the tail, defect of the mandible, deformity of the vertebral column and ribs, and microphthalmia were frequently observed. In this study, decreases in maternal weight gain and food consumption was observed at 7.5 mg/kg and higher. These results indicate that DBTCl produce teratogenic effects in the absence of overt maternal toxicity. However, the thymus weight was not determined. The susceptible gestational days to teratogenicity of DBTCl was determined after administration of relatively high doses of TBTCl on days 7 to 9, on days 10 to 12, or on days 13 to 15 of pregnancy (Ema et al. 1992). An increase in fetal malformations and postimplantation loss was detected after administration of DBTCl at 20 mg/kg on days 7 to 9, but neither was detected on days 10 to 12 nor on days 13 to 15. The data of the study in which pregnant rats were given a single dose of DBTCl by gavage showed that developing offspring were not susceptible to teratogenicity of DBTCl on day 6, and that day 7 was the earliest susceptible period, day 8 was the most susceptible period, and day 9 was no longer a susceptible period with respect to the teratogenicity of DBTCl (Ema et al. 1992). Occurrences of similar types of fetal malformations after administration of DBTCl on day 8 or on days 7 to 8 of pregnancy were also reported in rats (Noda et al. 1993, Ema et al. 1995b). Farr et al. (2001) also reported the developmental toxicity of DBTCl in rats. Wistar rats were administered DBTCl by gavage at 1, 2.5, 5, or 10 mg/kg on days 6 to 15 of pregnancy. Decreases in maternal weight gain, food consumption, and thymus weight, but not developmental indicators, were observed at the highest dose tested, 10 mg/kg. At this dose, four fetuses out of 262 fetuses had malformations, including ankyloglossia, mandible defects, tail anomaly, and deformity of the vertebrae, which were similar types of malformations to those previously reported after administration of DBTA (Noda et al. 1988, 1992a, b, 1993, 1994, 2001) and DBTCl (Ema et al. 1991, 1992, 1995b, Noda et al. 1993). They concluded that a slightly increased, but not statistically significant, number of malformations was associated with the onset of maternal toxicity, and that no increase in developmental defects was induced at dose levels that did not result in maternal toxicity.

The teratogenic effects of five DBTs with different anions, such as DBTA, DBTCl, dibutyltin maleate (DBTM), dibutyltin oxide (DBTO), and dibutyltin dilaurate (DBTL), were determined in Wistar rats given by gavage at 80 μmol/kg on the most susceptible day for teratogenicity of DBTA and DBTCl (Noda et al. 1993). Although the incidences of fetuses with malformations were different among DBTs, the types of malformations induced by these DBTs are similar to those in the previous studies with DBTA. Noda et al. (1993) suggest the importance of the dibutyl group rather than the anionic group in the production of fetal malformations. They also noted that butyl(3-hydroxybutyl)tin dilaurate (3-OHDBL), one of the main metabolites of DBTCl (Ishizaka et al. 1989), was not responsible for the teratogenicity of DBTCl because of weak potential for production of fetal malformations.

TeBT is metabolized to tri-, di-, and monobutyltin derivatives (Kimmel et al. 1977). The TBT compound is metabolized to di- and monobutyltin

derivatives, and DBT was metabolized to MBT in rats (Iwai et al. 1981). TeBT, TBTCl, DBTCl, and MBTCl were compared for their developmental toxicity to evaluate these butyltin compounds as potential toxicants in teratogenicity following administration of relatively high doses of butyltins to pregnant rats during the susceptible period to teratogenesis of TBTCl or during the susceptible period to teratogenesis of DBTCl. Pregnant rats were given TeBT, TBTCl, or DBTCl during the period of susceptibility to the teratogenesis of TBTCl, on days 13 to 15 of pregnancy (Ema et al. 1996a). TeBT caused an increased incidence of cleft palate at 1832 mg (5280μmol)/kg. TBTCl induced a markedly increased incidence of fetuses with cleft palate at 54 mg (165 μmol)/kg and higher, and decreased fetal weight at 108 mg (330 μmol)/kg. Following administration of DBTCl on days 13 to 15 of pregnancy, fetal weight was reduced at 54 mg (165 μmol)/kg and higher, but neither increase in postimplantation loss nor fetuses with malformations was found even at 100 mg (330 μmol)mg/kg. These results indicate that there are differences in the manifestation and degree of developmental toxicity among TeBT, TBT, and DBT. Pregnant rats received TBTCl, DBTCl, or MBTCl during the period of susceptibility to teratogenesis of DBTC, on days 7 to 8 of pregnancy (Ema et al. 1995b). TBTCl at 40 and 80 mg/kg caused an increase in postimplantation embryolethality, but no increase in fetal malformations. DBTCl caused a markedly high incidence of fetal malformations, lower fetal weight, and higher postimplantation embryonic loss at 10 mg/kg and higher. No increase in the incidences of postimplantation loss or malformed fetuses was observed after administration of MBTCl even at 1500 mg/kg. These results indicate that the developmental toxicity of DBTCl is different from that of TBTCl and MBTCl in the level of susceptibility and spectrum of toxicity. A lack of developmental toxicity of MBTCl was also reported by Noda et al. (1992a). MBTCl on days 7 to 17 of pregnancy did not affect maternal body weight and thymus weight, or fetal survival, growth, and morphological development, even at 400 mg/kg in Wistar rats. Their observations support the theory that MBTCl does not participate in the induction of the developmental toxicity of butyltins.

In Vitro *Dysmorphogenic Effects of Butyltin Compounds*

Krowke et al. (1986) evaluated the effects of TBTO on limb differentiation. In the organ culture system using mouse limb buds, TBTO interfered with morphogenetic differentiation at a concentration of 0.03 μg/mL. TBTO affected the differentiation of the paw skeleton and the development of the scapula. They concluded that the effects of TBTO on mouse limb differentiation should be interpreted as a cytotoxic effect rather than a specific dysmorphogenic action. Yonemoto et al. (1993) determined the relative teratogenic potencies of TBTO, TBTCl, (3-OH) hydroxybutyl dibutyltin chloride (3-OHHDBTCl), DBTCl, and MBTCl by comparing developmental hazard estimates using rat embryo limb bud cell cultures. The organotin compounds tested, except for MBTCl, were very strong inhibitors of cell differentiation

and cell proliferation. Fifty percent inhibition concentration for cell proliferation (IP50) and for cell differentiation (ID50), and the ratio of the former to the later (P/D ratio) of each compound was determined. Among TBTO, TBTCl, and its metabolites (i.e., 3-OHHDBTCl, DBTCl, and MBTCl), DBTCl showed the lowest ID50 and the highest P/D ratio, therefore the teratogenic potential of DBTCl was considered to be the highest. They noted that the proximate toxicant of DBT teratogenicity is DBT itself, TBT is rather embryolethal than teratogenic. These findings support the results of *in vivo* developmental toxicity studies on butyltins. The embryotoxicity and dysmorphogenic potential of DBTCl were determined for gestation day 8.5 rat embryos, which are highly susceptible to the teratogenic effects of DBTCl when administered to pregnant rats. Markedly decreased incidences in embryos with well-developed vascularization in the body and yolk sac, yolk sac diameter, crown-rump length, and number of somite pairs were found at 30 ng/mL (Ema et al. 1995c). A concentration-dependent decrease in the morphological score and increase in incidence of embryos with anomalies were noted, and the differences were significant for embryos exposed to DBTCl at concentrations of 10 and 30 ng/mL. Open anterior neuropore and craniofacial abnormalities were predominantly observed. These results indicate that DBTCl exerts dysmorphogenic effects on postimplantation embryos *in vitro*. Noda et al. (1994) reported that DBT was detected in rat maternal blood at 100 ng/g, and in embryos at 720 ng/g, at 24 hours after gavage administration of DBTA at 22 mg/kg, teratogenic dose, on day 8 of pregnancy. Their results show that DBT is transferred to embryos, and embryonic levels of DBT exceed those in maternal blood, suggesting that embryos may be able to accumulate DBT. The dysmorphogenic concentrations of DBTCl in embryos cultured from gestation day 8.5 were well within the range of levels detected in maternal blood after the administration of a teratogenic dose of DBT. These findings indicate that teratogenic effects of DBTCl may be due to a direct interference with embryos. The toxic effects of DTBCl were examined in rat embryos during three different stages of organogenesis (i.e., the primitive streak, neural fold, and early forelimb bud stages), using the rat whole embryo culture system (Ema et al. 1996b). Rat embryos were explanted on gestation day 8.5, 9.5, or 11.5 and cultured. Dysmorphogenesis in embryos cultured from gestation day 8.5, 9.5, or 11.5 was observed at concentrations of 10 ng/mL and higher, 50 ng/mL and higher, and 300 ng/mL, respectively. Incomplete turning and craniofacial defects in embryos cultured from gestation day 8.5 and day 9.5, and defects of the forelimb buds and tail in embryos cultured from gestation day 11.5, were frequently observed. These results show that *in vitro* exposure to DBTCl interferes with normal development of embryos during three different stages of organogenesis and that the susceptibility to the embryotoxicity, including dysmorphogenic potential, of DBTCl varies with developmental stage. These findings suggest that the phase specificity for the *in vivo* teratogenesis of DBTCl given to pregnant rats may be attributable to a decline in the susceptibility of embryos to the dysmorphogenesis of DBTCl with advancing development.

Summary of Developmental Toxicity of Butyltin Compounds

Maternal exposure during pregnancy to TBTs, such as TBTO, TBTA, and TBTCl, caused embryonic/fetal deaths and suppression of fetal growth at maternal toxic doses. At severely maternal toxic doses of TBTs, cleft palate was produced in fetuses. Behavioral changes were also reported in postnatal offspring of rats that received TBTs during pregnancy at doses that did not cause overt maternal toxicity. Significant effects on growth profiles in male and female offspring, and decreased liver weights in female offspring were noted after administration of TBTCl by gavage from day 8 of pregnancy until adulthood even at 0.025 mg/kg. Many reports showed that DBT is teratogenic when administered during organogenesis. DBT may increase the incidence of fetal malformations at marginal doses that induced maternal toxicity. Developing embryos were not susceptible to teratogenicity of DBTCl on day 6; day 7 was the earliest susceptible period, day 8 was the most susceptible period, and day 9 was no longer a period of susceptibility to the teratogenicity of DBTCl. There were differences in the manifestation and degree of developmental toxicity among TeBT, TBT, DBT, and MBT. The developmental toxicity studies on butyltins suggest that the teratogenicity of DBT is different from those of TeBT, TBT, and MBT in its mode of action, because the susceptible period for teratogenicity and types of malformations induced by DBT are different from those induced by tetra-, tri-, and monosubstituted organotins. DBTCl exerts dysmorphogenic effects on postimplantation embryos *in vitro*. The dysmorphogenic concentrations of DBTCl in embryos cultured were well within the range of levels detected in maternal blood after the administration of a teratogenic dose of DBT. The phase specificity for the *in vivo* teratogenesis of DBTCl may be attributable to a decline in the susceptibility of embryos to the dysmorphogenesis of DBTCl with advancing development. The findings of *in vivo* and *in vitro* studies suggest that DBT itself is a causative agent in DBT teratogenesis.

Developmental Toxicity of Miscellaneous Organotin Compounds

Table 3.5 presents the developmental toxicity studies on miscellaneous organotin compounds. Behavioral effects were determined in offspring of female SD rats given trimethyltin chloride (TMTCl) in drinking water at a concentration of 0.2, 0.8, or 1.7 mg/L, or monomethyltin trichloride (MMTCl) in drinking water at a concentration of 24.3, 80.9, or 243 mg/L from 12 days before mating, to day 21 of lactation, throughout the mating and pregnancy period (Noland et al. 1982). Only male pups were tested. Learning deficiency was detected in organotin-treated pups. Pups from dams exposed to TMTCl at 1.7 mg/L or MMTCl at 243 mg/L displayed an increased acquisition time in a runway learning test on PND 11. A higher escape time in a swim escape test on PND 21 was also observed in male pups exposed to prenatal MMTCl at 24 and 243 mg/L. In this study, there was no difference between the weights of control and experimental animals in suckling pups and their

Table 3.5 Developmental Toxicity of Miscellaneous Organotin Compounds

Compounds	Animals	Dose	Days of Administration	Route	Reproductive and Developmental Effects	Author(s)
TMTCl	SD rat	1.7 mg/L	14 days before mating to lactation day 21	Drinking water	Learning deficiency in male pups	Noland et al. (1982)
MMTCl	SD rat	243 mg/L	As above	As above	As above	
TMTCl	SD rat	5–9 mg/kg	Day 7, 12, or 17 of pregnancy	ip	Decreased postnatal wt. gain, decreased no. of pups, degenerative changes in hippocampus	Paule et al. (1986)
TMTCl	THA rat	5–7 mg/kg	Day 12 of pregnancy	ip	Disruption of learning acquisition	Miyake et al. (1989)
THTCl	SD rat	5 mg/kg	Day 6–20 of pregnancy	Gavage	Increased spontaneous activity, increased d-amphetamine-stimulate rearing	Gårdlund et al. (1991)
DMTCl	Wistar rat	15–20 mg/kg	Days 7–17 of pregnancy	Gavage	Decreased fetal wt., cleft palate	Noda (2001)
		40 mg/kg	Days 7–9 or 13–15 of pregnancy	Gavage	Skeletal variations	
Octyltin stabilizer ZK 30.434 (80% DOTTG and 20% MOTTG)						Faqi et al. (2001)
	Han:NMRI mouse	20–100 mg/kg	Days 5–16 of pregnancy	Gavage	Postimplantation loss, decreased fetal wt., bent forelimb, cleft palate, exencephaly, skeletal malformations and variations	

dams. Postnatal growth and neuronal alterations were evaluated in pups of SD rats intraperitoneally injected on either day 7, 12, or 17 of pregnancy with a single dose of TMTCl at 5, 7, or 9 mg/kg (Paule et al. 1986). Maternal body weight at term of pregnancy was lower in the TMTCl-treated groups. Prenatal TMTCl decreased pup weight at 7 mg/kg and higher. A decreased number of surviving pups was found only in the group treated TMTCl at 9 mg/kg on day 17 of pregnancy. Generative changes in the hippocampus were more frequently noted in pups exposed to TMTCl on day 12 or 17 than on day 7. Paule et al. (1986) concluded that prenatal exposure to TMTCl causes toxic effects in postnatal offspring, but only in the presence of maternal toxicity. Disruption of learning acquisition was reported in offspring of THA rats intraperitoneally injected with TMTCl at 5 or 7 mg/kg on day 12 of pregnancy (Miyake et al. 1989). No maternal toxicity was found at 5 mg/kg. No effects of TMTCl on body weight, survival, or physical and functional development of pups were detected. In the Sidman avoidance test, the avoidance rate of the TMTCl-treated offspring rats was lower when compared to that of the controls.

Postnatal behavioral changes in pups were determined in rats prenatally administered trihexyltin chloride (THTCl) (Gårdlund et al. 1991). Pregnant SD rats were gavaged THTCl at 5 mg/kg on days 6 to 20 of pregnancy and allowed to litter. An increase in spontaneous activity, including locomotion and total activity, and a marginally increased d-amphetamine–stimulated rearing behavior were observed in postnatal pups at 5 mg/kg. This dose level did not induce maternal toxicity.

Dimethyltin chloride (DMTCl) was given to Wistar rats by gavage at 5, 10, 15, or 20 mg/kg on days 7 to 17 of pregnancy (Noda 2001). At 20 mg/kg, severe clinical signs of toxicity, including death and marked decreases in body weight gain and food consumption in pregnant rats, and incidence of cleft palate in fetuses were observed. Decreases in maternal thymus weight and fetal weight were found at 15 mg/kg and higher. No increase in incidence of fetal malformations was detected following administration of DMTCl on days 7 to 9, on days 10 to 12, on days 13 to 15, or on days 16 to 17 of pregnancy at 20 or 40 mg/kg. Noda (2001) concluded that DMTCl produced fetal malformations at a severely maternal toxic dose.

The octyltin stabilizer ZK 30.434, a mixture of 80% dioctyltin diisooctylthioglycolate and 20% monooctyltin triisooctylthioglycolate (DOTTG/MOTTG) was gavaged to Han:NMRI mice at 20, 30, 45, 67, or 100 mg/kg on days 5 to 16 of pregnancy (Faqi et al. 2001). One death at 100 mg/kg and a decreased thymus weight at 45 and 100 mg/kg were observed in dams. An increase in resorptions and low fetal weight were found at 67 mg/kg and higher. An increase in number of external and skeletal anomalies, such as forelimb bent, cleft palate, exencephaly, clavicula bent, femur bent, and fused ribs, were observed at the highest dose. Incidences of cervical and lumbar ribs were increased at 20 mg/kg and higher. These results indicate that DOTTG/MOTTG is developmentally toxic in mice.

Summary of Developmental Toxicity of Miscellaneous Organotin Compounds

Prenatal and/or postnatal exposure to TMTCl possesses developmental neurotoxic effects in postnatal rat offspring, even at doses that induced no maternal toxicity. The learning deficiency induced by prenatal TMTCl may be due to hippocampal lesions. Prenatal treatment of maternal toxic doses of TMTCl adversely affected survival and growth of offspring. Prenatal treatment of THTCl is also reported to induce behavioral changes in postnatal offspring. An increased number of cleft palates were observed in fetuses of rats given DMTCl during organogenesis at a severely maternal toxic dose. A mixture of DOTTG and MOTTG is developmentally toxic and produces fetal malformations in mice.

Conclusions

Many studies on toxic effects of phenyltins and butyltins in aquatic organisms have been conducted. TBT or TPT causes the imposition of male sex organs (termed *imposex*) on female mud snails above the concentration of about 1 ng/L (Sn) in seawater, but DBT or MPT does not induce imposex. The intensity is characterized by a classification system based on the VDS index, and in advanced phases of imposex and sterilization with gross morphological changes would be irreversible. The biochemical mechanism studies suggested that the induction of either neurotropic hormone or androgen titers would lead to imposex induction at extremely low doses of TBT. Also TBT or TPT exposure in early life stages of fish causes altered embryonic development, impaired morphological development, and delayed or inhibited hatching, and induces reduced fecundity and sperm counts as reproductive effects. Such reproductive and developmental defects were also found in other species. The impaired reproduction and subsequent population decline in a variety of aquatic organisms by organotins are important issues in the aquatic ecosystem.

Many reports on reproductive and developmental toxic effects of phenyltins and butyltins in experimental animals have been published. While TPTs caused decreases in male fertility due to degenerative changes in testicular tissue, the female reproductive failure induced by TPTs is more prominent and the harmful effects of TPTs on the ovaries were presented after five days of treatment. TPTCl during early pregnancy caused implantation failure. Implantation failure due to TPTCl might be mediated by the suppression of uterine decidualization and correlated with the reduction in serum progesterone levels. These findings were also shown in rats given DPT, a major metabolite of TPT. Maternal exposure to TPTs during organogenesis caused embryonic/fetal death and suppression of fetal growth at maternal toxic doses. TPTs did not induce an increased number of fetal malformations, even at doses that produced overt maternal toxicity. Behavioral changes were reported in postnatal offspring of maternal rats that

received TPTs during pregnancy at doses that did not cause overt maternal toxicity. In a rat two-generation reproductive toxicity study, TBTCl at relatively low doses affected male and female reproductive systems, including decreased weights of the male reproductive organs, decreased counts of spermatids and sperms, decrease in serum estradiol levels, delayed vaginal opening, impaired estrous cyclicity, and increased female AGD. TBTCl and DBTCl during early pregnancy caused implantation failure in rats. Implantation failure due to TBTCl and DBTCl, at lower doses than TBTCl, may be mediated via the suppression of uterine decidualization and correlated with the reduction in serum progesterone levels. Administration of MBTCl during early pregnancy did not cause pre- or postimplantation loss. Maternal exposure during pregnancy to TBTs caused embryonic/fetal deaths, suppression of fetal growth, and cleft palate at maternal toxic doses. Significant effects on growth profiles and decreased liver weights were reported in offspring of rats given TBTCl by gavage, even at 0.025 mg/kg from day 8 of pregnancy until adulthood. Behavioral changes were also shown in postnatal offspring of rats that received TBTs during pregnancy at doses that did not cause overt maternal toxicity. Many reports demonstrated that DBT derivatives with different anions, such as dichloride, diacetate, maleate, dilaurate, and oxide, are teratogenic when administered during organogenesis in rats. Rat embryos are the most susceptible to teratogenic effects of DBT on day 8 of pregnancy after maternal exposure. The developmental toxicity studies on butyltins suggest that the teratogenic effects of DBT are different from those of TeBT, TBT, and MBT in its mode of action. DBTCl exerts dysmorphogenic effects on postimplantation embryos *in vitro*. The phase specificity for the in vivo teratogenic effects of DBTCl may be attributable to a decline in the susceptibility of embryos to the dysmorphogenesis of DBTCl with advancing development. The findings of *in vivo* and *in vitro* studies suggest that DBT itself is a causative agent in DBT teratogenesis. Because the teratogenicity of DTB has been reported in a single species, studies in additional species would be of great value in evaluating developmental toxicity of DBT. As for miscellaneous organotin compounds, several reports on developmental toxicity are published. Prenatal and/or postnatal exposure to TMTCl or THTCl caused behavioral changes in postnatal rat offspring. Behavioral changes in postnatal pups of rats given organotin prenatally and/or postnatally may be a sensitive parameter for reproductive and developmental toxicity. A mixture of DOTTG and MOTTG is developmentally toxic and produces fetal malformations in mice. An increased number of cleft palates was reported in fetuses of rats given DMTCl during organogenesis at severely maternal toxic dose.

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Reproductive and developmental toxicity screening test of basic rubber accelerator, 1,3-di-*o*-tolylguanidine, in rats

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Abstract

Twelve male and female rats per group were exposed to the rubber accelerator 1,3-di-*o*-tolylguanidine (DTG) by gavage at 0, 8, 20 or 50 mg/kg bw/day. Males were dosed for a total of 49 days beginning 14 days before mating. Females were dosed for a total of 40–49 days beginning 14 days before mating to day 3 of lactation throughout the mating and gestation period. At 50 mg/kg bw/day, deaths were observed in two males and three females. Lowered body weight gain and food consumption were noted in males at 50 mg/kg bw/day and females at 20 and 50 mg/kg bw/day. Mydriasis, decreased locomotor activity, bradypnea, prone position, tremor and/or salivation were observed in males and females at 20 and 50 mg/kg bw/day. No effects of DTG were found on the estrous cyclicity, precoital interval, copulation, fertility and gestational indices, numbers of corpora lutea and implantations, or gestation length. A significant decrease in the number, body weight and viability of offspring and increase in the incidence of fetuses with external malformations were found at 50 mg/kg bw/day. Oligodactyly, anal atresia and tail anomalies were observed. These data suggest that DTG may be teratogenic. The NOAELs of DTG for general and developmental toxicity in rats are 8 and 20 mg/kg bw/day, respectively.
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Keywords: Di-*o*-tolylguanidine; Rubber accelerator; Sigma ligand; Reproductive and developmental toxicity; Teratogenicity; Malformation; Rat

1. Introduction

The basic rubber accelerator 1,3-di-*o*-tolylguanidine (CAS No. 97-39-2; DTG) is produced in the million pound range annually in the United States [1,2]. DTG is known as a selective sigma ligand [3]. In this context, many pharmacological studies of DTG were performed [3–12]. Ligands that interact with sigma sites have been shown to produce hypothermia [4–6]. Hypothermia induced by DTG was detected following subcutaneous or intracerebroventricle injection in rats [5,6] and intraperitoneal injection in mice [4]. The intraperitoneal injection of DTG potently reduced the pain behavior in the acute but increased pain behavior in the tonic phase in the formalin test in mice [7]. Intraperitoneal injection of DTG produced significant but short-lived increases in the withdrawal latencies in mice [4]. Bastianetto et al. [8] showed that unilateral intranigral injection caused circulating behavior in rats and suggested that sigma sites play a role in movement and posture through their association with brainstem and forebrain motor control circuits. Decreased locomotor activity induced by intraperitoneal injection [9,10], increased bladder capacity induced by intravenous injection in the anaesthetized condition [11] and no change in immobility time in open field after intraperitoneal injection [12] were also reported in rats given DTG. Toxicological studies on DTG have given little information on acute animal toxicity [13]: intraperitoneal LD50 was 25 mg/kg bw in mice; oral LD50 was 500 mg/kg bw in rats; lowest published lethal dose of oral administration was 80 mg/kg bw in rabbits; and the lowest published lethal dose was 120 mg/kg bw after oral administration in mammals, species unspecified. At the present time, no information is available for the reproductive and developmental toxicity of DTG. It is generally assumed that the results of animal test on chemical toxicity are relevant to human health [14]. As such, the testing for reproductive and developmental toxicity

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in animal models is an important part of the overall toxicology. The present study was conducted to obtain information on the effects of DTG on reproductive and developmental parameters in rats.

2. Materials and methods

This study was performed in compliance with OECD guideline 421 Reproduction/Developmental Toxicity Screening Test [15] and in accordance with the principles for Good Laboratory Practice [16,17] and "Guidance for Animal Care and Use" of Panapharm Laboratories Co., Ltd.

2.1. Animals

International Genetic Standard (Crj: CD (SD) IGS) rats were used throughout this study. This strain was chosen because it is most commonly used in toxic studies, including reproductive and developmental toxicity studies, and historical control data are available. Males and females at 8 weeks of age were purchased from Atsugi Breeding Center, Charles River Japan, Inc. (Yokohama, Japan). The rats were acclimated to the laboratory for 13 days prior to the start of the experiment. Male and female rats found to be in good health were selected for use. Vaginal smears of each female were recorded and only females showing a 4-day estrous cycle were used in the experiment. Male and female rats were distributed on a random basis into four groups of 12 males and 12 females each. Rats were housed individually, except during the acclimation, mating and nursing periods. From day 0 of pregnancy to the day of sacrifice, individual dams and litters were reared using wooden chips as bedding (White Flake; Charles River Japan, Inc.).

Animals were reared on a sterilized basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and sterilized water ad libitum and maintained in an air-conditioned room at $24 \pm 2\,^{\circ}C$, with a relative humidity of $55 \pm 10\%$, a 12-h light/12-h dark cycle and ventilation with 13–15 air charges per hour.

2.2. Chemicals and dosing

DTG was obtained from Sumitomo Chemical Co., Ltd. (Tokyo, Japan). DTG, a white powder, is slightly soluble in hot water and alcohol, soluble in chloroform and very soluble in ether, and its melting point is 179 °C, specific gravity is 1.10 and molecular weight is 239.3 [2]. The DTG (Lot No. 30J08) used in this study was 99.6% pure, and it was kept in a dark place at room temperature. The purity and stability of the chemical were verified by analysis before the study. Rats were dosed once daily by gastric intubation with DTG at a dose of 0 (control), 8, 20 or 50 mg/kg bw. The dosage levels were determined based on the results of our previous dose-finding study, the 14-day repeated dose toxicity study in rats given DTG by gavage at 0, 10, 20, 40 or 80 mg/kg bw/day, in which deaths were found at 80 mg/kg bw/day, decreased locomotor activity, mydriasis, tremor and salivation were observed at 40 and 80 mg/kg bw/day, and no adverse effects were detected at 10 and 20 mg/kg bw/day (data not shown). DTG was suspended in 0.5% (w/v) carboxymethylcellulose-Na solution with 0.1% (w/v) Tween 80. Males (12 rats/group) were dosed for a total of 49 days beginning 14 days before mating. Females (12 rats/group) were dosed for a total of 40–49 days beginning 14 days before mating to day 3 of lactation throughout the mating and gestation period. The volume of each dose was adjusted to 10 ml/kg body weight based on the latest body weight during the re-mating and mating period in males and females or the body weight on day 0 of pregnancy in females after copulation. Control rats were given 0.5% (w/v) carboxymethylcellulose-Na solution with 0.1% (w/v) Tween 80. The stability of formulations has been confirmed for up to 8 days. During use, the formulations were maintained under such conditions for less than 7 days, and the target concentration was 96.5 to 101.4%.

2.3. Observations

All rats were observed daily for clinical signs of toxicity. The body weight was recorded twice a week in males, and twice a week during the pre-mating and mating periods, on days 0, 7, 14 and 21 of pregnancy and on days 0 and 4 of lactation in females. Food consumption was recorded twice weekly during the pre-mating period in males, and twice weekly during the pre-mating period, on days 1, 7, 14 and 21 of pregnancy and on days 1 and 4 of lactation in females. The rats were euthanized by exsanguination under anesthesia on the next day of the last administration in males and on day 4 of lactation in females. The external surfaces of the rats were examined. The abdomen and thoracic cavity were opened, and gross internal examination was performed. In males, the testes and epididymides were weighed. In females, the numbers of corpora lutea and implantation sites and weight of the ovaries were recorded. The testes and epididymides were fixed with Bouin's solution and preserved in 10% neutral buffered formalin, and the ovaries were stored in 10% neutral buffered formalin. Histopathological evaluations were performed on hematoxylin–eosin-stained tissue sections of these organs.

Daily vaginal lavage samples of each female were evaluated for estrous cyclicity throughout the pre-mating period. Each female rat was mated overnight with a single male rat of the same dosage group until copulation occurred or the mating period, 2 weeks, had elapsed. During the mating period, daily vaginal smears were examined for the presence of sperm. The presence of the sperm in the vaginal smear and/or a vaginal plug was considered evidence for successful mating. Once insemination was confirmed, the females were checked for signs of parturition before noon from day 20 of pregnancy. The females were allowed to deliver spontaneously and nurse their pups until postnatal day (PND) 4. The day on which parturition was completed by 12:00 was designated as PND 0. Litter size and numbers of live and dead pups were recorded. Gender was determined on live pups examined grossly and individually weighed on PNDs 0 and 4. On PND 4, the pups were euthanized by exsanguination under anesthesia and gross internal examinations were performed.

2.4. Data analysis

The statistical analysis of pups was carried out using the litter as the experimental unit. The body weight, body weight gain, food consumption, length of estrous cycles, precoital interval, gestation length, weight of the organs, relative organ weight, numbers of corpora lutea, implantations and live and dead pups, total number of pups and weight of live pups were analyzed with Bartlett's test for homogeneity of variance at the 5% level of significance. If homogeneous the data were analyzed using Dunnett's multiple comparison test to compare the mean of the control group with that of each dosage group. If not, the DTG-treated groups were compared with that of the control group with Steel's multiple comparison test. The implantation, delivery and viability indexes, and incidence of pups with anomalies and individual anomalies were analyzed with Wilcoxon's rank sum test. The mortality, copulation, fertility and gestation indexes, and sex ratio of pups were analyzed with Fisher's exact test. The 5% level of probability was used as the criterion for significant.

3. Results

Table 1 shows the findings in male rats given DTG. At 50 mg/kg bw/day, one male died after six administrations and one male died after seven administrations. These dead rats showed mydriasis, decreased locomotor activity, bradypnea, a prone position and tremor 10–20 min after the administration of DTG. In surviving males, mydriasis, decreased locomotor activity, bradypnea and prone position on days 1–9 of the administration period, tremor during the whole period of administration and salivation on days 22–49 of the administration period were also observed at 50 mg/kg bw/day. Salivation was noted on days 28–49 of the administration period at 20 mg/kg bw/day. A significant decrease in the body weight gain was found on days 1–8 (81% decrease) and days 15–22 (48% decrease) of the administration period at 50 mg/kg bw/day. At this dose, significantly lower food consumption on days 7–8 (20% decrease) and days 14–15 (7% decrease) of the administration period was also observed.

Table 1
Findings in male rats given DTG

	Dose (mg/kg bw/day)			
	0 (control)	8	20	50
No. of male rats	12	12	12	12
No. of deaths during pre-mating period	0	0	0	2
Initial body weight (g)[a]	381 ± 16	379 ± 16	378 ± 15	380 ± 16
Body weight gain (g)[a]				
Days 1–8	30 ± 7	33 ± 7	25 ± 7	6 ± 9**
Days 8–15	29 ± 5	32 ± 5	32 ± 7	24 ± 7
Days 15–22	23 ± 6	25 ± 8	23 ± 7	12 ± 11**
Days 22–29	19 ± 9	22 ± 7	25 ± 8	19 ± 5
Days 29–36	22 ± 6	22 ± 6	23 ± 7	18 ± 8
Days 36–43	15 ± 8	12 ± 9	13 ± 5	14 ± 7
Days 43–50	19 ± 8	19 ± 7	13 ± 4	13 ± 11
Food consumption (g/day/rat)[a]				
Days 7–8	25 ± 3	26 ± 3	26 ± 2	20 ± 3**
Days 14–15	29 ± 2	30 ± 2	29 ± 3	27 ± 3*
Days 29–30	27 ± 2	27 ± 3	28 ± 3	25 ± 2
Days 35–36	28 ± 2	29 ± 2	29 ± 2	27 ± 2
Days 42–43	26 ± 3	25 ± 3	27 ± 4	27 ± 3
Days 49–50	28 ± 4	29 ± 3	28 ± 2	28 ± 3

[a] Values are given as the mean ± S.D.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

Table 2 presents the findings in female rats given DTG. At 50 mg/kg bw/day, two females died after the first administration and one female died after normal delivery of her pups on day 22 of pregnancy. Mydriasis, decreased locomotor activity, bradypnea, prone position, and tremor and salivation 10–20 min after the administration of DTG were observed in females died after the first administration. These clinical signs and salivation were found during pregnancy and on day of parturition in a female which died after parturition. In surviving females, mydriasis, decreased locomotor activity, bradypnea and prone position on day 1 of the administration period to day 0 of lactation, tremor on day 1 of the administration period to day 5 of pregnancy and salivation on day 4 of pregnancy to day 3 of lactation were observed at 50 mg/kg bw/day. Mydriasis, decreased locomotor

Table 2
Findings in female rats given DTG

	Dose (mg/kg bw/day)			
	0 (control)	8	20	50
No. of female rats	12	12	12	12
No. of deaths during pre-mating period	0	0	0	2
No. of deaths during pregnancy	0	0	0	1
Initial body weight (g)[a]	381 ± 16	379 ± 16	378 ± 15	380 ± 16
Body weight gain (g)[a]				
Days 1–8	19 ± 8	17 ± 7	11 ± 6*	−1 ± 9**
Days 8–15	10 ± 7	15 ± 8	20 ± 5**	15 ± 10
Days 0–7 of pregnancy	34 ± 6	31 ± 6	33 ± 4	28 ± 8
Days 7–14 of pregnancy	34 ± 5	34 ± 4	36 ± 3	30 ± 10
Days 14–21 of pregnancy	85 ± 17	100 ± 14	105 ± 9*	42 ± 21**
Days 0–4 of lactation	20 ± 19	14 ± 16	22 ± 9	16 ± 13
Food consumption (g/day/rat)[a]				
Days 7–8	22 ± 3	21 ± 2	19 ± 2**	13 ± 3**
Days 14–15	20 ± 4	22 ± 3	22 ± 2	20 ± 2
Days 6–7 of pregnancy	22 ± 3	23 ± 2	23 ± 3	17 ± 3**
Days 13–14 of pregnancy	23 ± 2	24 ± 3	25 ± 2	22 ± 5
Days 20–21 of pregnancy	24 ± 4	26 ± 3	29 ± 3*	21 ± 5
Days 3–4 of lactation	41 ± 5	41 ± 3	46 ± 4*	32 ± 6**

[a] Values are given as the mean ± S.D.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).