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

Makoto Ema[1], Akihiro Arima[2], Katsuhiro Fukunishi[2], Mariko Matsumoto[1], Mutsuko Hirata-Koizumi[1], Akihiko Hirose[1], and Toshio Ihara[2]

[1]*Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, Tokyo, Japan, and* [2]*Shin Nippon Biomedical Laboratories, Ltd., Kagoshima, Japan*

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

Address for Correspondence: Makoto Ema, Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan; Fax: +81-3-3700-1408; E-mail: ema@nihs.go.jp

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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

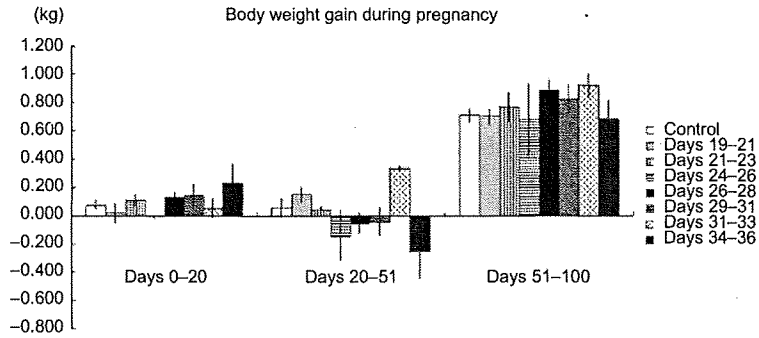

Figure 1. Maternal body weight gain during pregnancy in cynomolgus monkeys given DBTCl on three consecutive days during organogenesis.

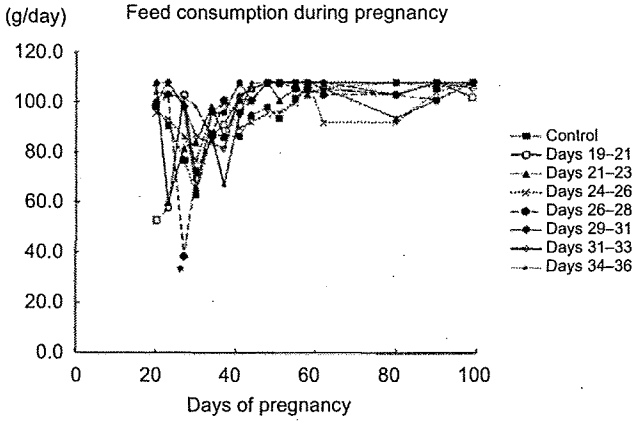

Figure 2. Maternal feed consumption during pregnancy in cynomolgus monkeys given DBTCl on three consecutive days during organogenesis. *Significantly different from the control group; $P<0.05$.

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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Fetal malformations and early embryonic gene expression response in cynomolgus monkeys maternally exposed to thalidomide☆

Makoto Ema[a,*], Ryota Ise[b], Hirohito Kato[c], Satoru Oneda[d], Akihiko Hirose[a], Mutsuko Hirata-Koizumi[a], Amar V. Singh[e], Thomas B. Knudsen[f], Toshio Ihara[c]

[a] Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[b] Shin Nippon Biomedical Laboratories (SNBL), Ltd., Tokyo, Japan
[c] Shin Nippon Biomedical Laboratories (SNBL), Ltd., Kagoshima, Japan
[d] SNBL USA, Ltd., Everett, WA, USA
[e] Contractor to NCCT, Lockheed-Martin, Research Triangle Park, NC 27711, USA
[f] National Center for Computational Toxicology (NCCT), U.S. Environmental Protection Agency, Research Triangle Park, NC 27711, USA

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ABSTRACT

The present study was performed to determine experimental conditions for thalidomide induction of fetal malformations and to understand the molecular mechanisms underlying thalidomide teratogenicity in cynomolgus monkeys. Cynomolgus monkeys were orally administered thalidomide at 15 or 20 mg/kg-d on days 26–28 of gestation, and fetuses were examined on day 100–102 of gestation. Limb defects such as micromelia/amelia, paw/foot hyperflexion, polydactyly, syndactyly, and brachydactyly were observed in seven of eight fetuses. Cynomolgus monkeys were orally administered thalidomide at 20 mg/kg on day 26 of gestation, and whole embryos were removed from the dams 6 h after administration. Three embryos each were obtained from the thalidomide-treated and control groups. Total RNA was isolated from individual embryos, amplified to biotinylated cRNA and hybridized to a custom Non-Human Primate (NHP) GeneChip® Array. Altered genes were clustered into genes that were up-regulated (1281 genes) and down-regulated (1081 genes) in thalidomide-exposed embryos. Functional annotation by Gene Ontology (GO) categories revealed up-regulation of actin cytoskeletal remodeling and insulin signaling, and down-regulation of pathways for vasculature development and the inflammatory response. These findings show that thalidomide exposure perturbs a general program of morphoregulatory processes in the monkey embryo. Bioinformatics analysis of the embryonic transcriptome following maternal thalidomide exposure has now identified many key pathways implicated in thalidomide embryopathy, and has also revealed some novel processes that can help unravel the mechanism of this important developmental phenotype.

1. Introduction

Thalidomide (α-phthalimidoglutarimide) was synthesized in West Germany in 1953 by the Chemie Grünenthal pharmaceutical firm, and was marketed from October 1957 into the early 1960s. It was used for treating nausea and vomiting late during pregnancy and was also said to be effective against influenza. The first case of the phocomelia defect, although not recognized at the time as drug-related, was presented by a German scientist in 1959; subsequently, malformed children were reported in 31 countries [1]. A pattern of defects of limbs as well as the ocular, respiratory, gastrointestinal, urogenital, cardiovascular and nervous systems caused by maternal thalidomide exposure during early pregnancy was observed. Limb defects such as phocomelia, amelia, micromelia, oligodactyly, and syndactyly were the most common malformations [2]. After removal from the global market in 1962, thalidomide was reintroduced in 1998 by the biotechnology firm Celgene as an immunomodulator for the treatment of erythema nodosum leprosum, a serious inflammatory condition of Hansen's disease, and in orphan status for treating Crohn's disease and several other diseases [1].

Animal species are not equally susceptible or sensitive to the teratogenicity of chemical agents, and some species respond more readily than others [3]. For thalidomide, a variety of developmental toxic effects were reported in 18 animal species, but the responses have been highly variable across species. Limb defects that mimic

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* Corresponding author. Tel.: +81 3 3700 9878; fax: +81 3 3700 1408.
E-mail address: ema@nihs.go.jp (M. Ema).

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human thalidomide embryopathy have only been observed and replicated in a few strains of rabbits and in primates [1,3,4]. Eight of nine subhuman primates treated with thalidomide showed characteristic limb reduction malformations ranging from amelia to varying degrees of phocomelia at a dosage and timing comparable to those observed in human thalidomide embryopathy [3,5]. Since the first report of thalidomide embryopathy appeared 50 years ago, considerable information regarding the therapeutic applications of this drug has accumulated, but the mechanisms by which thalidomide produce congenital malformations are still not well understood [2,3,5].

The non-human primate *Macaca fascicularis* (cynomolgus monkey) is widely used in prenatal developmental studies because of year-round rather than seasonal breeding behavior [6]. Kalter [5] noted that non-human primates, especially macaques and baboons, are favorable for mechanistic studies; however, only two full reports of the teratogenicity of thalidomide in cynomolgus monkeys are available [7,8]. In those studies, cynomolgus monkeys were given thalidomide by gavage at doses of 5–30 mg/kg-d during gestation days 20–30, and fetuses were examined morphologically. The findings of these studies determined the critical period and doses of thalidomide required for the production of fetal malformations in this macaque species. Although amounts taken were not always accurately recorded in humans, available documents show that typical malformations resulted from the ingestion of as little as 25 mg three times a day or 100 mg/day for 3 days during the sensitive period, equivalent to an astonishingly small dosage of about 1 mg/kg-d [5]. In teratology studies using cynomolgus monkeys, the timing of dosing was comparable to the human one and the doses were estimated to be 5–30 times higher than those which produced typical malformations in humans [5,7,8].

Knowledge of the patterns of altered gene expression in embryonic target organs on a global scale is an important consideration for understanding the mechanisms of teratogenesis [9–13]. The application of cDNA microarray technology, a genome-wide analysis technique, to cynomolgus monkeys facilitates the rapid monitoring of a large number of gene alterations in this species [14]. In order to obtain information about the molecular mechanisms underlying the detrimental effects of thalidomide teratogenicity, the present study has determined the experimental conditions required to produce thalidomide-induced fetal defects that mimicked human abnormalities in cynomolgus monkeys and then profiled altered patterns of gene expression in these embryos during the critical period. The dosing used in the present study was 15 or 20 mg/kg-d thalidomide given by gavage to pregnant dams at days 26–28 of gestation for teratological evaluation, and 20 mg/kg given on day 26 for gene expression profiling 6 h post-treatment.

2. Materials and methods

2.1. Teratological evaluation

The teratology study was performed at SNBL USA, Ltd. (Everett, WA, USA) in compliance with the Animal Welfare Act and recommendations set forth in The Guide for the Care and Use of Laboratory Animals [15]. Only females showing 25–32-day menstrual cycles were used in these experiments. Each female monkey was paired with a male of proven fertility for 3 days between days 11 and 15 of the menstrual cycle. When copulation was confirmed, the median day of the mating period was regarded as day 0 of gestation. Pregnancy was confirmed on day 20 or day 25 by ultrasound (SSD-4000, Aloka Co., Mitaka, Japan) under sedation induced by intramuscular injection of 5% ketamine hydrochloride (Sigma Chemical Co., St Louis, MO, USA). The monkeys were given (±)-thalidomide (Lot no. SEH7050, Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 15 or 20 mg/kg-d by oral administration using gelatin capsules (Japanese Pharmacopiae grade) on days 26–28 of gestation. The dosage was adjusted to the body weight on day 25 of gestation. Cesarean section was performed on day 100–102 of gestation under deep anesthesia induced by intramuscular injection of 5% ketamine hydrochloride (0.1–0.2 ml/kg) and inhalation of isoflurane (0.5–2.0%, Baxter, Liberty Corner, NJ, USA). Salivation was inhibited by atropine (0.01 mg/kg, Phenix Pharmaceutical, St. Joseph, MO, USA). Fetal viability was recorded, and the fetuses were euthanized by intraperitoneal injection of pentobarbital and phenytoin solution (Euthasol®, Virbac Corp., Fort Worth, TX, USA). Fetuses were sexed and examined for external anomalies after confirmation of the arrested heartbeat. After the completion of external examinations, fetuses were examined for internal abnormalities.

2.2. Microarray experiments

The animal experiments were performed at Shin Nippon Biomedical Laboratories (SNBL), Ltd. (Kagoshima, Japan) 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.

Each female monkey was paired with a male of proven fertility for 1 day between day 11 and day 15 of the menstrual cycle. Pregnant females, aged 5–8 years and weighing 2.84–3.76 kg on day 22 of gestation, were allocated randomly to two groups, each with three monkeys, and housed individually. The monkeys were orally dosed with (±)-thalidomide (Lot no. SDH7273/SDJ3347, Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 0 or 20 mg/kg by oral administration of a gelatin capsule on day 26 of gestation, which was during the critical period for thalidomide-induced teratogenesis [7,8]. Dosage was adjusted to the body weight on day 22 of gestation. Control monkeys received the capsule only.

2.3. RNA sample collection

Hysterectomy was performed under terminal anesthesia at 6 h after the administration of thalidomide on day 26 of gestation. Whole embryos were rapidly removed from the uterus using a stereomicroscope and immersed in sterilized physiological saline. Three embryos each in the thalidomide-treated and control groups were obtained for RNA analysis and stored at −70 °C until further processing. General factors of maternal age, weight and date of processing these samples are shown in Table 1. Embryos were processed simultaneously, and aside from the blocking factors in Table 1, all six samples were handled concurrently through RNA isolation and hybridization.

2.4. RNA preparation and labeling

Total RNA was isolated from each day-26 embryo, amplified to cRNA, and biotin-labeled for analysis on the Affymetrix NHP GeneChip® Array at Gene Logic Inc. (Gaithersburg, MD, USA) using the TRIzol method and RNeasy columns according to protocols from Affymetrix (Santa Clara, CA, USA). The 28S/18S rRNA ratio of isolated RNA was assessed using a Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and found to be of sufficiently high quality. Biotinylated cRNA was finally cleaned up and fragmented by limited hydrolysis to a distribution of cRNA fragment sizes below 200 bases.

2.5. Affymetrix NHP GeneChip® Array and hybridization

Biotinylated cRNA samples from control and exposed embryos ($n = 3$ each) were hybridized using Biogen Idec's (NASDAQ: BIIB) proprietary Affymetrix NHP GeneChip® Array platform. This microarray chip contains a comprehensive representation of the Cynomolgus genome derived from Biogen Idec's proprietary sequencing efforts, from which Gene Logic (www.genelogic.com/) subsequently obtained the exclusive rights to provide as a service (personal communication, Jun Mano, Gene Logic). The steps for hybridization followed a protocol described in the Gene Logic GeneChip® Analysis Manual (Gaithersburg, MD, USA). Probe-sets for this analysis consisted of cynomolgus expressed sequence tags (ESTs), published rhesus monkey ESTs, predictive coding sequences from the rhesus genome, and human genes not represented by monkey sequences. Because of the incomplete state of annotation for the cynomolgus genome at the time this study was undertaken, we used human, mouse and rat gene annotations to characterize monkey genes on the NHP GeneChip® Array. This reasonably assumes that most cynomolgus sequences are well-annotated by human ortholog information. After hybridization the GeneChip® Arrays were scanned and raw signal values were subjected to subsequent normalization and processing.

2.6. Microarray data processing and analysis

Probe-level data normalization from the six *.cel files used the robust multi-chip average (RMA) method with perfect-match (PM) but not mismatch (MM) data from the microarrays. RMA returns a single file containing the 51,886 probes in six columns of normalized data, representing the log 2-intensity of each probe. To query differential transcript abundance between sample groups, the log 2 ratio of treated (Q) to reference (R) was computed for all six samples, with R being the average of the three controls. The six columns were centered to MEDIAN = 0.00 and scaled to STDEV = 0.50 [10,12]. These data were loaded to GeneSpring GX7.3 software (Agilent Technologies, Redwood City, CA, USA) for one-way analysis of variance (ANOVA) by treatment group. Due to the small sample size ($n = 3$) and limited annotation of the cynomolgus genome for this preliminary analysis we relaxed the selection criterion

Table 1
Procurement of cynomolgus embryos at SNBL for microarray study.

Group	Embryo	Maternal age in years	Maternal bw in kg (day 22)	Date of embryo collection (day 26)	*.cel filename (NIHS)
Control	001	6	3.76	November 2, 2006	137255bpcyna11.cel
	002	7	2.84	December 2, 2006	137256bpcyna11.cel
	003	8	3.68	December 2, 2006	137257bpcyna11.cel
Thalidomide	101	5	2.97	October 30, 2006	137258bpcyna11.cel
	102	6	3.01	November 6, 2006	137259bpcyna11.cel
	103	8	3.14	November 24, 2006	137260bpcyna11.cel

by not applying a false-discovery rate filter. Genes or probes passing the statistical (ANOVA) filter at a P value of 0.05 were subjected to K-means clustering, with cluster Set 1 and Set 2 that were up-regulated and down-regulated, respectively, in the thalidomide-exposed versus control embryos. Entrez gene identifiers were used for bioinformatics evaluation (http://www.ncbi.nlm.nih.gov/).

3. Results

3.1. Teratological evaluation

To confirm thalidomide embryopathy in the cynomolgus colony under the conditions used for this study, pregnant dams were given thalidomide at 15 and 20 mg/kg on days 26–28 of gestation. Four fetuses were obtained at each dose for teratological evaluation (Table 2). Although we did not observe a clear dose–response in this limited number of fetuses, we did observe a number of cases with limb defects consistent with human thalidomide embryopathy. Fig. 1 shows external appearance of fetuses of dams exposed to thalidomide on days 26–28 of gestation. Bilateral amelia in the fore-/hindlimbs was noted in one female fetus at 20 mg/kg, and bilateral micromelia in the hindlimbs was observed in four fetuses at 15 mg/kg. Deformities of the paw and/or foot including hyperflexion, ectrodactyly, polydactyly, syndactyly, brachydactyly, and/or malpositioned digits, were observed in all fetuses at 15 mg/kg and in two fetuses at 20 mg/kg. Tail anomalies were found in one fetus at 15 mg/kg and three fetuses at 20 mg/kg. Small penis was noted in one fetus each in both thalidomide-treated groups. No internal abnormalities were noted in any of the thalidomide-treated fetuses examined here. This confirmed the relevant sensitivity of cynomolgus embryos to thalidomide, based on a maternally administered dose of 15–20 mg/kg during days 26–28 of gestation.

3.2. Genes altered by thalidomide

The embryonic transcriptome was evaluated at 6 h after 20 mg/kg maternal thalidomide exposure on day 26. For this analysis, we used a proprietary Non-Human Primate (NHP) microarray having representation of the cynomolgus genome (see Section 2 for details). The NHP array includes 18,293

Table 2
Morphological findings in fetuses of cynomolgus monkeys given thalidomide on days 26–28 of gestation.

Target	Dose	15 mg/kg				20 mg/kg			
Findings	Fetus no.	1	2	3	4	5	6	7	8
	Gender	Female	Male	Female	Female	Male	Male	Male	Female
Forelimb									
Amelia		–	–	–	–	–	–	–	B
Paw									
Hyperflexion		B	–	–	–	–	–	–	–
Ectrodactyly		L	–	–	–	–	–	–	–
	Accessory digit(s)[a]	L	–	–	–	–	–	–	–
Polydactyly[a]		–	R	–	–	–	–	–	–
	Brachydactyly	–	–	–	B	–	–	–	–
Hindlimb									
Micromelia		B	B	B	B	–	–	–	–
Amelia		–	–	–	–	–	–	–	B
Foot									
Hyperflexion		–	B	B	B	–	–	–	–
Ectrodactyly		–	B	R	R	–	–	–	–
Polydactyly		–	–	–	–	B	B	–	–
Syndactyly		R	–	B	–	–	–	–	–
	Brachydactyly	–	–	–	L	–	–	–	–
	Malpositioned digit(s)	–	–	L	–	–	–	–	–
Craniofacial		–	–	–	–	–	–	–	–
Trunk		–	–	–	–	–	–	–	–
Tail									
	Bent or curled tail	–	–	–	+	–	+	+	+
Short tail		–	–	–	–	–	–	+	–
External genital organs									
Small penis		–	+	–	–	+	–	–	–

–: No anomaly was observed.
+: Anomaly was observed.
B: Bilateral anomaly was observed.
R: Unilateral (right side) anomaly was observed.
L: Unilateral (left side) anomaly was observed.
[a] Polydactyly means (almost) complete extra digits existed, and accessory digit incomplete "digit like tissue" attached to a normal digit.

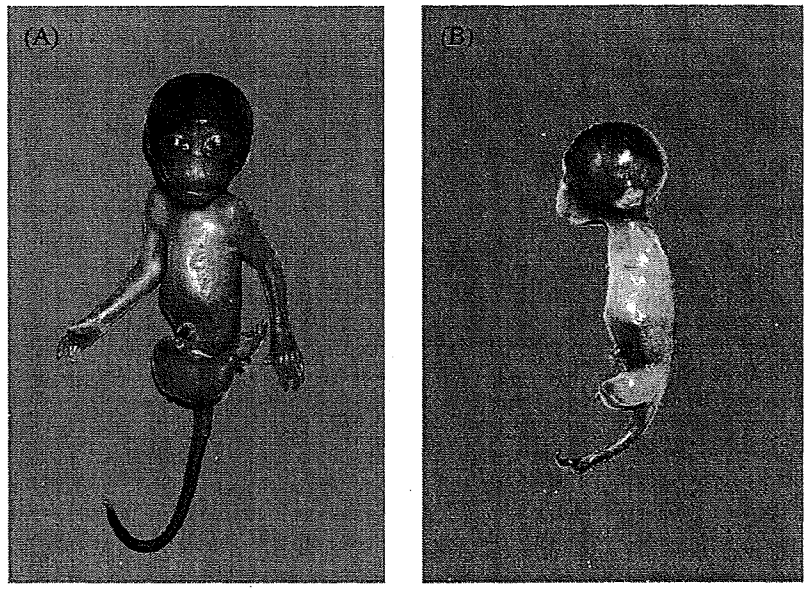

Fig. 1. Malformed fetuses of cynomolgus monkeys exposed to thalidomide on days 26–28 of gestation. (A) The fetus of maternal monkey given thalidomide at 15 mg/kg-d exhibiting brachydactyly in the paw, micromelia in the hindlimb, hyperflexion, ectrodactyly and brachydactyly in the foot and curled tail. (B) The fetus of maternal monkey given thalidomide at 20 mg/kg-d exhibiting amelia in the fore- and hindlimb and bent tail.

cynomolgus genes and 8411 Rhesus genes as well as genes from several other species. The six-array dataset conforming to MIAME standards resides in the Gene Expression Omnibus repository (www.ncbi.nlm.nih.gov/geo/) under platform accession number GPL8393 (series GSM389350–GSM389355). A thalidomide-sensitive subset of genes in the embryonic transcriptome was reflected in the high-percentage of present calls for genes whose expression levels showed ≥1.5-fold difference between thalidomide-treated and control embryos.

Statistical (ANOVA) analysis identified 2362 genes that differed significantly between control and thalidomide groups ($P \leq 0.05$). The heat map for these genes showed a clear pattern (Fig. 2). *K*-means clustering partitioned them into primary sets of up-regulated (1281) genes and down-regulated (1081) genes for thalidomide relative to control embryos.

3.3. Annotation systems

Ranking functional categories of genes in an expression cluster is an important step to unravel the cellular functions and pathways represented in the differentially expressed gene list. To derive the highest ranking biological themes across the up-/down-regulated gene lists, Entrez gene IDs were annotated by Gene Ontology (GO) category using the Database for Annotation, Visualization, and Integrated Discovery (http://apps1.niaid.nih.gov/david/). Table 3 lists the significantly over-represented themes when the 1281 up-regulated genes (Table 3A) and 1081 down-regulated genes (Table 3B) were mapped by GO category. We used level-4 annotation for Biological Processes, Cellular component and Molecular Function as well as curated pathways from the KEGG (Kyoto Encyclopedia of Genes and Genomes) open source pathway resource to obtain categories passing by Fisher exact test ($P \leq 0.05$). For clarity and greater specificity we limited the categories in Table 3 to those having at least 10 hits for sensitivity and no more than 50 hits to improve specificity.

Integrated biological processes evident across the up-regulated categories addressed the regulation of cellular growth, including cell cycle progression, DNA repair and nucleic acid transport. Other up-regulated biological processes addressed the regulation of metabolism, the cytoskeletal cycle, heart development and vesicle transport. Many of these processes were logically reflected in the ontologies for cellular components addressing the nucleo-ribosomal system, the microtubule network, and molecular functions for GTPase activity and actin binding. Up-regulated signaling pathways (KEGG) included several oncogenic growth pathways as well as the TGF-beta, GnRH and insulin signaling pathways.

Integrated biological processes evident across the down-regulated categories addressed ion homeostasis and cellular secretion. These processes were logically reflected in the ontologies for cellular components addressing the endoplasmic reticulum, GTPase activity and transferases. Other down-regulated biological processes addressed cell growth, muscle and vasculature development, and the inflammatory response—consistent with KEGG pathways for hematopoietic cells and antigen processing.

4. Discussion

The results from this study show that a teratogenic dose of thalidomide (20 mg/kg) significantly alters global gene expression profiles in the cynomolgus monkey embryo within 6 h of exposure on day 26 of gestation. Bioinformatics analysis of the embryonic transcriptome following maternal thalidomide exposure revealed up-regulation in several signaling pathways with roles in morphogenesis and oncogenesis (e.g., TGF-beta, insulin signaling), and down-regulation of the endoplasmic reticulum and inflammatory response. As might be anticipated, this implies a broad reaction of the embryo to the mechanism of thalidomide and a generalized reprogramming of pathways known to be important in development and teratogenesis.

The dosing scenario used in the present study was 15 or 20 mg/kg-d thalidomide given by gavage to pregnant dams on days 26–28 of gestation for teratological evaluation, and 20 mg/kg given on day 26 for gene expression profiling 6 h post-treatment. The teratological exposure induced limb malformations consistent with earlier studies with thalidomide in pregnant macaques. For example, it was previously reported that two fetuses with amelia were obtained from two of four cynomolgus monkeys given thalidomide by gavage at 10 mg/kg-d on days 32–42 after commencement of menses (approximately equivalent to days 20–30 of gestation)

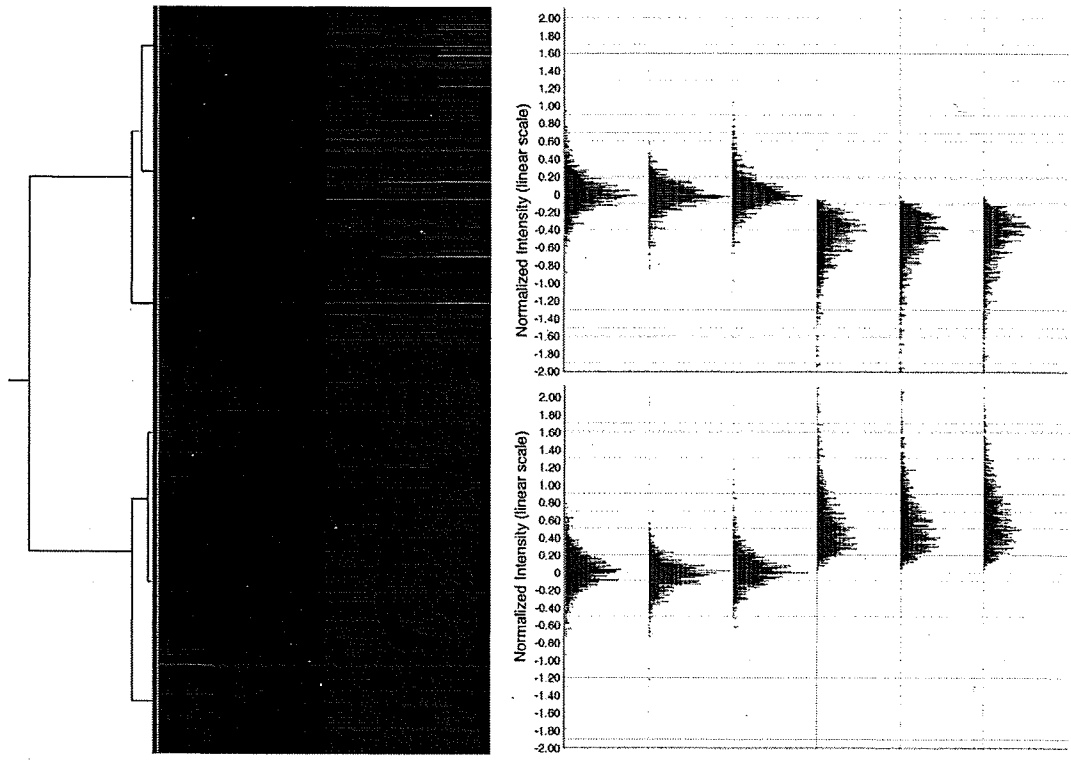

Fig. 2. Molecular abundance profiles of the thalidomide-sensitive genes in the cynomolgus embryonic transcriptome on day 26 of gestation. RNA was isolated from day 26 embryos 6 h after maternal exposure to 20 mg/kg thalidomide or vehicle control. Values represent log 2 ratios of treated/reference, where the reference is an average of all three controls for each gene. ANOVA returned 2362 genes that were significantly different between the groups ($n = 3$, $P \leq 0.05$). The heat map visualizes the genes in rows and the embryos in columns, and the histogram shows the distribution of genes in each cluster. Columns left to right: 1–3 from control embryos (#001, #002, #003) and 4–6 from thalidomide embryos (#101, #102, #103). Genes were partitioned by *K*-means clustering into two primary expression clusters with 1281 up-regulated genes (red) and 1081 down-regulated genes (green).

and that the fetal malformations were similar to malformations reported in children whose mothers had taken thalidomide during pregnancy [7]. Forelimb malformations in the cynomolgus fetus were noted following a single oral administration of thalidomide on days 25, 26 or 27 of gestation at 10 and 30 mg/kg and daily administration on days 25–27 of gestation at 5 mg/kg, and both fore- and hindlimb malformations were observed following a single oral administration on day 25 or 28 of gestation at 30 mg/kg [8]. The present study, taken together with the previous studies [7,8], indicate that orally administered thalidomide induces fetal malformations in cynomolgus monkeys similar to human pregnancies and furthermore localizes the vulnerable period to days 25–28 of gestation and the effective doses to 5–30 mg/kg-d.

Given the limitations of working with this species the preliminary application of a custom NHP microarray, the analysis at one dose and time point, and the incomplete state of annotation of the macaque genome, the current study design focused on RNA collected from individual embryos rather than the specific target organ system (forelimb, hindlimb). Ideally a follow-up study on focused gene expression analysis should be performed for specific embryonic limbs in which malformations have been induced with thalidomide; however, the present study is among the first to provide genomic information on the initial changes in gene expression occurring in macaque embryos during the critical events following a teratogenic dose of thalidomide. A total of 43 and 26 functional categories of redundant genes were up- and down-regulated, respectively, based on the GO annotation system for human Locus Link identifiers.

Statistically, the top-ranked 20 up-regulated genes included 4 hits to cell shape and polarity genes: KIAA0992 (twice), FNML2, FMNL3. Palladin, encoded by the KIAA0992 gene, plays a role in cytoskeletal organization, embryonic development, cell motility, and neurogenesis [16]. Formin-related proteins play a role in Rho GTPase-dependent regulation of the actin cytoskeletal cycle and have been implicated in morphogenesis, cell movement and cell polarity [17]. Several genes in the focal adhesion/actin cytoskeleton pathway were up-regulated. Guanine nucleotide exchange factors (GEFs) *DOCK1*, which forms a complex with RhoG, and *VAV2* and *ARHGEF7* that act on Rho family GTPases, play a fundamental role in small G-protein signaling pathways that regulate numerous cellular processes including actin cytoskeletal organization [18–22]. To further understand the mechanisms of thalidomide-induced teratogenicity the regional and developmental stage of expression for these genes and corresponding proteins should be determined; however, these preliminary findings suggest that thalidomide perturbs a general program involving the up-regulation of Rho family GTPases and their GEFs.

One candidate pathway for the control of cytoskeletal remodeling evident in studies of early induction of the Fetal Alcohol Syndrome (FAS) in mouse embryos is the receptor tyrosine kinase (RTK) signaling pathway, mediating insulin-like growth factors [12]. Genes in the RTK insulin signaling pathway were significantly up-regulated by thalidomide treatment as in FAS. AKT1 and GSK3β, which were up-regulated by thalidomide, are key genes in this pathway. AKT1, a serine–threonine protein kinase, is regulated by PDGF and insulin through PI-3 kinase signaling [23–25]. GSK3β, a substrate of AKT, is a proline-directed serine–threonine kinase that was initially identified as a phosphorylating and inactivating glycogen synthase [26]. IGF-I and IGF-II are expressed in the anterior and posterior mesodermal cells of the developing limbs [27–29]. IGF-I can influence chick limb outgrowth [29–31] and regulate mus-

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

Mutsuko Hirata-Koizumi[1], Kiyomi Matsuno[2], Mitsuhiko Kawabata[2], Kanako Yajima[2], Takashi Matsuyama[2], Akihiko Hirose[1], Eiichi Kamata[1], and Makoto Ema[1]

[1]*Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, Tokyo, Japan, and* [2]*Shin Nippon Biomedical Laboratories, Ltd. (SNBL), Tokyo, Japan*

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,

Address for Correspondence: Mutsuko Hirata-Koizumi, Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan; Fax: +81-3-3700-1408; E-mail: mkoizumi@nihs.go.jp

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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 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 × *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