previous 28-day repeated-dose toxicity study (Hirata-Koizumi et al., 2007). In this previous study, male and female rats were given HDBB by gavage at 0.5, 2.5, 12.5, or 62.5 mg/kg/day, and adverse effects, mainly on the liver, were found at all doses in males and at 12.5 mg/kg and above in females.

Dosing solutions were prepared as a suspension in corn oil. The volume of each dose was adjusted to 10 mL/kg of body weight, based on the latest body weight. The formulations were kept cool in a light-resistant, tight container until dosing and were used within 7 days after preparation. Stability under refrigerated conditions was confirmed up to 7 days in the previous 28-day repeated-dose toxicity study (Hirata-Koizumi et al., 2007).

All males and females were observed twice-daily for clinical signs of toxicity, and body weight was measured on days 1, 7, 14, 21, and 28 of administration. Blood samples (approximately 0.2 mL/animal) were collected from the jugular vein at 1, 2, 5, 8, and 24 hours after the 1st dose, just before the 7th, 14th and 28th doses, and at 1, 2, 5, 8, and 24 hours after the 28th dose. All surviving animals were euthanized by ether anesthesia after the completion of final blood sampling.

The blood samples were centrifuged at 4°C and 1,710 × $g$ for 15 minutes to obtain plasma. The plasma (0.05 mL) was mixed with acetonitrile (0.05 mL) and internal standard solution (DBHCB, 0.05 mL) and centrifuged at 12,000 rpm for 5 minutes at 4°C. The

supernatant (10 μL) was analyzed by using a CAPCELL PAK C8 DD column [2.0 (inner diameter) × 75 mm, 3 μm; Shiseido Co., Ltd., Tokyo, Japan] on a Shimadzu LC-10A HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a photodiode array detector (SPD-M20A; Shimadzu Corporation) set at 200–400 nm and a triple quadrupole mass spectrometer (API 3000; Applied Biosystems Japan, Tokyo, Japan). The mobile phase consisted of acetonitrile and 0.1% formic acid (75:25, v/v) at a flow rate of 0.2 mL/minute for 15 minutes. Under this condition, the retention time of HDBB was about 9 minutes and the lower limit of qualification was 0.02 μg/mL.

Toxicokinetic parameters of HDBB, maximum plasma concentration ($C_{max}$), time to $C_{max}$ ($T_{max}$), and area under the plasma concentration-time curve from time zero to 24 hours ($AUC_{0-24h}$), were assessed by standard noncompartmental analysis, using WinNonlin version 4.0 (Pharsight Corporation, Mountain View, California, USA).

### In vitro *metabolism reaction*

Since the metabolic products of HDBB have not been elucidated, metabolic activity of hepatic S-9 fractions and microsomes from male and female rats was determined by measuring the disappearance rate of HDBB after incubation. HDBB was dissolved in acetonitrile at 4.5 mmol/L, and 0.005 mL of the HDBB solution was incubated with 0.05 mL of S-9 fractions or microsomes (20 mg/mL) in 0.1 mol/L of phosphate buffer (pH 7.4) containing 0.05 mmol/L of EDTA. Final HDBB concentration in the incubation mixtures was 45 μmol/L. The incubation was carried out at 37°C in air. After a 5-minute preincubation, the reaction was initiated by adding the NADPH-generating system (15.5 mmol/L NADP+, 33 mmol/L glucose-6-phosphate, 4 U/mL glucose-6-phosphate dehydrogenase, and 33 mmol/L MgCl₂), and incubated for 60 minutes. The reaction was terminated by the addition of 1 mL of ice-cold acetonitrile, and the solution was centrifuged for 15 minutes at 10,000 × $g$ and 4°C. The supernatant (0.05 mL) was eluted by using the above-mentioned HPLC system, and the elution was monitored at 346 nm with a Shimadzu SPD-10A or 20A UV detector (Shimadzu Corporation). All experiments were performed in duplicates. The residual HDBB ratio was calculated by dividing the peak area of HDBB after a 60-minute incubation with that of the control, in which the incubation system was inactivated by the addition of 1 mL of acetonitrile prior to incubation ($n = 1$).

To examine the role of individual CYP isoforms involved in the metabolism of HDBB, each of the

recombinant CYPs (200 pmol of CYP/mL) was incubated with HDBB, using the same method as mentioned above, except that potassium phosphate buffer was used instead of phosphate buffer. Microsomes from insect cells infected with wild-type baculovirus (BD Gentest), which contains negligible amounts of CYP, served as controls.

### Effect of HDBB on Hepatic CYP Activity

HDBB was administered by gavage to male and female rats (5/sex/group) at 0.5, 2.5, or 12.5 mg/kg/day for 28 days. Control groups (5 males and 5 females) received the vehicle only. Preparation of the dosing solutions, observation of the clinical signs of toxicity and measurement of body weight, was performed in the same way as the above-mentioned study for determining plasma HDBB levels. The day after the last administration, the animals were euthanized by exsanguination under deep anesthesia by the intraperitoneal (i.p.) injection of pentobarbital sodium. The surface of the body, organs, and tissues of the entire body were grossly observed. The liver was then collected and weighed. After perfusion to remove blood, the right lobe was homogenized in a 9-fold volume of ice-cold Tris buffer (50 mmol/L Tris-hydrochloric acid buffer containing 0.25 mol/L sucrose; pH 7.4) and centrifuged at $9,000 \times g$ for 30 minutes. The supernatant was centrifuged at $105,000 \times g$ for 60 minutes, the pellet was suspended in Tris buffer, and centrifugation was repeated. These preparations were performed at 4°C. The resulting pellet was suspended in Tris buffer in an amount equal to the liver weight and used as hepatic microsomes.

The concentration of hepatic microsomal protein was determined by using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Inc., Hercules, California, USA) with bovine serum albumin (BSA) as a standard. The total CYP content was measured by the CO difference spectrum method (Omura and Sato, 1964). Seven types of MFO activities (i.e., aminopyrine *N*-demethylation, ECOD, EROD, testosterone 6β-, 2α- and 16α-hydroxylation, and lauric acid 12-hydroxylation activity) in hepatic microsomes were determined by standard procedures. Briefly, aminopyrine *N*-demethylase activity was assayed by determining the formation of monomethylol dimethylhydantoin from aminopyrine spectrophotometrically. ECOD and EROD activities were measured as the rate of conversion of 7-ethoxycoumarine to 7-hydroxycoumarin, and of 7-ethoxyresorufin to resorufin, respectively, using a spectrophotofluorometer. Testosterone 6β-, 2α-, and 16α-hydroxylase activities and lauric acid 12-hydroxylase activity

were assayed by using $^{14}$C-labeled substrates, and the respective activities were determined by quantifying the formed amount of 6β-, 2α-, and 16α-hydroxytestosterone and 12-hydroxylauric acid by radio-HPLC. Each type of MFO activity was assayed by using NADPH as the sole electron source.

### Data analysis

Body weight and absolute and relative liver weight were analyzed by Bartlett's test for homogeneity of variance ($P < 0.05$). When homogeneity was recognized, Dunnett's test was applied to compare the mean value in the control group with that in each test article group ($P < 0.01$ or 0.05). If not homogenous, the data were rank-converted and a Dunnett-type test was applied ($P < 0.01$ or 0.05).

For metabolic enzyme activity, Bartlett's test was similarly performed ($P < 0.05$). When the variance was homogenous, Williams' test, assuming a dose-related trend, was applied ($P < 0.05$). If significant differences were not detected by the Williams' test, the data were further analyzed by Dunnett's test to compare between control and individual treatment groups ($P < 0.05$). When the variances were heterogeneous, the Shirley-Williams' test, assuming a dose-related trend, was performed ($P < 0.05$). If no significant differences were found, Steel's test was applied ($P < 0.05$).

## Results

HDBB administration for 28 days did not induce any clinical signs or affect the body weight, except for significantly increased body weight on day 14 of administration in males in the 0.5 mg/kg group. At the completion of 28-day administration, the liver was grossly enlarged in 5/5 males and 1/5 females at 12.5 mg/kg, 5/5 males at 2.5 mg/kg, and 1/5 males at 0.5 mg/kg. In the liver, white focus was found in 4/5 males and 2/5 females at 12.5 mg/kg and in 4/5 males at 2.5 mg/kg. Absolute and relative liver weight was significantly increased at 2.5 mg/kg and above in males and at 12.5 mg/kg in females, as shown in Figure 1. There was also an increase in absolute and relative liver weight at 0.5 mg/kg in males, but no statistically significant difference was found from the control.

### Plasma HDBB concentration

The time course for levels of HDBB in male and female plasma after the first intragastric administration is shown in Figure 2A. HDBB was rapidly absorbed and
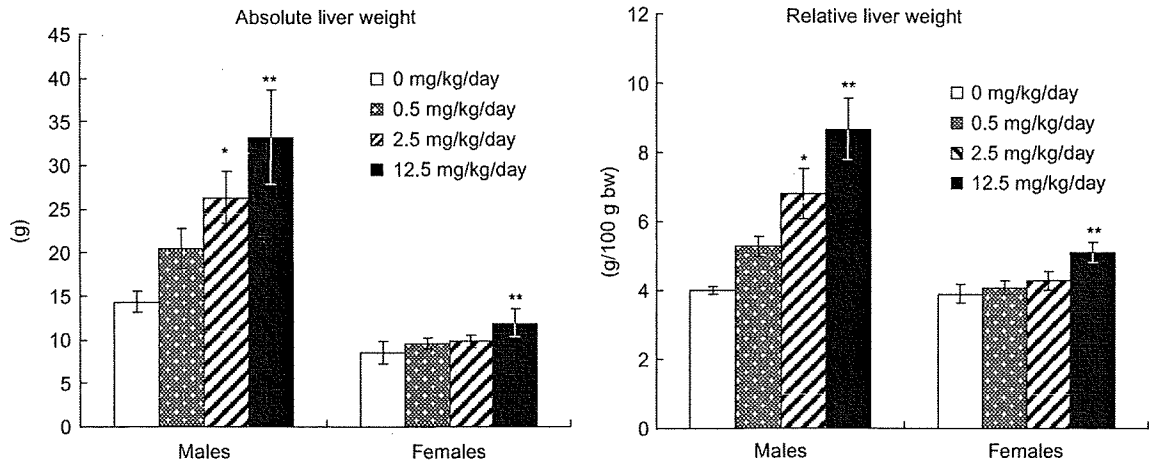
**Figure 1.** Absolute and relative liver weight of male and female rats given HDBB by gavage for 28 days. Data are expressed as the mean ± standard deviation (SD). 'Significantly different from the control, $P < 0.05$; "Significantly different from the control, $P < 0.01$.
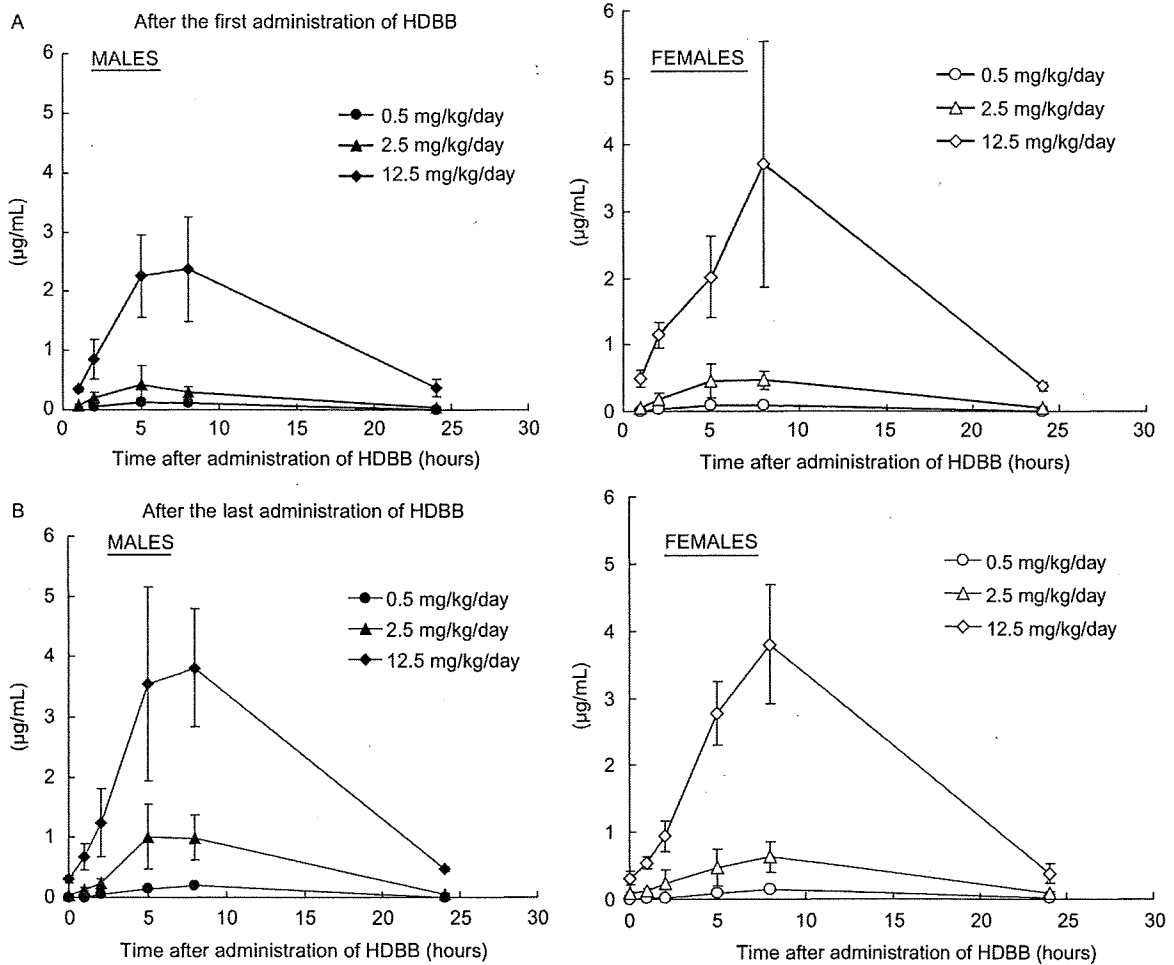


**Figure 2.** Plasma HDBB concentrations against time after the administration of HDBB to male and female rats. Data are expressed as the mean ± SD.

eliminated from the plasma in both sexes. No clear gender-related differences were found in the plasma profiles of HDBB at any doses. After 28-day repeated-dose administration, similar plasma HDBB profiles were observed, and there were no gender-related differences in the profiles (Figure 2B). In all dose groups, HDBB metabolites were not detected in the plasma at any sampling times in either sex.

The calculated values of $C_{max}$, $T_{max}$, and $AUC_{0-24h}$ for HDBB in plasma are given in Table 1. The data showed that $AUC_{0-24h}$ as well as $C_{max}$ values increased in rat plasma with the higher HDBB dose. Comparison of data for males and females indicated no gender-related differences.

### In vitro metabolism reaction

After a 60-minute incubation using the liver S-9 fraction prepared from male or female rats with the NADPH-generating system, the concentration of HDBB in the incubation mixture was hardly changed, as shown in Figure 3. The mean residual ratio of HDBB was 98.1% with male rat hepatic microsomes and 91.4% with female rat hepatic microsomes. On the other hand, when incubated with male and female hepatic microsomes, HDBB concentration in the incubation mixture decreased to 73.4 and 76.1% of the control, respectively. In either male or female microsomes, another peak was found around a retention time of 1-2 minutes.

Figure 4 represents residual ratios of HDBB after a 60-minute incubation with microsomes containing cDNA-expressed individual rat CYP enzymes in the presence of the NADPH generating system. Among the 14 types of CYP isoforms tested here, CYP1A1 exhibited the greatest metabolic activity of HDBB (mean residual HDBB ratio: 61.8%). CYP1A2, 2A2, 2B1, 2C6, 2C11, and 2D2 also metabolized 10-20% of HDBB. Other CYP isoforms, CYP2A1, 2C12, 2C13, 2D1, 2E1, 3A1, and 3A2, showed no significant metabolism of the chemical (mean residual ratio of HDBB: >95%). After incubation with CYP1A1, 1A2, 2A2, 2C6, 2C11, 2D2, 3A1, or 3A2, some peaks other than HDBB were detected.

### Effect of HDBB on hepatic CYP activity (Table 2)

While microsomal protein content showed no significant differences between HDBB-treated and control groups, the total CYP content was significantly increased in males of the 2.5 and 12.5 mg/kg groups. In these groups, aminopyrine N-demethylase activity, and testosterone 2α- and 16α-hydroxylase activity, decreased significantly. EROD activity showed a

**Table 1.** Toxicokinetic parameters of HDBB.

| Doses | Sexes | $C_{max}$ (μg/mL) | $T_{max}$ (h) | $AUC_{0-24h}$ (μg·h/mL) |
|---|---|---|---|---|
| After the first administration of HDBB | | | | |
| 0.5 mg/kg/ day | Males | 0.145±0.031 | 5.75±1.50 | 1.59±0.32 |
| | Females | 0.116±0.036 | 5.75±1.50 | 1.25±0.10 |
| 2.5 mg/kg/ day | Males | 0.484±0.276 | 5.75±1.50 | 4.99±1.45 |
| | Females | 0.573±0.165 | 7.25±1.50 | 6.65±1.61 |
| 12.5 mg/kg/ day | Males | 2.85±0.64 | 6.50±1.73 | 34.4±7.1 |
| | Females | 3.84±1.71 | 7.25±1.50 | 47.1±15.7 |
| After the last administration of HDBB | | | | |
| 0.5 mg/kg/ day | Males | 0.214±0.054 | 6.50±1.73 | 2.49±0.62 |
| | Females | 0.154±0.009 | 8.00±0.00 | 1.98±0.15 |
| 2.5 mg/kg/ day | Males | 1.14±0.42 | 5.75±1.50 | 13.6±5.0 |
| | Females | 0.636±0.221 | 7.25±1.50 | 8.89±3.25 |
| 12.5 mg/kg/ day | Males | 4.27±0.96 | 5.75±1.50 | 54.0±11.4 |
| | Females | 3.80±0.89 | 8.00±0.00 | 50.1±9.8 |

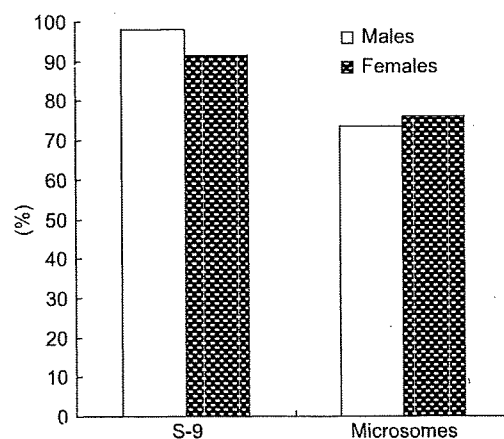Values are expressed as the mean ± SD.



**Figure 3.** Residual ratios of HDBB after incubation with male and female rat liver S-9 and microsomes in the presence of a NADPH-generating system. Data are expressed as the mean values of two determinations. Residual HDBB ratio was calculated by dividing the peak area of HDBB after a 60-minute incubation by that of the control, in which the incubation system was inactivated by the addition of 1 mL of acetonitrile prior to incubation.

significant decrease in males in all HDBB-treated groups. These changes were not detected in females. Lauric acid 12-hydroxylase activity was significantly increased at 0.5 mg/kg and above in males and at 12.5 mg/kg in females. No significant changes were found in ECOD activity or testosterone 6β-hydroxylase activity in either sex.

### Discussion

The current study was conducted to clarify the mechanism of marked gender-related differences in HDBB
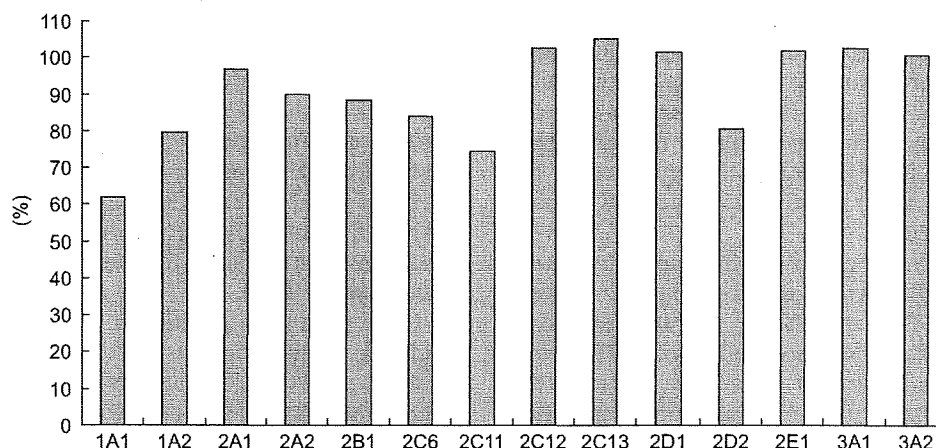
**Figure 4.** Residual ratios of HDBB after incubation with recombinant rat CYP isoforms in the presence of a NADPH-generating system. Data are expressed as the mean values of two determinations. Residual HDBB ratio was calculated by dividing the peak area of HDBB after incubation with microsomes containing cDNA-expressed individual rat CYPs by that after incubation with control microsomes containing negligible amounts of CYP.

**Table 2.** Protein content, total CYP contents, and enzyme activities in hepatic microsomes of male and female rats given HDBB by gavage for 28 days.

| | Dose (mg/kg/day) | | | |
| --- | --- | --- | --- | --- |
| | 0 (control) | 0.5 | 2.5 | 12.5 |
| No. of males | 5 | 5 | 5 | 5 |
| Microsomal protein content (mg/g liver) | 27.2±5.3 | 21.6±2.3 | 22.0±6.0 | 23.5±4.3 |
| Total CYP content (nmol/mg protein) | 0.670±0.119 | 0.783±0.075 | 0.885±0.052[r] | 0.738±0.119[r] |
| Aminopyrine N-demethylase activity (nmol/ min/mg protein) | 6.700±0.443 | 6.942±0.812 | 4.902±0.484[r] | 4.713±0.743[r] |
| ECOD activity (nmol/min/mg protein) | 1.420±0.140 | 1.713±0.354 | 1.528±0.253 | 1.213±0.202 |
| EROD activity (nmol/min/mg protein) | 0.0627±0.0083 | 0.0459±0.0045[r] | 0.0233±0.0054[r] | 0.0237±0.0020[r] |
| Testosterone 6β-hydroxylase activity (nmol/ min/mg protein) | 2.67±0.44 | 3.18±0.96 | 2.89±0.41 | 2.53±0.36 |
| Testosterone 2α-hydroxylase activity (nmol/ min/mg protein) | 1.562±0.170 | 1.385±0.495 | 0.179±0.207[r] | 0.000±0.000[r] |
| Testosterone 16α-hydroxylase activity (nmol/ min/mg protein) | 2.165±0.439 | 1.714±0.451 | 0.432±0.278[r] | 0.119±0.137[r] |
| Lauric acid 12-hydroxylase activity (nmol/ min/mg protein) | 1.60±0.47 | 7.80±2.14[s] | 9.99±0.58[s] | 11.09±2.26[s] |
| No. of females | 5 | 5 | 5 | 5 |
| Microsomal protein content (mg/g liver) | 11.4±3.5 | 11.7±3.5 | 16.2±6.7 | 16.5±3.0 |
| Total CYP content (nmol/mg protein) | 0.637±0.132 | 0.674±0.168 | 0.646±0.131 | 0.600±0.076 |
| Aminopyrine N-demethylase activity (nmol/ min/mg protein) | 4.157±0.534 | 4.362±0.630 | 4.403±1.007 | 5.133±0.680 |
| ECOD activity (nmol/min/mg protein) | 0.657±0.105 | 0.649±0.099 | 0.647±0.128 | 0.693±0.095 |
| EROD activity (nmol/min/mg protein) | 0.0869±0.0266 | 0.0882±0.0145 | 0.0904±0.0144 | 0.1142±0.0237 |
| Testosterone 6β-hydroxylase activity (nmol/ min/mg protein) | 0.121±0.023 | 0.138±0.019 | 0.150±0.040 | 0.159±0.047 |
| Testosterone 2α-hydroxylase activity (nmol/ min/mg protein) | 0.000±0.000 | 0.000±0.000 | 0.000±0.000 | 0.000±0.000 |
| Testosterone 16α-hydroxylase activity (nmol/ min/mg protein) | 0.066±0.123 | 0.018±0.025 | 0.054±0.076 | 0.083±0.073 |
| Lauric acid 12-hydroxylase activity (nmol/ min/mg protein) | 1.37±0.15 | 1.40±0.23 | 1.51±0.38 | 3.20±3.01[s] |

Values are expressed as the mean ± SD.
[r]Significantly different from the control by the Williams test, $P < 0.05$.
[s]Significantly different from the control by the Shirley-Williams test, $P < 0.05$.

toxicity. Following 28-day HDBB administration, macroscopic changes in the liver and/or increased liver weight were found at 0.5 mg/kg and above in males and at 12.5 mg/kg in females. This showed about a 25 times higher susceptibility of male rats to the hepatotoxicity of HDBB and demonstrated the reproducibility of our previous 28-day study (Hirata-Koizumi et al., 2007).

Unexpectedly, we could not find sexual variation in plasma HDBB levels and toxicokinetic parameters (i.e., $C_{max}$, $T_{max}$, and $AUC_{0-24h}$) in rats orally given HDBB for 28 days. No metabolites of HDBB were detected in the plasma of either sex. Although the *in vitro* study using hepatic microsomal preparation from male and female rats showed evidence of some HDBB metabolism, no sexual differences were found in the residual HDBB ratio after a 60-minute incubation with an NADPH-generation system. *In vitro* results using recombinant CYP enzymes suggest the contribution of multiple CYP isozymes (i.e., CYP1A1, 1A2, 2A2, 2C6, 2C11, and 2D2) to the overall metabolism of HDBB in rat liver microsomes. Among these isozymes, gender-related difference was reported in CYP2C11, which is known to be a male-specific isoform (Waxman and Chang, 2005). However, considering our present result that 28-day HDBB administration markedly reduced CYP2C11-dependent testosterone 2α- and 16α-hydroxylation in the liver, male-specific metabolism catalyzed by this enzyme is unlikely to contribute to the higher susceptibility of male rats to HDBB toxicity. These findings show that gender-related differences in HDBB toxicity do not come from the variation in plasma concentration of causative substances (i.e., HDBB or its metabolites) and hepatic metabolism.

HDBB exerted sexually different effects on hepatic metabolic activities. Of particular note is the change in CYP4A-specific activity and lauric acid 12-hydroxylation, which increased at 0.5 mg/kg and above in males and at 12.5 mg/kg in females. The dose responsiveness was consistent with that of liver-weight change and macroscopic findings. Hepatic CYP4A expression is known to be highly inducible by a diverse group of compounds referred to as peroxisome proliferators, which include the widely prescribed lipid-lowering drug of the fibrate class, phthalate ester plasticizer, the endogenous steroid, dehydroepiandrosterone, and chlorinated phenoxy and benzoic acid herbicides (Bacher and Gibson, 1988; Espandiari et al., 1995; Okita et al., 1993; Sundseth and Waxman, 1992; Wu et al., 1989). In the previous 52-week repeated dose toxicity study of HDBB, we observed the centrilobular hypertrophy of hepatocytes with eosinophilic granular cytoplasm (Hirata-Koizumi et al., 2008a), which is known to

be a characteristic change found in rodents administered with peroxisome proliferators (Cattley and Popp, 2002). Other hepatic changes observed in the previous study, such as altered hepatocellular foci and lipofuscin deposition in hepatocytes, could be also induced by peroxisome proliferators (Hirata-Koizumi et al., 2008a; IARC, 1995). The present result provided additional evidence that peroxisome proliferation was involved in the mechanism of the hepatotoxicity of HDBB.

Other changes in hepatic metabolic activities included marked decreases in the above-mentioned male-specific CYP2C11 activity (testosterone 2α- and 16α-hydroxylation) and in CYP1A1-dependent EROD activity. These changes in metabolic enzyme activities would lead to little metabolism of HDBB *in vivo* despite significant metabolism by these enzymes *in vitro*. These changes in CYP2C11 and 1A1 activities *in vivo* might have resulted from the peroxisome proliferative effects of HDBB because it is reported that well-known peroxisome proliferators, WY-14643, clofibrate, gemfibrozil, and/or di-n-butyl phthalate, downregulated hepatic CYP2C11 and 1A1 expressions (Corton et al., 1998; Shaban et al., 2004). HDBB-induced hepatic changes in aminopyrine *N*-demethylase activity, which is known to be catalyzed by multiple CYP isoforms (Guengerich et al., 1982; Imaoka et al., 1988), and total CYP content are considered to be attributed to changes in the expression of various CYP isozymes, including CYP4A, 2C11, and 1A1.

Peroxisome proliferators are considered to exert biological effects via activation of a nuclear receptor, peroxisome proliferator-activated receptor-alpha (PPARα) (Green, 1995). This is strongly supported by the findings that various biological effects of peroxisome proliferators were not observed in mice that lack a functional PPARα gene (Lee et al., 1995; Ward et al., 1998). Recently, Sparatore et al. (2006) investigated the capacity of various [4-(2H-1,2,3-benzotriazol-2-yl) phenoxy]alkanoic acids to activate PPAR-modulated transcription, using transiently transfected mammalian cells (Hep G2) with a modified variant of the transactivation assay, named the Gal 4-PPAR transactivation assay. They showed that some of these compounds displayed 56–96% maximum activity of the reference drug, Wy-14643, on PPARα. The structural similarity with these compounds suggested the possible agonistic action of HDBB on PPARα. Further, in the above-mentioned Gal 4-PPAR transactivation assay, the introduction of chlorine substituent to the benzotriazole nucleus markedly decreased the activity on PPARα (Sparatore et al., 2006). This is consistent with our previous findings on the toxicity of DBHCB; this structural analog of HDBB with a chlorine substituent exerted much less of an effect on the liver than

HDBB (Ema et al., 2008). In order to further clarify the mechanism of HDBB hepatotoxicity, we are planning a PPARα transactivation assay of HDBB. In this assay, we will also determine the activity of various other benzotriazole UV absorbers, including DBHCB.

Several studies have reported that, in rats, males are more responsive than females to various effects of peroxisome proliferators, including increased liver weight, peroxisome proliferation, and peroxisomal β-oxidation, as well as changes in various enzyme activities (Amacher et al., 1997; Gray and de la Iglesia, 1984; Kawashima et al., 1989a, 1989b; Yamada et al., 1991; Svoboda et al., 1969). Male rats have higher levels of hepatic PPARα mRNA and protein than female rats (Jalouli et al., 2003), which is considered to explain the sex differences in the effects of peroxisome proliferators, at least in part. Previously, we showed that gender-related differences in HDBB toxicity were markedly reduced by castration of male and female rats (Hirata-Koizumi et al., 2008b). Similar phenomena were reported in the hepatic PPARα mRNA expression in rats (Jalouli et al., 2003); therefore, gender-related differences in HDBB toxicity might also come from such a variation in hepatic PPARα expression. In the previous study, we also showed that the gender-related difference in the toxicity of HDBB was not observed in preweaning rats (Hirata-Koizumi et al., 2008c). PPARα expression in the liver is known to be developmentally regulated; it was first detected on embryonic day 13.5 and increased during the suckling period, followed by a decrease postsuckling (Balasubramaniyan et al., 2005; Braissant and Wahli, 1998; Panadero et al., 2000). However, unfortunately, these data are based on a study conducted without separating males and females, and therefore, it has yet to be revealed when and how gender-related differences develop in rats. In order to clarify the role of hepatic PPARα expression in gender-related differences in HDBB toxicity, there is a need to investigate the ontogeny of hepatic PPARα expression with the sexes separated.

In our previous repeated-dose toxicity studies of HDBB, gender-related differences were observed not only in hepatic changes, but also in the inhibition of body-weight gain, anemia, and histopathological changes in the heart, thyroid, spleen, or kidneys (Hirata-Koizumi et al., 2007, 2008a). Since most changes were found at higher doses than the exerted hepatic effects, they could be considered to be secondary effects due to hepatic changes caused by peroxisome proliferative effects of HDBB. On the other hand, in our previous studies, HDBB caused cystic/vacuolar degeneration of hepatocytes, focal necrosis, and bile duct proliferation in the liver, which are not considered to be necessarily associated with the mechanism of peroxisome proliferation (Hirata-

Koizumi et al., 2007, 2008a); therefore, the possible involvement of other mechanisms could not be ruled out. In the above-mentioned Gal 4-PPAR transactivation assay, some structural analogs of HDBB exhibited moderate activity on either PPARγ or -δ (Sparatore et al., 2006), suggesting the possible involvement of these PPAR isoforms in the development of HDBB toxicity. Investigating HDBB agonistic activity to various nuclear receptors, including PPARγ and -δ, might provide useful information for understanding the mechanism of HDBB toxicity.

Finally, we selected rats as study animals exclusively in our series of toxicity experiments on HDBB because it is most commonly used in toxicity studies. Although the current result suggested that HDBB exerts toxicity via peroxisome proliferation, it has been reported that rodents are much more sensitive to peroxisome proliferators than primates (Elcombe and Mitchell, 1986; Blaauboer et al., 1990). In order to clarify the toxicity of HDBB, it would be important to conduct studies using primates or PPARα knockout mice. Such studies would clarify the possible involvement of other mechanisms in the HDBB toxicity.

## Conclusion

The current results showed no sexual variation in the plasma concentration of HDBB or its metabolites in rats orally given HDBB and in the *in vitro* hepatic metabolism of HDBB. HDBB increased hepatic CYP4A activity more markedly in male rats than in females and decreased hepatic CYP1A1 and 2C11 activity only in males. These results suggest that HDBB exerts toxicity via peroxisome proliferation, and the difference in susceptibility of male and female rats to this effect might lead to marked gender-related differences in HDBB toxicity.

## Acknowledgment

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## REVIEW ARTICLE

# Disappearance of gender-related difference in the toxicity of benzotriazole ultraviolet absorber in juvenile rats

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**ABSTRACT**    2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (HDBB) is an ultraviolet absorber used in plastic resin products, such as building materials and automobile components. In oral repeated dose toxicity studies using 5- or 6-week-old rats, this chemical induced hepatic histopathological changes, such as hypertrophy accompanied with eosinophilic granular changes and focal necrosis of hepatocytes, and male rats showed nearly 25 times higher susceptibility to the toxic effects than females. Castration at approximately 4 weeks of age markedly reduced the sex-related variation in HDBB toxicity, but some difference, less than five times, remained between male and female castrated rats. Following oral HDBB administration to male and female juvenile rats from postnatal days 4–21, such gender-related difference in toxic susceptibility was not detected; therefore, it is speculated that the determinants of susceptibility to HDBB toxicity are differentiated between sexes after weaning. In young rats given HDBB, there was no gender-related difference in plasma HDBB concentration, and no metabolites were detected in the plasma of either sex. HDBB induced lauric acid 12-hydroxylase activity in the liver and this change was more pronounced in males than in females. These findings indicate that HDBB could show hepatic peroxisome proliferation activity, and the difference in the susceptibility of male and female rats to this effect might lead to marked gender-related differences in toxicity.

**Key Words:** benzotriazole ultraviolet absorber, gender-related difference, hepatotoxicity, juvenile rat, peroxisome proliferation

## INTRODUCTION

Benzotriazole ultraviolet (UV) absorbers, which have a phenolic group attached to the benzotriazole structure, have excellent absorption capacity with a full spectrum of UV absorption (Toyota Tsusho Corporation and Toyotsu Plachem Corporation 2009); therefore, they are used in a variety of polymers to improve their long-term weather resistance and stability. However, as these compounds are generally poorly biodegradable and some are also highly bioaccumulative (US EPA 2008; METI, MHLW and MOE 2009), there are growing concerns about their effects on human health through the environment.

Regarding toxicity, only limited information has been published in scientific journals; however, in 1999, the Phenolic Benzotriazole Association voluntarily agreed to participate in the US High Production Volume Chemical Challenge Program, and reviewed the existing industrial data on four kinds of benzotriazole UV absorbers (US EPA 2008). In Japan, six compounds in this group were selected as object substances in an existing chemical testing program by the government, and some results of screening toxicity tests have already been released to the public (METI, MHLW and MOE 2009). These data indicate a consistent trend of the toxicity of benzotriazole UV absorbers; low acute toxicity and a lack of reproductive/developmental toxicity and genotoxicity. As for repeated dose toxicity, the main target seems to be the liver, but the severity varies in each compound. In rats, the lowest observed adverse effect levels range from 0.5 mg/kg per day to more than 1000 mg/kg per day.

2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (CAS no. 3846-71-7; HDBB) is a benzotriazole UV absorber that has severe hepatotoxicity. The structural formula of this compound is presented in Figure 1. Recently, we showed a marked gender-related difference in the toxicity of HDBB in young rats (Hirata-Koizumi *et al.* 2007, 2008a). Interestingly, the sex-related difference was markedly reduced by castration and abolished in preweaning rats (Hirata-Koizumi *et al.* 2008b,c). In the present review, we summarize the toxicity of HDBB and its marked gender-related difference in young rats, and then describe the toxicity in castrated and preweaning rats. Finally, we discuss the possible mechanism for the gender-related difference in young rats, based on our most recent work (Hirata-Koizumi *et al.* 2009).

## SUMMARY OF TOXICITY INFORMATION

In a single oral dose toxicity study, HDBB induced no mortality in male and female CD(SD)IGS rats (6 weeks of age), even at a dose of 2000 mg/kg (MHLW 2003). No clinical signs of toxicity or changes in body weight were found at this dose. These findings indicate that the acute toxicity of HDBB is very low.

However, in repeated-dose toxicity studies, severe toxicity is observed at relatively low doses. In a 28-day repeated-dose toxicity study, HDBB was administered by gavage to male and female CD(SD)IGS rats from 5 weeks of age, and toxic effects were found mainly in the liver, such as hypertrophy and vacuolar degeneration of hepatocytes, focal necrosis and bile duct proliferation (Hirata-Koizumi *et al.* 2007). Other changes included anemia, degeneration and hypertrophy of the myocardium in the heart, and hypertrophy of the tubular epithelium in the kidneys. These adverse effects were found even at the lowest dose of 0.5 mg/kg in males, but in females, they were detected only at 12.5 mg/kg and above. After a 14-day recovery period, these changes were mostly recovered in females, but not in males. In a 52-week repeated-dose toxicity study, CD(SD)IGS rats were given HDBB by gavage at 0.1, 0.5 or

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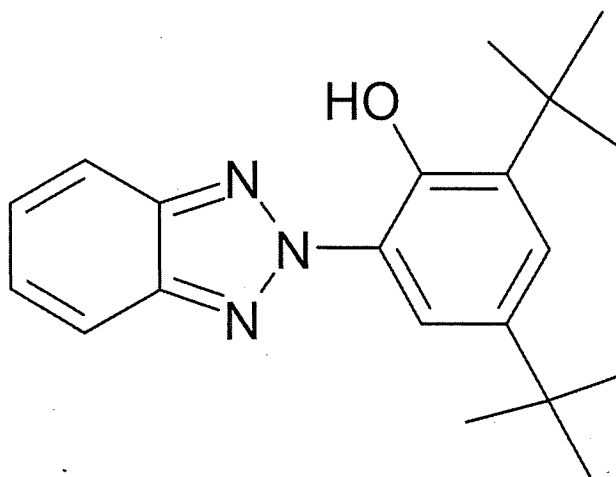
**Fig. 1** Structural formula of 2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl) benzotriazole (HDBB).

2.5 mg/kg per day in males and at 0.5, 2.5 or 12.5 mg/kg per day in females, starting from 6 weeks of age (Hirata-Koizumi *et al.* 2008a). At the completion of administration, the liver was principally affected at 0.5 mg/kg and above in males and at 12.5 mg/kg in females, and the histopathological findings included precancerous changes (i.e. altered hepatocellular foci). Lowered body weight and anemia were also found at 0.5 mg/kg and above only in males. Based on these findings, the no observed adverse effect level (NOAEL) for repeated dose toxicity of HDBB was concluded to be 0.1 mg/kg per day in males and 2.5 mg/kg per day in females.

HDBB did not induce reverse mutation in *Salmonella typhimurium* and *Escherichia coli* in the absence or presence of an exogenous metabolic activation system (MHLW 2003). As the substance also gave negative responses in the chromosomal aberration test using cultured Chinese hamster lung (CHL/IU) cells, either with or without metabolic activation, it was not considered to be genotoxic.

Although no data are available on the reproductive/developmental toxicity of HDBB, reproductive and developmental toxicity studies of its structural analogues are reported. In a combined repeated dose and reproductive/developmental toxicity screening test using rats (combined tests), 2-(2′-hydroxy-3′-*sec*-butyl-5′-*tert*-butylphenyl)benzotriazole (HBBB) exerted no effects on reproductive/developmental parameters, even at the highest dose of 12.5 mg/kg, while hepatic changes were observed in parental animals at this dose (METI 2007a). A similar result was reported in the combined test of 2-(3′,5′-di-*tert*-butyl-2′-hydroxyphenyl)-5-chlorobenzotriazole (DBHCB); hepatic effects were found at 25 mg/kg and higher, but reproductive/developmental parameters were not affected even at the highest dose of 250 mg/kg (Ema *et al.* 2008). The latter compound also did not show any adverse effects on maternal rats and embryonic/fetal development in the prenatal developmental toxicity study (Ema *et al.* 2006).

## GENDER-RELATED DIFFERENCE IN TOXICITY

As mentioned above, male rats were much more susceptible to the toxicity of HDBB than females. Comparing the toxic susceptibility between sexes, the NOAEL must be a useful parameter, although it

is very dependent on dose-selection. Based on the NOAEL, the difference could be considered to be approximately 25 times.

For other benzotriazole UV absorbers, while the above-mentioned structural analogues, HBBB and DBHCB, also caused male predominant hepatic effects, no clear gender-related difference was found in the hepatic effects of 2-(2′-hydroxy-5′-methylphenyl)benzotriazole in the combined tests (METI 2007a,b; Ema *et al.* 2008). Review documents of the US High Production Volume Chemical Challenge Program suggest that the toxic susceptibility of males to 2-(2′-hydroxy-3′,5′-di-*tert*-amylphenyl)benzotriazole is higher than females, but conversely, a higher susceptibility of females to the toxicity of 2-(2H-benzotriazole-2-yl)-4,6-bis(1-methyl-1-phenylethyl)phenol was reported (US EPA 2008). Although the available data are limited, they indicate that the branched alkyl group attached to both meta positions of the phenolic group to the benzotriazole ring might play an important part in male predominance in the toxicity of benzotriazole UV absorbers.

### Gonadal influence on gender-related differences

Gender-related differences in the toxic susceptibility of rats have been documented for many other industrial chemicals (Muraoka & Itoh 1980), environmental pollutants (Knuckles *et al.* 2004), insecticides (Carlson & DuBois 1970; Agarwal *et al.* 1982) and pharmaceuticals (McGovren *et al.* 1981; Coleman *et al.* 1990; Wang *et al.* 2001; Stern *et al.* 2007). As it is known that sex hormones play important roles in such sex-related differences in toxic responses, we examined the effects of castration on gender-related differences in the toxicity of HDBB.

Male and female castrated CD(SD) rats were given HDBB by gavage at 0.5, 2.5 or 12.5 mg/kg per day for 28 days (Hirata-Koizumi *et al.* 2008b). The rats were castrated at approximately 4 weeks of age under ether anesthesia, and were subjected to HDBB treatment at 6 weeks of age. At the completion of administration, hepatic histopathological changes, including nucleolar enlargement, increased mitosis, hypertrophy and/or focal necrosis of hepatocytes, were observed with blood biochemical changes, such as increased levels of albumin, aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase, at 0.5 mg/kg and above in males, and at 2.5 mg/kg and above in females. Hepatic changes at 0.5 mg/kg in males were slightly milder than at 2.5 mg/kg in females, showing that the difference in susceptibility of male and female castrated rats was less than five times. As shown in Figure 2, comparison of the rate of changes in relative liver weight provided a clear description of nearly 25 times difference in the susceptibility of male and female intact rats to HDBB hepatotoxicity and marked reduction by castration.

### Lack of gender-related differences in juvenile rats

Although the castration of male and female rats markedly reduced the sex-related variation in HDBB toxicity, some difference remained between male and female castrated rats. It is speculated that the determinants of susceptibility to HDBB toxicity are already differentiated between sexes by 4 weeks of age, when the castration was performed; therefore, we determined the sex-related difference in the susceptibility to HDBB toxicity in juvenile rats, which were considered to be under more limited influence of sex hormones.

Male and female CD(SD) rats were given HDBB by gavage at 0.1, 0.5, 2.5 or 12.5 mg/kg per day on postnatal days 4–21 (Hirata-Koizumi *et al.* 2008c). Blood biochemical changes, including increases in the levels of albumin, AST and ALP, were found in both sexes at 12.5 mg/kg. Histopathological examination of the liver revealed hepatocellular changes, such as nucleolar enlargement,
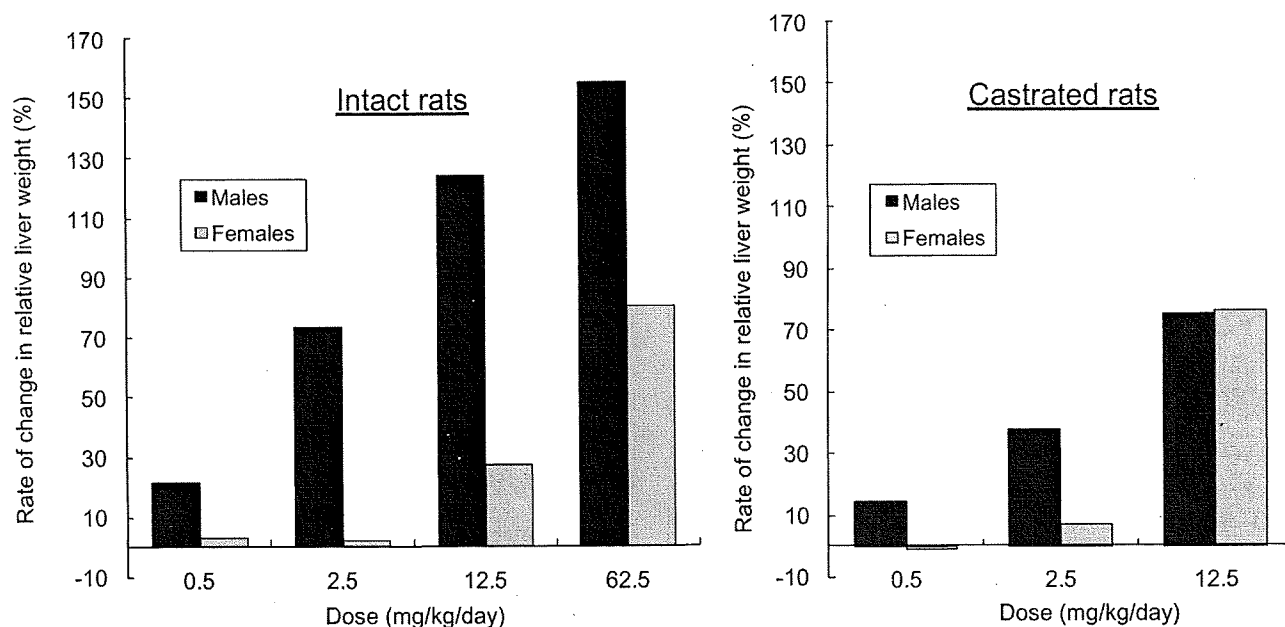
**Fig. 2** Comparison of change in the relative liver weight of male and female intact and castrated rats administered 2-(2'-hydroxy-3',5'-di-*tert*-butylphenyl)benzotriazole (HDBB). Male and female intact or castrated rats were administered HDBB by gavage for 28 days, starting at 5 or 6 weeks of age (Hirata-Koizumi *et al.* 2007, 2008b). Castration was performed at approximately 4 weeks of age under ether anesthesia. Reproduced from Hirata-Koizumi *et al.* (2008b) with permission.

anisokaryosis, increased mitosis and/or hypertrophy, at 2.5 mg/kg and above, and the incidence and degree was similar in both sexes. These results indicate no gender-related differences in the susceptibility to toxic effects of HDBB in preweaning rats. The rate of changes in the relative liver weight clearly showed a lack of gender-related differences in HDBB hepatotoxicity in preweaning rats (Fig. 3).

**Possible mechanism of gender-related differences in young rats**

Gender-related differences in HDBB toxicity were found not only for hepatic effects, but also for the body weight reduction, anemia and histopathological changes in the heart and kidneys of young rats; therefore, we first speculated that sex-related variation in HDBB toxicity might be explained by the difference in blood concentration of their causative substances (HDBB or these metabolites). In fact, it is well-known that some metabolic enzymes and membrane transporters are expressed in a sex-dependent manner in rats (Buist *et al.* 2002, 2003; Morris *et al.* 2003; Waxman & Chang 2005). It is also reported that sex-related variation in such toxicokinetic determinants is under the control of sex hormones and is not apparent until puberty; however, following 28-day oral administration of HDBB to 5-week-old CD(SD) rats, no sex-related variation was found in plasma HDBB levels, as shown in Figure 4 (Hirata-Koizumi *et al.* 2009). No metabolites of HDBB were detected in the plasma of either sex. Although an *in vitro* study using hepatic S9 fractions and microsomes prepared from male and female SD rats showed evidence of some HDBB metabolism, no sex-related differences were found in the residual HDBB ratio after 60-min incubation with an NADPH generation system (Fig. 5). These findings suggest that toxicodynamic factors would contribute to gender-related differences in the toxicity of HDBB.
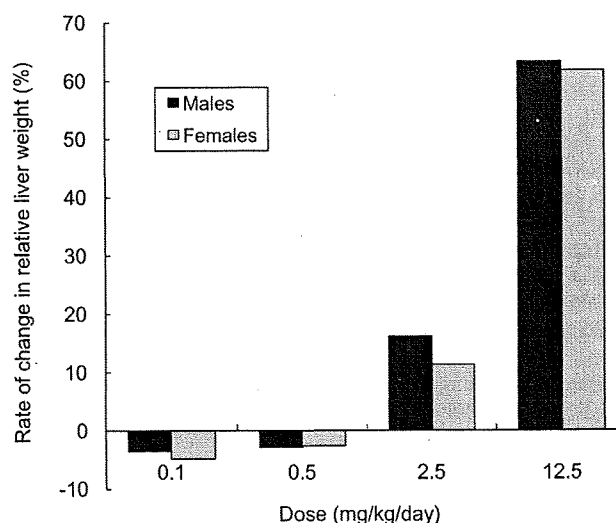


**Fig. 3** Comparison of change in the relative liver weight of male and female preweaning rats administered with 2-(2'-hydroxy-3',5'-di-*tert*-butylphenyl)benzotriazole (HDBB). Male and female rats were administered HDBB by gavage from postnatal days 4 to 21 (Hirata-Koizumi *et al.* 2008c).

In repeated dose toxicity studies using young and preweaning rats, the centrilobular hypertrophy of hepatocytes caused by HDBB was accompanied with eosinophilic granular changes (Hirata-Koizumi *et al.* 2008a,c). As this is known to be a characteristic change in rodents administered peroxisome proliferators, such as
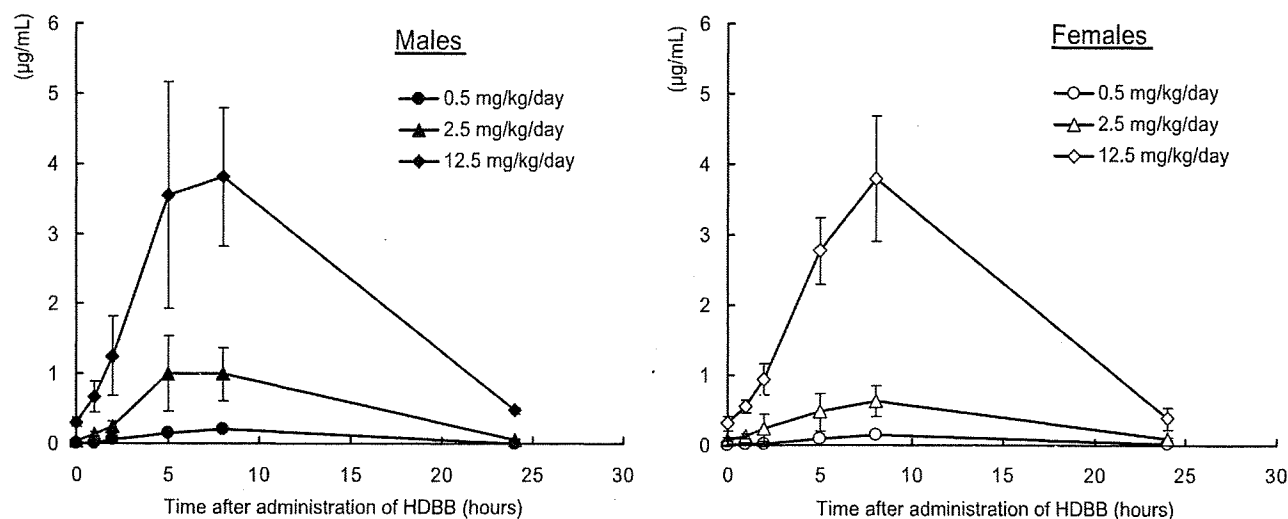
**Fig. 4** Plasma 2-(2'-hydroxy-3',5'-di-*tert*-butylphenyl)benzotriazole (HDBB) concentrations against time after HDBB administration to male and female rats. HDBB was given by gavage to male and female rats for 28 days, starting at 5 weeks of age, and blood samples were collected from the jugular vein before and 1, 2, 5, 8, and 24 hours after the 28th dose (Hirata-Koizumi *et al.* 2009). Data are expressed as the mean ± SD. Reproduced from Hirata-Koizumi *et al.* (2009) with permission.
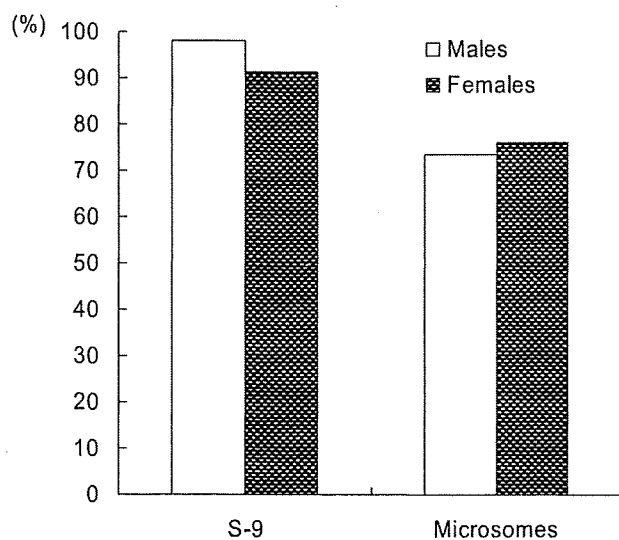


**Fig. 5** Residual ratios of 2-(2'-hydroxy-3',5'-di-*tert*-butylphenyl) benzotriazole (HDBB) after incubation with male and female rat liver S9 or microsomes in the presence of NADPH-generating system. HDBB was dissolved in acetonitrile and incubated with hepatic S9 fractions or microsomes prepared from male and female rats in the presence of NADPH-generating system for 60 min (Hirata-Koizumi *et al.* 2009). The residual HDBB ratio was calculated by dividing the peak area of HDBB after incubation with that of the control, in which the incubation system was inactivated by the addition of acetonitrile prior to incubation. Reproduced from Hirata-Koizumi *et al.* (2009) with permission.

fibrate hypolipidemic drugs and phthalate plasticizers (Cattley & Popp 2002), peroxisome proliferation might be involved in the mechanism of HDBB hepatotoxicity. Supporting this speculation, our recent study revealed that HDBB induced hepatic cytochrome

P450 (CYP) 4A-specific activity, lauric acid 12-hydroxylation (Hirata-Koizumi *et al.* 2009), which is highly inducible by peroxisome proliferators (Bacher & Gibson 1988; Sundseth & Waxman 1992; Okita *et al.* 1993; Espandiari *et al.* 1995). The dose-responsiveness was consistent with that of liver weight change and macroscopic findings.

Several studies have reported that in rats, males are more responsive than females to various effects of peroxisome proliferators, including increased liver weight, peroxisome proliferation, and peroxisomal β-oxidation, as well as changes in various enzyme activities (Svoboda *et al.* 1969; Gray & de la Iglesia 1984; Kawashima *et al.* 1989a,b; Yamada *et al.* 1991; Amacher *et al.* 1997). Peroxisome proliferators are considered to exert biological effects through activation of a nuclear receptor, peroxisome proliferator-activated receptor (PPAR) α (Green 1995; Lee *et al.* 1995; Ward *et al.* 1998). Male rats have higher levels of hepatic PPARα mRNA and protein than females (Jalouli *et al.* 2003), which is considered to explain the sex-related differences in the effects of peroxisome proliferators, at least in part. Jalouli *et al.* (2003) reported that castration of male and female rats markedly reduced the gender difference in hepatic PPARα mRNA expression. We showed similar phenomena in HDBB toxicity (Hirata-Koizumi *et al.* 2008b), suggesting that the gender-related difference in HDBB toxicity might also come from such variation in hepatic PPARα expression. In our future studies, we plan to investigate HDBB agonistic activity with various nuclear receptors, including PPARα.

Our findings that male and female preweaning rats showed similar susceptibility to HDBB toxicity (Hirata-Koizumi *et al.* 2008c) suggests a lack of sex-related variation in hepatic PPARα expression during the early postnatal period. PPARα expression in the liver is known to be developmentally regulated (Braissant & Wahli 1998; Panadero *et al.* 2000; Balasubramaniyan *et al.* 2005); however, unfortunately, the available developmental data on PPARα expression are based on a study conducted that did not separate males and females, and therefore, it has yet to be revealed when and how gender-related differences develop in rats. To clarify the role of hepatic PPARα expression in sex-related differences in

HDBB toxicity, we are currently investigating the ontogeny of hepatic PPARα expression in each sex.

## CONCLUSION

HDBB exerts male predominant hepatotoxicity in young rats. The gender-related difference was markedly reduced by castration, and completely abolished in preweaning rats. In young rats, HDBB induced lauric acid 12-hydroxylase activity in the liver and this change was more pronounced in males than females. These findings suggest that HDBB would have hepatic peroxisome proliferation activity, and the difference in the susceptibility of male and female rats to this effect might lead to marked gender-related differences in toxicity.

## ACKNOWLEDGMENTS

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