In the present study, cervical priming using laminaria was performed before gemeprost was administered. Laminaria absorbs water from proteoglycan complexes in the cervix, leading to dissociation of the complexes, thereby softening and dilating the cervix.[13] Cervical preparation using laminaria is considered very important to prevent cervical lacerations in second-trimester medical abortions, as well as in surgical abortions. In the present study, cervical lacerations as the main cause of excessive bleeding were not observed. In addition to acting as a mechanical modulator, laminaria also acts as a chemical modulator and sensitizes the myometrium to gemeprost.[14] Gemeprost alone can induce cervical ripening as well as labor. Nonetheless, the gemeprost dose can be reduced by exploiting both the mechanical and chemical effects of laminaria. The three-step cervical dilation using laminaria may decrease the total dose of gemeprost required, and TOP using this procedure may be faster and safer than that using gemeprost alone. We consider our regimen is effective and safe for midtrimester TOP in women who have previously undergone CS.

Conclusion

In summary, our findings show that cervical dilation with laminaria followed by vaginal administration of gemeprost can be used effectively and safely for TOP in women who have previously undergone CS as well as for those who have not.

Ethical Approval

The protocol of the present study is in accordance with the Maternal Protection Law (1996) of Japan and the approval of the ethical review committee of the University of Tsukuba Hospital.

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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,

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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 ($AUC_{0\text{-}24h}$), 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 $MgCl_2$), 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 duplicate. 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 observed grossly. 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 × *g* for 30 minutes. The supernatant was centrifuged at 105,000 × *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

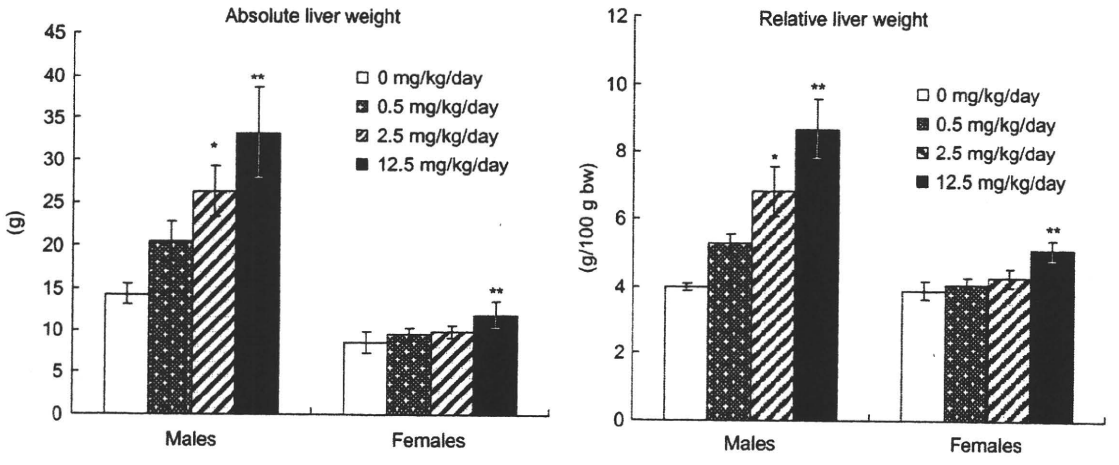

Figure 1. Absolute and relative liver weight of male and female rats given HDBB by gavage for 28 days. Data are expressed as the mean ± standard deviation (SD). *Significantly different from the control, $P < 0.05$; **Significantly different from the control, $P < 0.01$.

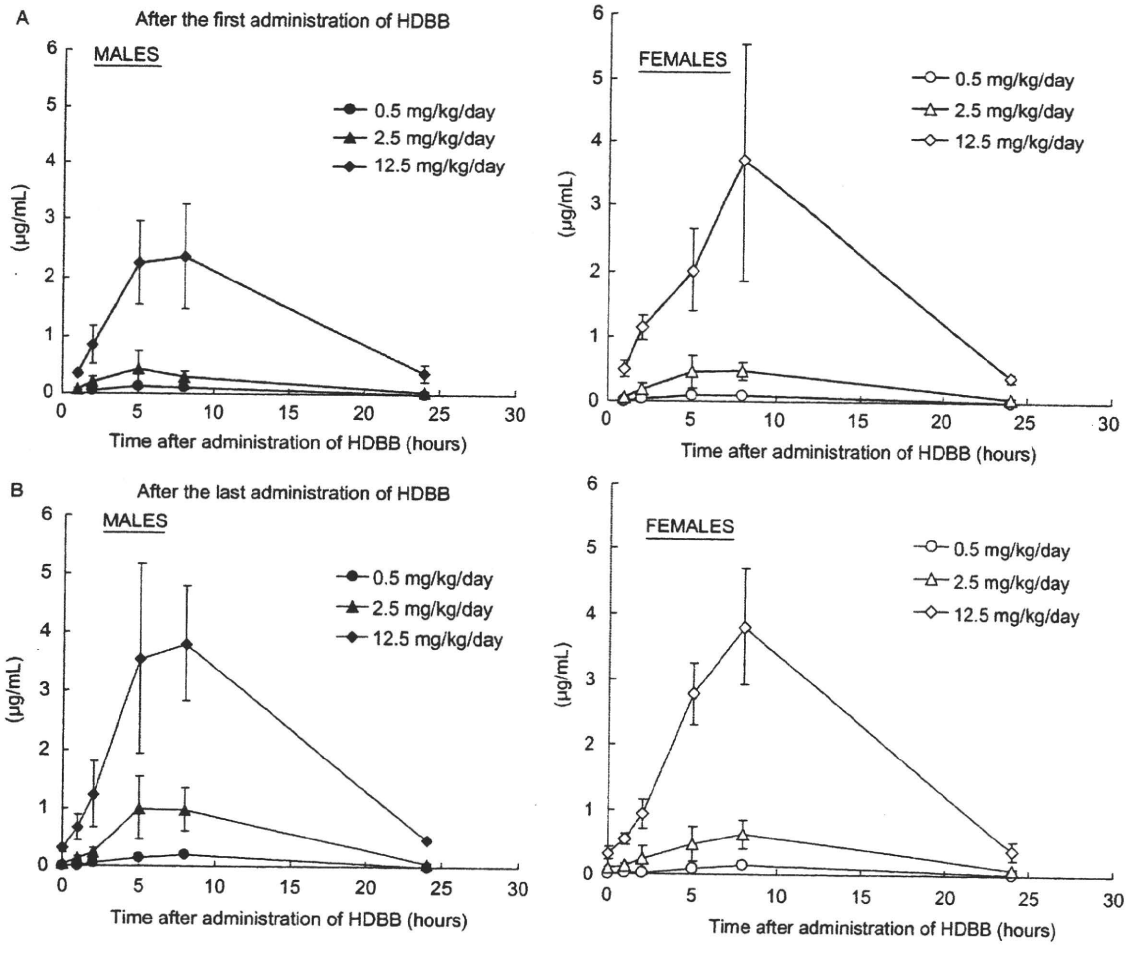

Figure 2. Plasma HDBB concentrations against time after the administration of HDBB to male and female rats. Data are expressed as the mean ± SD.

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.

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.

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.

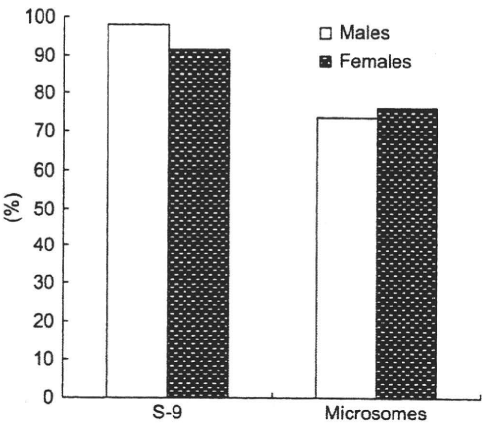

Figure 3. Residual ratios of HDBB after incubation with male and female rat liver S-9 and microsomes in the presence of a NADPH-generating system. Data are expressed as the mean values of two determinations. Residual HDBB ratio was calculated by dividing the peak area of HDBB after a 60-minute incubation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

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 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

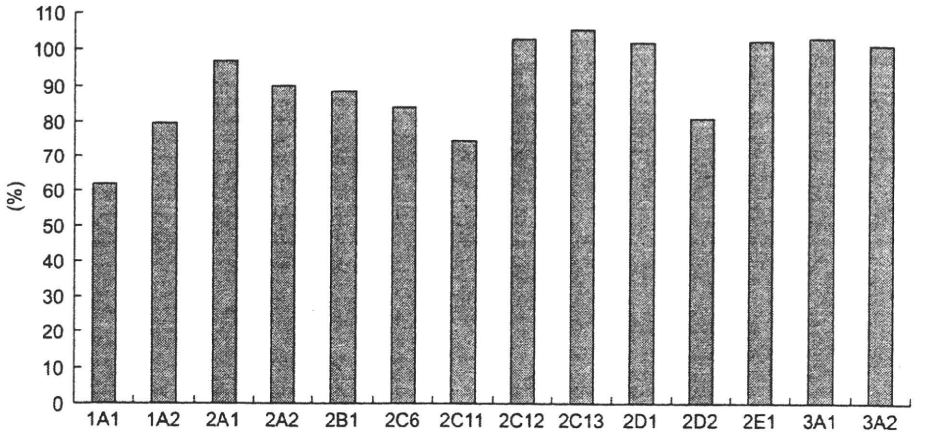

Figure 4. Residual ratios of HDBB after incubation with recombinant rat CYP isoforms in the presence of a NADPH-generating system. Data are expressed as the mean values of two determinations. Residual HDBB ratio was calculated by dividing the peak area of HDBB after incubation with microsomes containing 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#	0.738±0.119#
Aminopyrine *N*-demethylase activity (nmol/min/mg protein)	6.700±0.443	6.942±0.812	4.902±0.484#	4.713±0.743#
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§	0.0233±0.0054#	0.0237±0.0020#
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#	0.000±0.000#
Testosterone 16α-hydroxylase activity (nmol/min/mg protein)	2.165±0.439	1.714±0.451	0.432±0.278#	0.119±0.137#
Lauric acid 12-hydroxylase activity (nmol/min/mg protein)	1.60±0.47	7.80±2.14§	9.99±0.58§	11.09±2.26§
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§

Values are expressed as the mean ± SD.
#Significantly different from the control by the Williams test, $P < 0.05$.
§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 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.

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RESEARCH ARTICLE

Developmental toxicity of dibutyltin dichloride given on three consecutive days during organogenesis in cynomolgus monkeys

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Abstract

We previously reported that the administration of dibutyltin dichloride (DBTCl) by nasogastric intubation during the entire period of organogenesis, days 20–50 of pregnancy, was embryolethal, but not teratogenic, in cynomolgus monkeys. The present study was conducted to further evaluate the developmental toxicity of DBTCl given to pregnant monkeys on 3 consecutive days during organogenesis. Cynomolgus monkeys were given DBTCl at 7.5 mg/kg body weight/day by nasogastric intubation on days 19–21, 21–23, 24–26, 26–28, 29–31, 31–33, or 34–36 of pregnancy, and the pregnancy outcome was determined on day 100 of pregnancy. Embryonic/fetal loss was observed in 1 female given DBTCl on days 19–21, 2 females given DBTCl on days 24–26, and 1 female given DBTCl on days 34–36. There were no effects of DBTCl on developmental parameters in surviving fetuses, including fetal body weight, crown-rump length, tail length, or placental weight. No external, internal, or skeletal malformations were detected in fetuses in any group. DBTCl did not affect the incidence of fetuses with skeletal variation or skeletal ossification of fetuses. These data confirm our previous findings that DBTCl was embryolethal, but not teratogenic, in cynomolgus monkeys.

Keywords: *Developmental toxicity; embryolethality; dibutyltin; monkey*

Introduction

Organotin compounds are widely used in agriculture and industry (Quevauviller et al., 1991). Disubstituted organotin compounds are commercially the most important derivatives and are mainly used in the plastics industry, particularly as heat and light stabilizers for polyvinyl chloride (PVC) plastics to prevent degradation of the polymer during melting and forming of the resin into its final products, as catalysts in the production of polyurethane foams, and as vulcanizing agents for silicone rubbers (Piver, 1973; WHO, 1980). The most important nonpesticidal routes of entry for organotin compounds into the environment are through their use as PVC stabilizers (Quevauviller et al., 1991) and their use as antifouling agents, which introduces them to the aquatic environment (Maguire, 1991). Tributyltin (TBT) and dibutyltin (DBT) have been found in aquatic marine organisms (Lau, 1991; Sasaki et al., 1988) and marine products (Suzuki et al., 1992). TBT is degraded spontaneously and biochemically to DBT in the environment via a debutylation pathway (Seligman et al., 1988; Stewart and de Mora, 1990). These findings suggest that organotin compounds could be introduced into food products and subsequently consumed by humans.

We previously showed that dibutyltin dichloride (DBTCl) was embryolethal when orally administered during early pregnancy in rats (Ema and Harazono, 2000a, 2000b; Ema et al., 2003) and mice (Ema et al., 2007a). DBTCl was teratogenic when orally administered during organogenesis in rats (Ema et al., 1991); rat embryos were highly susceptible to the teratogenic effects of DBTCl when orally administered on days

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7 and 8 of pregnancy (Ema et al., 1992; Noda et al., 1993). Dibutyltin diacetate (DBTA) (Noda et al., 1992, 1993, 1994), dibutyltin maleate, dibutyltin oxide, and dibutyltin dilaurate were also teratogenic when orally administered during organogenesis in rats (Noda et al., 1993). Developmental toxicity studies on butyltins suggest that the teratogenicity of DBT is different from that of tetrabutyltin (TeBT), TBT, and monobutyltin (MBT) in its mode of action because the period of susceptibility and the types of induced malformations are different (Ema et al., 1995a, 1996a). DBTCl showed dysmorphogenic potential in a rat whole-embryo culture system (Ema et al., 1995b, 1996b). DBT was detected in maternal blood at 100 ng/g and embryos at 720 ng/g at 24 hours after gavage of DBTA at 22 mg/kg on day 8 of pregnancy in rats (Noda et al., 1994). The dysmorphogenic concentrations of DBTCl in cultured embryos were within the range of levels detected in maternal blood after the administration of a teratogenic dose of DBT at 20–40 mg/kg. These findings suggest that DBT itself causes DBT teratogenesis, possibly via direct interference with embryos.

The developmental toxicity of organotin compounds has been extensively investigated in rodents (Ema and Hirose, 2006). We previously assessed the prenatal developmental toxicity of DBT in cynomolgus monkeys and reported that nasogastric intubation of DBTCl at 2.5 or 3.8 mg/kg body weight/day during the entire period of organogenesis (days 20–50 of pregnancy) was embryolethal but is unlikely to be teratogenic (Ema et al, 2007b). However, the treatment regimen in our previous study, which was designed to screen for embryofetal lethality/teratogenicity and included a longer duration of treatment, might have masked or diminished some effects. A shorter administration period can provide more information about developmental toxicity because it permits increased doses and reduces maternal toxicity. However, there have been no studies on developmental toxicity in monkeys after shorter durations of treatment with organotin compounds. Therefore, the present study was conducted to further evaluate the developmental toxicity of DBTCl given to pregnant monkeys on 3 consecutive days during organogenesis and to determine if phase specificity could be observed with the shorter duration of administration.

Materials and methods

Animal experiments were performed at Shin Nippon Biomedical Laboratories, Ltd. (SNBL; Kagoshima, Japan) during 2004–2007 in compliance with the Guideline for Animal Experimentation (1987) and in accordance with the Law Concerning the Protection and Control of Animals (1973) and the Standards Relating to the Care and Management of Experimental Animals (1980). This study was approved by the Institutional Animal Care and Use Committee of SNBL and performed in accordance with the ethics criteria contained in the bylaws of the SNBL committee.

Animals

Cynomolgus monkeys (*Macaca fascicularis*) were used in this study. The monkeys were obtained from Guangxi Primate Center of China (Guangxi, China) through Guangdong Scientific Instruments and Materials Import/Export Co. (Guangzhou, China). The monkeys were quarantined for 4 weeks and confirmed to be free from tuberculosis, *Salmonella*, and *Shigella*. The animals were maintained in an air-conditioned room at 23.0–29.0°C, with a relative humidity of 35–75%, a controlled 12-12-light and dark cycle, and a ventilation rate of 15 air changes/hour. Monkeys were housed individually, except during the mating period and fed 108 g/day of diet (Teklad global 25% protein primate diet; Harlan Sprague-Dawley Inc., Madison, Wisconsin, USA) and *ad libitum* tap water from an automatic supply (Edstrom Industries Inc., Waterfold, WI, USA). Healthy male and female monkeys were selected for use. Only females showing 25–32-day menstrual cycles were used in the experiment. Each female monkey was paired with a male of proven fertility for 3 consecutive days between days 11–15 of the menstrual cycle. Visual confirmation of copulation and/or the presence of sperm in the vagina were considered evidence of successful mating. When copulation was confirmed, the median day of the mating period was regarded as day 0 of pregnancy. Pregnancy was confirmed 18–23 days after copulation by ultrasound (SSD-4000; Aloka Co., Mitaka, Japan) under anesthesia induced by an intramuscular injection of 5% ketamine hydrochloride (Sigma Chemical Co., St Louis, Missouri, USA). Pregnant females, weighing 2.51–4.50 kg on day 0 of pregnancy, were allocated randomly to seven groups, each with 5 monkeys, and housed individually.

Dosing

Monkeys were dosed once-daily with DBTCl (Lot no. GG01, 98% pure; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) at 7.5 mg/kg by nasogastric intubation on either days 19–21, 21–23, 24–26, 26–28, 29–31, 31–33, or 34–36 of pregnancy. The dosage levels were determined in previous studies where the administration of DBTCl at 2.5 or 3.8 mg/kg body weight/day

by nasogastric intubation during the entire period of organogenesis caused embryolethality (Ema et al., 2007b). DBTCl was dissolved in olive oil (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The dose volume was adjusted to 0.5 mL/kg body weight, based on the most recent body weight. The present study was performed almost at the same time as the previous study, in which the control monkeys were given olive oil on days 20–50 of pregnancy (Ema et al, 2007b), and the administration period in the previous study covered the administration period in the present study. Therefore, cynomolgus monkeys that received only olive oil in our previous study were used as the control group for this study and compared with the DBTCl-treated groups.

Observations

The pregnant monkeys were observed for clinical signs of toxicity twice a day during the administration period and once a day during the nonadministration period. Body weight was recorded on days 0, 20, 27, 34, 41, 51, 60, 70, 80, 90, and 100 of pregnancy. Food consumption was recorded on days 20, 23, 27, 30, 34, 37, 41, 44, 48, 51, 55, 58, 62, 80, 90, and 99 of pregnancy. Embryonic/fetal heartbeat and growth were monitored by using ultrasound under anesthesia on days 18, 19, 22, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, and 99 of pregnancy. For dams in which embryonic/fetal cardiac arrest was confirmed by ultrasound, the uterus, including the embryo/fetus and placenta and ovaries, were removed from the maternal body and stored in 10% neutral buffered formalin. The dead embryos/fetuses and placentae were morphologically examined.

Terminal caesarean sectioning was performed on day 100 of pregnancy, under anesthesia induced by an intramuscular injection of 5% ketamine hydrochloride (0.1–0.2 ml/kg) and inhaled isoflurane (0.5–2.0%; Dainippon Pharmaceutical Co., Ltd., Osaka, Japan). Salivation was inhibited by atropine (0.01 mg/kg; Tanabe Seiyaku Co., Ltd., Osaka, Japan). The fetus and placenta were removed from the dams. The placenta was morphologically examined, weighed, and stored in neutral buffered 10% formalin. Dams that underwent caesarean sectioning were not necropsied.

After fetal viability was recorded, fetuses were anesthetized by an intraperitoneal injection of pentobarbital sodium and euthanized by submersion in saline for 30–40 minutes at room temperature. Fetuses were weighed, sexed, and examined for external anomalies after confirmation of the arrested heartbeat. The anogenital distance (AGD), crown-rump length (CRL), head width, tail length, chest circumference, paw and foot length, distance between the eyes, umbilical cord length, volume of amniotic fluid, and diameters of the primary and secondary placentae were measured. After completion of the external examinations, the fetuses were examined for internal anomalies. The peritoneal cavity was opened, and the organs were grossly examined. The brain, thymus, heart, lungs, spleen, liver, kidneys, adrenal glands, and testes/uterus and ovaries were weighed and stored in 10% neutral buffered formalin. The eyeballs, stomach, small and large intestine, head skin, and auricles were stored in neutral buffered 10% formalin. Fetal carcasses were fixed in alcohol, stained with alizarin red S (Dawson, 1926), and examined for skeletal anomalies. The number of ossification centers in the vertebral column and the lengths of each humerus, radius, ulna, femur, tibia, and fibula were recorded.

Data analysis

The data were analyzed by using MUSCOT statistical analysis software (Yukums Co., Ltd., Tokyo, Japan). Data were analyzed by using Bartlett's test (Snedecor and Cochran, 1980) for the homogeneity of variance. When the variance was homogeneous, Dunnett's test (Dunnett, 1996) was performed to compare the mean value of the control group with that of each DBTCl group. When the variance was heterogeneous, the data were rank-converted and a Dunnett-type test (Miller, 1981) was performed to compare the mean value of the control group with that of each DBTCl group. The incidence of females showing toxicological signs was analyzed by Fisher's exact test. The fetal parameters were not statistically analyzed because the size of the groups was limited to a small number.

Results

Table 1 shows maternal findings for monkeys given DBTCl on 3 consecutive days during organogenesis. No maternal death occurred in any group. Soft stool and/or diarrhea in all groups, including the control group and vomiting in all DBTCl-treated groups, were observed. Significant increases in the incidence of females showing soft stool and/or diarrhea after the administration of DBTCl on days 19–21, 21–23, 24–26, or 26–28 of pregnancy, and females showing vomiting after the administration of DBTCl on days 19–21 of pregnancy were noted.

Figure 1 presents maternal body weight gain during pregnancy in monkeys given DBTCl on 3 consecutive days during organogenesis. Body weight gain on days 0–20 (during the preadministration

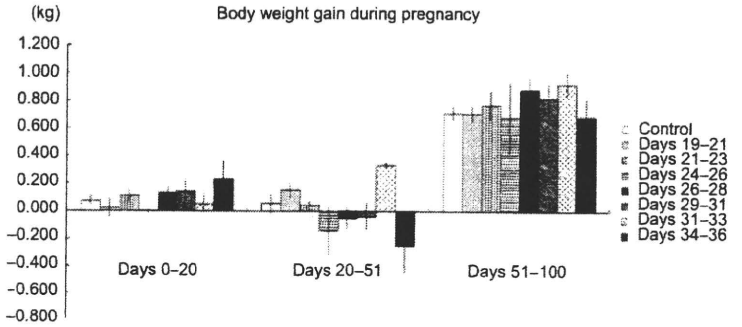

Figure 1. Maternal body weight gain during pregnancy in cynomolgus monkeys given DBTCl on three consecutive days during organogenesis.

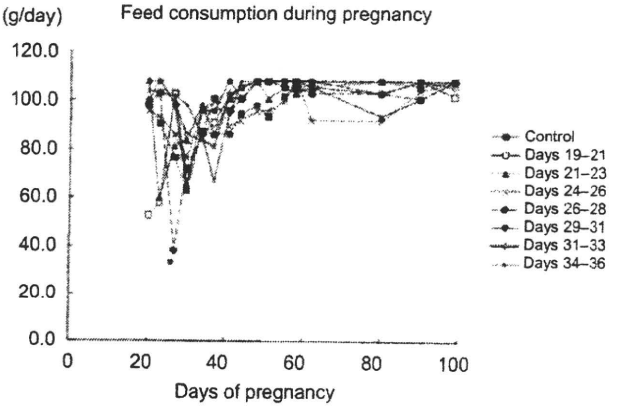

Figure 2. Maternal feed consumption during pregnancy in cynomolgus monkeys given DBTCl on three consecutive days during organogenesis. *Significantly different from the control group; $P<0.05$.

Table 1. Maternal findings in cynomolgus monkeys given DBTCl on three consecutive days during organogenesis.

Dose (mg/kg)	0 (Control)	7.5						
Dosing days of pregnancy	20–50	19–21	21–23	24–26	26–28	29–31	31–33	34–36
No. of pregnant females	12	5	5	5	5	5	5	5
No. of females showing toxicological sign								
Death	0	0	0	0	0	0	0	0
Soft stool/diarrhea	1	4*	4*	5*	4*	3	3	3
Yellowish white stool	0	2	0	2	0	1	0	0
External genital bleeding	0	1	0	0	1	0	0	0
Vomiting	0	3*	2	2	1	1	1	2
Initial body weight (kg)[a]	3.53±0.59	3.42±0.60	3.20±0.48	3.71±0.63	3.71±0.63	3.38±0.41	3.26±0.17	4.06±0.61

[a]Values are given as the mean±SD
*Significantly different from the control group, $P<0.05$

period) did not significantly differ between the control and DBTCl-treated groups. Although body weight gain on days 20–51 was reduced in groups given DBTCl on days 24–26, 26–28, 29–31, and 34–36 of pregnancy, there were no statistically significant differences between the control and DBTCl-treated groups. No significant decreases in body weight gain on days 51–100 were found in the DBTCl-treated groups.

Figure 2 illustrates maternal feed consumption during pregnancy in monkeys given DBTCl on 3 consecutive days during organogenesis. Significantly

Table 2. Reproductive and developmental findings for cynomolgus monkeys given DBTCl on threee consecutive days during organogenesis.

Dose (mg/kg)	0 (Control)	7.5						
Dosing days of pregnancy	20–50	19–21	21–23	24–26	26–28	29–31	31–33	34–36
No. of pregnant females	12	5	5	5	5	5	5	5
No. of females with embryonic/fetal loss	1	1	0	2	0	0	0	1
No. of females with live fetuses	11	4	5	3	5	5	5	4
No. of live fetuses	11	4	5	3	5	5	5	4
Sex ratio of live fetuses (male/female)	6/5	3/1	4/1	2/1	3/2	2/3	1/4	3/1
Body weight of live fetuses (g)[a]	126±14	122±12	124±16	100±12	110±7.5	117±21	111±16	124±13
Crown-rump length (cm)[a]	12.7±0.5	12.3±0.3	12.6±0.2	11.9±0.7	12.5±0.3	12.3±0.9	12.1±0.5	12.4±0.4
Tail length (cm)[a]	11.8±1.0	11.8±0.6	11.2±0.2	10.5±0.2	11.5±0.6	11.6±0.9	10.6±0.9	12.1±0.8
Anogenital distance (cm)[a]								
Male	4.2±0.5	4.3±0.1	4.2±0.2	3.8	4.0±0.3	4.2	3.4	4.5±0.3
Female	1.0±0.1	0.8	1.0	0.9	1.0	0.9±0.2	0.9±0.1	0.9
Placental weight (g)[a]	42.1±7.0	41.3±9.4	38.9±4.2	39.8±15.2	37.1±4.08	42.3±6.7	44.7±7.2	50.0±14.3
No. of single placentae	1	0	1	1	0	0	0	0
No. of fused placentae	0	0	1	0	0	0	1	1

[a]Values are given as the mean±SD.

Table 3. Summary of morphological examinations for fetuses of cynomolgus monkeys given DBTCl on three consecutive days during organogenesis.

Dose (mg/kg)	0 (Control)	7.5						
Dosing days of pregnancy	20–50	19–21	21–23	24–26	26–28	29–31	31–33	34–36
No. of fetuses examined	11	4	5	3	5	5	5	4
External examinations								
No. of fetuses with malformations	0	0	0	0	0	0	0	0
Internal examinations								
No. of fetuses with malformations	0	0	0	0	0	0	0	0
No. of fetuses with variations	0	0	0	0	0	0	0	0
Skeletal examination								
No. of fetuses with malformations	0	0	0	0	0	0	0	0
No. of fetuses with variations	0	1	2	2	0	0	1	0
Full supernumerary ribs	0	1	2	2	0	0	0	0
Shortening of 12th ribs	0	0	0	0	0	0	1	0
Cervical ribs	0	0	0	0	0	0	1	0
Ossification								
No. of ossified centers of vertebral column[a]	53.6±0.8	53.0±1.4	53.4±1.3	53.7±1.5	53.4±1.1	53.2±1.6	52.8±1.3	53.8±0.5
Skeletal length (mm)[a]								
Humerus	23.6±0.8	23.1±1.3	23.3±1.3	21.2±0.4	22.8±0.5	23.2±1.5	22.2±1.3	23.3±0.8
Radius	23.0±1.0	22.4±1.7	22.9±1.6	20.7±0.4	22.2±1.0	22.2±1.6	21.6±1.2	22.3±1.1
Ulna	24.6±1.0	24.0±1.1	24.3±1.1	22.4±0.5	23.9±0.8	23.5±1.5	23.0±1.1	22.8±2.2
Femur	22.3±1.2	22.0±1.4	21.7±1.5	20.2±0.6	21.3±0.3	22.2±1.6	20.9±1.7	22.5±1.3
Tibia	21.5±1.3	21.2±1.9	21.2±1.6	19.6±0.5	20.3±0.6	21.1±1.1	19.9±1.6	21.4±1.4
Fibula	19.8±1.0	20.0±1.8	19.6±1.4	18.1±0.1	18.7±0.4	19.6±1.0	18.5±1.5	19.6±1.0

[a]Values are given as the mean±SD

reduced feed consumption was only found on days 27–28 in the group given DBTCl on days 26–28 of pregnancy.

Table 2 shows the reproductive and developmental findings for monkeys given DBTCl on 3 consecutive days during organogenesis. There was an abortion on day 90 for 1 female given DBTCl on days 19–21, an abortion on day 35, and an embryonic loss on day 35 for females given DBTCl on days 24–26, and fetal death on day 90 for 1 female given DBTCl on days 34–36. No embryonic/fetal loss or abortions were found for females given DBTCl on days 21–23, 26–28, 29–31, or 31–33. No difference was observed in the incidence of embryonic/fetal loss between the control and DBTCl-treated groups. A shortened tail length was detected in fetuses of dams given DBTCl on days 31–33 of pregnancy. There were no changes in sex ratio, body weight, AGD or CRL of live fetuses, or placental weight. A single placenta was observed for one dam each from the control group and groups given DBTCl on days 21–23 and 24–26, and a fused placenta was found in one dam each in the groups given DTBCl on days 21–23, 31–33, and 34–36. There were no differences in head width, chest circumference, paw and foot length, or distance between the eyes of fetuses. There were also no differences in umbilical cord length, volume of amniotic fluid, or diameters of the primary and secondary placentae between the control and DBTCl-treated groups (data not shown).

Table 3 summarizes the results of morphological examinations of monkey fetuses given DBTCl on 3 consecutive days during organogenesis. No external, internal, or skeletal malformations were found in fetuses in any group. No internal variations were observed of any group. Skeletal examinations revealed full supernumerary ribs in 1 fetus in the groups given DBTCl on days 19–21 and 2 fetuses each in the groups given DBTCl on days 21–23 and 24–26, as well as shortening of the 12th and cervical ribs in 1 fetus of the group given DBTCl on days 31–33. There were no differences in the number of ossified centers of the vertebral column or the length of the radius, femur, tibia, or fibula between the control and DBTCl-treated groups. However, the humerus, radius and ulna all were shortened in the group given DBTCl on days 24–26. Although a decrease was observed in the absolute brain weight of monkey fetuses given DBTCl on days 24–26, 26–28, and 31–33, and a decrease was also observed in the weights of the hearts of fetuses given DBTCl on days 24–26, there was no difference between the control and DBTCl-treated groups in the relative weight of any organ (data not shown).

Discussion

Many studies on the developmental toxicity of DBT have been performed using rodents, primarily rats (Ema and Hirose, 2006). No single species has yet clearly emerged as a superior model for the testing of developmental toxicity (Schardein, 2000). Nonhuman primates appear to provide an especially appropriate model for the testing of teratogenicity because of their high ranking on the evolutionary scale (Hendrickx and Binkerd, 1979). The close phylogenetic relationship of old-world monkeys to humans may render them the most desirable models for teratology studies (Schardein, 2000). The similarities in placentation and embryonic development between monkeys and humans are of considerable value for investigating the developmental toxicity of chemicals (Poggel and Günzel, 1988). Therefore, we previously determined prenatal developmental toxicity in monkeys given DBTCl during the entire period of organogenesis (Ema et al., 2007b). In the present study, relatively high doses of DBTCl were administered to monkeys during the early and middle periods of organogenesis, because teratogenic effects have been noted following the administration of DBTCl to rats during early organogenesis (Ema et al., 1992; Noda et al., 1993).

The doses of DBTCl used in the present study were expected to induce maternal toxicity, thereby allowing the characterization of the effects of DBTCl on embryonic/fetal development. Maternal toxicity, as evidenced by the increased incidence of pregnant females showing soft stool/diarrhea and vomiting, was found in all groups given DBTCl and was observed after the administration of DBTCl on days 19–28 of pregnancy. These findings indicate that more severe general toxicity is induced by DBTCl administration at earlier time points during pregnancy in cynomolgus monkeys.

In our previous study in which DBTCl was given to cynomolgus monkeys during the entire period of organogenesis, DBTCl at 2.5 mg/kg was sufficient to induce embryonic/fetal loss around days 35–60 of pregnancy (Ema et al., 2007b). In the present study, embryonic/fetal loss was found in females given DBTCl on days 19–21 and 34–36 and in 2 females given DBTCl on days 24–26 of pregnancy. It is, therefore, likely that days 24–26 of pregnancy may be more susceptible to the lethal effect of DBTCl on embryos/fetuses.

Decreased absolute weights of the brain and/or heart observed in fetuses of monkeys given DBTCl on days 24–26, 26–28, and 31–33 were not thought to be due to toxic effects on embryonic/fetal development because the changes were small and the

relative weights were not decreased. Short tail length was observed in fetuses of dams given DBTCl on days 24–26 and 31–33 of pregnancy. The tail lengths in the background control data during 1994–2006 in the laboratory performing the current study were 8.6–15.1 mm (mean ± SD = 12.3 ± 0.6) in 239 fetuses. The short tails observed in the present study are unlikely to have toxicological significance, because the change was small and within the range of the control background data. However, the embryonic/fetal changes observed in the 24–26-day group may be associated with the adverse maternal effects observed at these dosage levels. Collectively, these findings suggest that DBTCl is not toxic to embryonic/fetal growth *per se* at 7.5 mg/kg when administered on 3 consecutive days during organogenesis, but that the delays in development may be associated with maternal toxicity.

Although a single placenta was found in 1 female in the control group and 1 female each in groups given DBTCl on days 21–23 and 24–26, and a fused placenta was found in 1 female each in groups given DBTCl on days 21–23, 31–33, and 34–36, the appearance of a single placenta or fused placenta is not uncommon in developmental toxicity studies in cynomolgus monkeys. The incidences in our historical control data during 1994–2006 were 0–66.7% (mean = 12.6% of 255 pregnancies) for a single placenta and 0–11.1% (mean = 4.2% of 255 pregnancies) for a fused placenta. The incidences of females with a single or fused placenta in the present study were within the range of or slightly higher value than that of the background control data, respectively. We are unaware of any studies of the relationship between these types of placenta and the development of monkey embryos/fetuses, and we do not have any evidence suggesting that these types of placenta adversely affect the normal development of embryos/fetuses in cynomolgus monkeys.

On morphological examination, fetuses with full supernumerary ribs as well as shortened 12th ribs and cervical ribs were found in the DBTCl-treated groups. A recent survey of international experts in the field of reproductive/developmental toxicology resulted in high agreement that full supernumerary ribs and cervical ribs should be considered as variations, and in poor agreement that shortened 12th ribs should be considered as malformations (Solecki et al., 2001). Therefore, our findings would be classified as skeletal variations, based on the above survey. Chahoud et al. (1999) noted that variations are unlikely to adversely affect survival or health and might result from delayed growth or morphogenesis; the fetuses otherwise follow a normal pattern of development. The incidences in our historical control data were 0–33.3% (mean = 9.5%, 24 of 239 fetuses) for full supernumerary ribs and 0–18.2% (mean = 2.0%, 5 of 239 fetuses) for cervical ribs. In the present study, a relatively higher incidence of full supernumerary ribs was observed after the administration of DBTCl on days 19–26 of pregnancy. We defined the ribs present in the lateral potion of the first lumbar vertebra and the distal cartilagious portion as full supernumerary ribs. Full supernumerary, but not rudimentary, ribs are thought to be an indicator of toxicity during the embryonic development of rats (Kimmel and Wilson, 1973) and mice (Rogers et al., 2004). Branch et al. (1996) noted that supernumerary ribs might be induced in embryos on gestation day 8, prior to any morphological differentiation of the axial skeleton, and cartilaginous supernumerary ribs were visible in fetuses on gestation day 14 prior to ossification in mice. These findings may be consistent with the present findings that full supernumerary ribs were found in cynomolgus monkeys given DBTCl during early organogenesis. In monkeys, however, the toxicological significance of supernumerary ribs is still unknown.

Shorter lengths of the humerus, radius, and ulna were observed in fetuses of dams given DBTCl on days 24–26. The lengths of the humerus and ulna in our background control data during 1994–2006 were 19.1–26.6 mm (mean ± SD = 23.4 ± 0.6) and 18.4–28.9 mm (mean ± SD = 24.7 ± 0.7), respectively, for 239 fetuses. The shortened lengths of these bones observed in the present study are probably associated with maternal toxic exposure. Morphological examinations of dead embryos/fetuses in the DBTCl-treated groups revealed no anomalies.

Collectively, these findings suggest that the morphological alterations observed in fetuses in the present study do not indicate a teratogenic response, and that DBTCl possesses no teratogenic potential in cynomolgus monkeys, although it does retard development and increase variations at maternally toxic doses.

Conclusion

In conclusion, the administration of DBTCl to pregnant cynomolgus monkeys on 3 consecutive days during organogenesis had an adverse effect on embryonic/fetal survival, retarded fetal growth, and produced a slight increase in skeletal variations, but no malformations.

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