Summary of Reproductive Toxicity of Phenyltin Compounds

TPTs caused a decrease in male fertility due to degenerative changes in testicular tissue, which were associated with a marked decrease in food consumption. Complete recovery of fertility and impairment of the spermatogenesis was noted following withdrawal of treatment. Female reproductive failure induced by TPTs is more prominent. The harmful effects of TPTs on the ovaries were present after 5 days of treatment, before any significant effects on body weight gain. TPTCl during early pregnancy caused implantation failure at relatively low doses, and TPTCl had greater antiimplantation effects when administered during the preimplantation period. The implantation failure due to TPTCl might be mediated by suppression of uterine decidualization and correlated with the reduction in serum progesterone levels. Implantation failure and suppression of uterine decidualization accompanied with decreased levels of serum progesterone were also observed in rats given DPT, a major metabolite of TPT.

Developmental Toxicity of Phenyltin Compounds

Table 3.2 presents the developmental toxicity studies on phenyltin compounds given to female animals during pregnancy. Several reports on the adverse effects of phenyltins on development of offspring following maternal exposure have been published. Female SD rats were given TPTA by gavage at 5, 10, or 15 mg/kg on days 6 to 15 of pregnancy (Giavini et al. 1980). TPTA caused a decrease in maternal body weight gain at 10 mg/kg and higher, an increase in postimplantation loss at 15 mg/kg, and a reduction of fetal ossification at 5 mg/kg and higher. Teratogenic effects of TPTA were not found even at doses resulting in clear maternal toxicity. Depression of maternal body weight gain and food intake at 9.0 mg/kg and higher, and increase in postimplantation embryonic loss and decrease in fetal ossification at 9.0 mg/kg and higher, but not teratogenic effects, were observed in Wistar rats after administration of TPTA at 1.5, 3.0, 6.0, 9.0, or 12.0 mg/kg by gavage on days 7 to 17 of pregnancy (Noda et al. 1991a). Behavioral effects of prenatal exposure to TPTA were reported. A transient increase in spontaneous locomotor activity and increased mortality during the lactation period were found in pups of CFY rats given TPTA by gavage at 6 mg/kg on days 6 to 14 of pregnancy (Lehotzky et al. 1982). In this study, maternal rats were free of any overt signs of toxicity. Disruptions of learning acquisition, as evidenced by low avoidance rate in the Sidman avoidance test, and prolonged swimming time to the goal, and an increased number of errors in a reversed test in the water E-maze, were observed in postnatal offspring of Tokai High Avoiders (THA) rats received TPTA by gavage on days 6 to 20 of pregnancy at 4 or 8 mg/kg (Miyake et al. 1991). Maternal deaths and decreased weight gain were found at 8 mg/kg, no maternal toxicity was observed at 4 mg/kg, and no malformed offspring appeared in any group.

Table 3.2 Developmental Toxicity of Phenyltin Compounds

Compounds	Animals	Dose	Days of Administration	Route	Reproductive and Developmental Effects	Author(s)
TPTA	Wistar rat	5–15 mg/kg	Days 6–15 of pregnancy	Gavage	Postimplantation loss, delayed ossification	Giavini et al. (1980)
TPTA	Wistar rat	9–12 mg/kg	Days 7–17 of pregnancy	Gavage	Postimplantation loss, delayed ossification	Noda et al. (1991a)
TPTA	CFY rat	6 mg/kg	Days 6–14 of pregnancy	Gavage	Postnatal death, transient increase in spontaneous locomotor activity	Lehotzky et al. (1982)
TPTA	THA rat	4–8 mg/kg	Days 6–20 of pregnancy	Gavage	Disruption of learning acquisition	Miyake et al. (1991)
TPTH	SD rat	20 mg/kg 15 mg/kg	Days 1–7 of pregnancy Days 8–14 of pregnancy	Gavage Gavage	Decreased pregnancy rate Postimplantation loss, decreased fetal wt.	Winek et al. (1978)
		15 mg/kg	Days 14–12 of pregnancy	Gavage	Effects as above	
TPTH	SD rat	13 mg/kg	Days 6–15 of pregnancy	Gavage	Postimplantation loss	Chernoff et al. (1990)
TPTCl	Wistar rat	6.3–12.5 mg/kg 9.4–12.5 mg/kg	Days 7–9 of pregnancy Days 10–12 or 13–15 of pregnancy	Gavage Gavage	Postimplantation loss Postimplantation loss, decreased fetal wt.	Ema et al. (1999c)

Winek et al. (1978) noted that (1) SD rats given Vancide KS (TPTH) by gavage at 20 mg/kg on days 1 to 7 of pregnancy did not produce pups nor did they exhibit any resorption sites, (2) that only two of the six rats given TPTH at 15 mg/kg on days 14 to 20 of pregnancy produced viable pups, and (3) that four of the six rats given TPTH at 15 mg/kg on days 14 to 20 of pregnancy produced viable pups. Their study was conducted on only a small number of animals and the design of the study was not described in detail. Chernoff et al. (1990) observed a significant decrease in maternal body weight gain and an increase in postimplantation embryonic loss, but not fetal malformations, after administration of TPTH by gavage on days 6 to 15 of pregnancy at 13 mg/kg in SD rats. They stated that there was a correlation between maternal toxicity and fetal weight and/or lethality.

Following administration of TPTCl by gavage to pregnant Wistar rats, the maternal body weight gain and food consumption were decreased at 3.1 mg/kg and higher on days 7 to 9 of pregnancy, and at 6.3 mg/kg and higher on days 10 to 12 or on days 13 to 15 of pregnancy (Ema et al. 1999c). An increase in the incidence of postimplantation embryonic loss was found in pregnant rats given TPTCl at 6.3 mg/kg and higher on days 7 to 9, and at 9.4 mg/kg and higher on days 10 to 12 and on days 13 to 15. A decreased fetal weight was observed at 12.5 mg/kg on days 10 to 12 and at 9.4 mg/kg and higher on days 13 to 15. No increase in the incidence of fetuses with malformations was detected after administration of TPTCl regardless of the days of administration. These results indicate that TPTCl is developmentally toxic and that TPTCl has greater embryolethal effects when administered during earlier than later stages of organogenesis.

Summary of Developmental Toxicity of Phenyltin Compounds

Maternal exposure to TPTs caused embryonic/fetal death and suppression of fetal growth at maternal toxic doses. TPTs may cause reduction of fetal ossification at doses that are nontoxic to the mother. TPTs did not induce an increased number of fetal malformations even at doses producing 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.

Reproductive Toxicity of Butyltin Compounds

Table 3.3 shows reproductive toxicity studies on butyltins. A decrease in the sperm head count and vacuolization of Sertoli cells were found in ICR mice gavaged with TBTO at 2 and 10 mg/kg twice a week for 4 weeks (Kumasaka et al. 2002). The male reproductive toxicity of tributyltin chloride (TBTCl) was reported in a two-generation reproductive toxicity study using Wistar rats (Omura et al. 2001). F0 females were fed a diet containing TBTCl at 5, 25, or 125 ppm (estimated to be 0.4, 2.0, or 10.0 mg/kg) from day 0 of pregnancy to the day of weaning of F1 rats. Feeding of TBTCl was continued

Table 3.3 Reproductive Toxicity of Butyltin Compounds

Compounds	Animals	Dose	Days of Administration	Route	Reproductive and Developmental Effects	Author(s)
TBTO	ICR mouse	2–10 mg/kg	4 weeks (twice a week)	Gavage	Decreased sperm head count, vacuolization of Sertoli cells	Kumasaka et al. (2002)
TBTCl	Wistar rat	25–125 ppm	2 generations	Diet	Decreased wt of testis and epididymis, decreased spermatid count, decreased levels of serum estradiol, decreased wt. gain of male offspring	Omura et al. (2001)
TBTCl	Wistar rat	5–125 ppm	2 generations	Diet	Decreased birth index, decreased no. and wt. of pups, delayed vaginal opening, increased female AGD, decreased wt. gain of female offspring	Ogata et al. (2001)
TBTCl	Wistar rat	12.2–16.3 mg/kg	Days 0–7 of pregnancy	Gavage	Decreased pregnancy rate, decreased fetal wt.	Harazono et al. (1996)
TBTCl	Wistar rat	16.3–32.5 mg/kg	Days 0–3 of pregnancy	Gavage	Decreased pregnancy rate, decreased fetal wt.	Harazono et al. (1998b)
		16.3–65.1 mg/kg	Days 4–7 of pregnancy	Gavage	Effects as above, postimplantation loss	
TBTCl	Wistar rat	16.3–32.5 mg/kg	Days 0–3 of pseudopregnancy	Gavage	Suppression of uterine decidualization, decreased levels of serum progesterone, increased levels of serum estradiol	Harazono and Ema (2000)
		16.3–65.1 mg/kg	Days 4–7 of pseudopregnancy	Gavage	Suppression of uterine decidualization, decreased levels of serum progesterone	
DBTCl	Wistar rat	7.6–15.2 mg/kg	Days 0–3 or 4–7 of pregnancy	Gavage	Decreased pregnancy rate, pre- and postimplantation loss, decreased fetal wt.	Ema and Harazono (2000)
DBTCl	Wistar rat	7.6–15.2 mg/kg	Days 0–3 or 4–7 of pseudopregnancy	Gavage	Suppression of uterine decidualization, decreased levels of serum progesterone	Harazono and Ema (2003)
MBTCl	Wistar rat	903 mg/kg	Days 0–3 or 4–7 of pregnancy	Gavage	Decreased fetal wt.	Ema and Harazono (2001)

throughout the premating, mating, gestation, and lactation periods, for two generations. TBTCl affected the male reproductive system. The effects of TBTCl in the F2 generation were greater than those in the F1 generation. Body weight gain was consistently suppressed at 125 ppm in F1 and F2 males. The weights of the testis and epididymis were decreased and homogenization-resistant spermatid and sperm counts were reduced mainly at 125 ppm. Ventral prostate weight and spermatid count were decreased at 125 ppm in F1 males and at 25 and 125 ppm in F2 males. The serum 17-estradiol levels were decreased at 125 ppm in F1 and F2 males, but serum levels of luteinizing hormone and testosterone were not decreased. Omura et al. (2001) note that these changes corresponded with those caused by aromatase inhibitor and suggest that TBTCl might cause a weak aromatase inhibition in male rats.

Regarding female reproductive toxicity, the results with female rats in the above-mentioned two-generation reproduction study were reported by Ogata et al. (2001). Decreases in body weight gain during pregnancy, total number and average body weight of pups, and live birth index were observed at 125 ppm in F0 and F1 dams. Body weight gain was consistently suppressed at 125 ppm in F1 and F2 females. Delayed vaginal opening and impaired estrous cyclicity were found at 125 ppm in F1 and F2 females. The normalized anogenital distance (AGD) was increased at 5 ppm and higher in F1 females on postnatal day 1, and at 125 ppm in F1 and F2 females on postnatal days (PNDs) 1 and 4. These results show that a whole-life exposure to TBTCl affects the sexual development and reproductive function of female rats. They noted that TBTCl-induced increase in female AGD seems to suggest that it may exert a masculinizing (androgenic) effect on female pups.

Female Wistar rats were administered TBTCl by gavage at 8.1, 12.2, or 16.3 mg/kg on days 0 to 7 of pregnancy, and the adverse effects of TBTCl on implantation and maintenance of pregnancy were determined (Hrazono et al. 1996). Decreases in maternal body weight gain at 12.2 mg/kg and higher, and food consumption at 8.1 mg/kg and higher, were found. Implantation failure was found at doses that also produced maternal toxicity. The pregnancy rate was significantly decreased at 12.2 mg/kg and higher. In females having implantations, the numbers of corpora lutea, implantations, and postimplantation loss, were comparable across all groups. To examine whether pregnancy failure was the result of the effects of TBTCl or maternal malnutrition from reduced food consumption, a pair-feeding study was performed. The results show that the pregnancy failure observed in the TBTCl-treated group is due to the effects of TBTCl, not to the maternal malnutrition from reduced food consumption (Harazono et al. 1998a). The adverse effects of TBTCl on implantation and maintenance of pregnancy after administration during the pre- or periimplantation period were evaluated. Female Wistar rats were given TBTCl by gastric intubation on days 0 to 3 of pregnancy at 4.1, 8.1, 16.3, or 32.5 mg/kg, or on days 4 to 7 of pregnancy at 8.1, 16.3, 32.5, or 65.1 mg/kg, and pregnancy outcome was determined on day 20 of pregnancy (Harazono et al. 1998b). TBTCl on days

0 to 3 at 16.3 mg/kg and higher and on days 4 to 7 at 65.1 mg/kg caused a decrease in pregnancy rate and an increase in preimplantation embryonic loss. TBTCl on days 4 to 7 of pregnancy caused a significant increase in the incidence of postimplantation loss at 16.3 mg/kg and higher. The results show that the manifestation of adverse effects of TBTCl varies with gestational stage at the time of maternal exposure, and that TBTCl during the preimplantation period causes implantation failure, while TBTCl during the periimplantation period adversely affects the viability of implanted embryos.

Female Wistar rats were given TBTCl by gavage on days 0 to 3 or on days 4 to 7 of pseudopregnancy, and the effects of TBTCl on the uterus, as a cause of implantation failure, were evaluated according to the same procedures described above. After administration of TBTCl on days 0 to 3 of pseudopregnancy, a decrease in the uterine weight was detected at 16.3 mg/kg and higher (Harazono and Ema 2000). Decreased levels of serum progesterone occurred on day 9 at 16.3 mg/kg and higher and on day 4 at 8.1 mg/kg and higher, and increased levels of serum estradiol at 32.5 mg/kg were observed after administration on days 0 to 3. Following administration of TBTCl on days 4 to 7 of pseudopregnancy, uterine weight and serum progesterone levels on day 9 decreased at 16.3 mg/kg and higher. The doses that induced decreases in uterine weight and serum progesterone levels in pseudopregnant rats are consistent with those that induced pre- and postimplantation loss in pregnant rats. These results indicate that TBTCl suppresses uterine decidualization correlated with a reduction in serum progesterone levels, and suggest that the decline in uterine decidualization and serum progesterone levels participate in the induction of implantation failure induced by TBTCl.

TBT compound is reported to be metabolized to di- and MBT derivatives, and DBT was metabolized to MBT in rats (Fish et al. 1976, Kimmel et al. 1977, Ishizaka et al. 1989, Iwai et al. 1981). The adverse effects of dibutyltin dichloride (DBTCl) on the implantation and maintenance of pregnancy, and the role of DBT in the reproductive toxicity of TBT were evaluated after maternal exposure during the pre- or periimplantation period (Ema and Harazono 2000). Female Wistar rats were given DBTCl by gastric intubation at 3.8, 7.6, or 15.2 mg/kg on days 0 to 3 or on days 4 to 7 of pregnancy. The pair-feeding study was also performed. After administration of DBTCl on days 0 to 3, the pregnancy rate in the 7.6 mg/kg group was lower than in the control group, and that in the 15.2 mg/kg group was lower than in the control and pair-fed groups. The incidence of postimplantation embryonic loss in the groups given DBTCl on days 4 to 7 at 7.6 and 15.2 mg/kg was higher than in the control and pair-fed groups. Early embryonic loss was considered to be due to the effects of DBTCl, not to maternal malnutrition from reduced feed consumption, and the lowest dose of DBTCl inducing early embryonic loss was conservatively estimated at 7.6 mg (25 μmol)/kg. An increase in the incidence of implantation failure was observed after administration of TBTCl, the parent compound of DBTCl, at 16.3 mg (50 μmol)/kg and higher on days 0 to 3 and on days 4 to 7 of pregnancy,

respectively. The doses of DBTCl that caused early embryonic loss were lower than those of TBTCl (Harazono et al. 1998b). Thus, it is likely that DBTCl and/or its metabolites can be considered the agents responsible for early embryonic loss induced by TBTCl. Suppression of uterine decidualization accompanied by reduced levels of serum progesterone was found in pseudopregnant rats given DBTCl at doses that caused implantation failure (Harazono and Ema 2003), and administration of progesterone protected, at least in part, against the DBTCl-induced implantation failure (Ema et al. 2003). These results suggest that the decline in progesterone levels is a primary mechanism for the implantation failure due to DBTCl. Administration of butyltin trichloride (MBTCl) on days 0 to 3 or on days 4 to 7 of pregnancy did not cause pre- or postimplantation loss, even at 903 mg (equivalent to 3200 μmol)/kg in Wistar rats (Ema and Harazono 2001). It is unlikely that MBTCl and/or metabolites are actively involved in the early embryonic loss due to butyltins. The dose levels of DBTCl that suppressed the DCR were lower than the effective doses of TBTCl on a molar base. The similarity of effects and equivalent or greater effectiveness of DBTCl may suggest that DBTCl participates in the inhibition of DCR and in the decrease in serum progesterone levels associated with TBTCl. Although increased levels of serum estradiol on day 9 of pseudopregnancy was observed in rats given TBTCl on days 0 to 3 (Harazono and Ema 2000), administration of DBTCl did not affect serum estradiol levels. Thus, the mechanisms of TBTCl and DBTCl adversely affecting ovarian function might be different. Further studies are needed to determine the effects of TBTCl and DBTCl on the maternal endocrine system, including ovarian function.

Summary of Reproductive Toxicity of Butyltin Compounds

In a rat two-generation reproductive toxicity study, TBTCl affected the male and female reproductive system. TBTCl caused decreases in weight of the testis, epididymis, and ventral prostate, and spermatid and sperm counts in male offspring. The serum estradiol levels decreased in male offspring, but serum levels of luteinizing hormone and testosterone did not decrease. Total number and average body weight of pups, and live birth index decreased. Delayed vaginal opening and impaired estrous cyclicity were found in female offspring. The AGD increased even at 0.4 mg/kg in female offspring. TBTCl during early pregnancy caused implantation failure in rats. Implantation failure due to TBTCl may be mediated via the suppression of uterine decidualization and correlated with the reduction in serum progesterone levels. Implantation failure was also observed following administration of DBTCl, at lower doses than TBTCl, during early pregnancy. Suppression of uterine decidualization, accompanied by reduced levels of serum progesterone, was also observed in pseudopregnant rats given DBTCl at doses that induced implantation failure. Administration of progesterone protected, at least in part, against the DBTCl-induced implantation failure. Administration of MBTCl during early pregnancy did not cause pre- or postimplantation

loss even at 903 mg/kg. These results suggest that DBT may be responsible for the TBT-induced implantation failure, and that the decrease in serum progesterone levels may be a primary factor in implantation failure due to butyltins.

Developmental Toxicity of Butyltin Compounds

In Vivo *Developmental Toxic Effects of Butyltin Compounds*

Studies on developmental toxicity of butyltins are shown in Table 3.4. Several studies concerning the developmental toxicity of TBTO have been conducted in mice and rats. Davis et al. (1987) reported that an increased incidence of resorptions, reduced fetal weight, and an increased incidence of cleft palate were accompanied by a marked decrease in maternal weight gain after administration of TBTO by gavage to NMRI mice on days 6 to 15 of pregnancy. TBTO at 11.7 mg/kg was the lowest dose resulting in reduced maternal weight with no indication of decreases in litter size and fetal weight. At 35 mg/kg, the incidence of resorptions was 59% and fetal weight was markedly lowered. Doses lower than 11.7 mg/kg did not cause clear-cut teratogenic effects, and the incidences of cleft palate were 7% at 11.7 mg/kg and 48% at 35 mg/kg. They concluded that cleft palate might be a nonspecific toxic effect and not a teratogenic effect of TBTO. Swiss albino mice received TBTO by gavage at on days 6 to 15 of pregnancy (Baroncelli et at. 1990, 1995). In the prenatal study, a decrease in maternal weight gain and fetal weight were found, along with high embryolethality, but no increased incidence of fetal malformations were found at 40 mg/kg (Baroncelli et al. 1990). In the postnatal study, reduced litter size and pup weight at 20 mg/kg and higher, increased percentage of dams that had not built a nest at 10 mg/kg and higher, and decreased maternal weight gain and increased number of early or late deliveries at 5 mg/kg were detected. No malformations in pups were observed (Broncelli et al. 1995). Nonspecific alterations of hematological parameters and thymus or spleen weights were noted in dams and offspring of Swiss mice after administration of TBTO by gavage at 5, 10, or 20 mg/kg on days 6 to 15 of pregnancy (Karrer et al. 1995). A high incidence of cleft palate (11.4 percent) was found at 27 mg/kg in Han:NMRI mice given TBTO by gavage on days 6 to 17 of pregnancy (Faqi et al. 1997). At this dose, two fetuses exhibited a bent radius, eight fetuses were observed with a short mandible, and five fetuses showed a fusion of the occipital bones with their basal parts. In this study, no signs of toxicity in maternal and fetal mice were detected up to the dose of 13.5 mg/kg. Long Evans rats were given TBTO by gavage at 2.5, 5, 10, 12, or 16 mg/kg on days 6 to 20 of pregnancy, allowed to give birth, and pups were examined (Crofton et al. 1989). Maternal body weight gain, and pup litter size, weight, and viability on PNDs 1 and 3 were decreased at 10 mg and higher. A 3% incidence of cleft palate was detected at 12 mg/kg. There were no pups born with malformations at 10 mg/kg and lower. Vaginal opening was delayed in females exposed to 10 mg/kg. Motor activity was decreased on PND 14 at all doses. Adult brain weight

TBTCl	SD rats	1–5 mg/kg	Days 6–20 of pregnancy	Gavage	Increased spontaneous activity, retarded aquisition of the radial arm maze task, potentiation of d-amphetamine-induced hyperactivity	Gårdlung et al. (1991)
DBTA	Wistar rats	15 mg/kg	Days 0–19 of pregnancy	Gavage	Postimplantation loss, decreased fetal wt., manibular dysplasia, ankyloglossia, schistoglossia, skeletal variation	Nada et al. (1988)
DBTA	Wistar rat	5–15 mg/kg	Days 7–17 of pregnancy	Gavage	Postimplantation loss, decreased fetal wt., cleft mandible, cleft lower lip, ankyloglossia, schistoglossia, tail anomaly, deformity of ribs and vertebrae, skeletal variations	Noda et al. (1992a)
MBTCl	Wistar rat	50–400 mg/kg	Days 7–17 of pregnancy	Gavage	No effects	
DBTA	Wistar rat	15 mg/kg	Days 7–9 of pregnancy	Gavage	Effects as above	Noda et al. (1992b)
		22 mg/kg	Day 8 of pregnancy	Gavage	Malformations as above	
DBTA	Wistar rat	10–22 mg/kg	Day 8 of pregnancy	Gavage	Malformations as above	Noda et al. (2001)
DBTCl	Wistar rat	5–10 mg/kg	Days 7–15 of pregnancy	Gavage	Postimplantation loss, decreased fetal wt., cleft jaw, cleft palate, ankyloglossia, omphalocere, tail anomaly, deformity of ribs and vertebrae	Ema et al. (1991)
DBTCl	Wistar rat	20 mg/kg	Days 7–9, 10–12, or 13–15 of pregnancy	Gavage	Decreased fetal wt., postimplantation loss, malformations as above after p.o. on days 7–9	Ema et al. (1992)

Table 3.4 Developmental Toxicity of Butyltin Compounds

Compounds	Animals	Dose	Days of Administration	Route	Reproductive and Developmental Effects	Author(s)
TBTO	NMRI mouse	11.7–35 mg/kg	Days 6–15 of pregnancy	Gavage	Postimplantation loss, decreased fetal wt., cleft palate	Davis et al. (1987)
TBTO	Swiss mouse	40 mg/kg	Days 6–15 of pregnancy	Gavage	Postimplantation loss, decreased fetal wt.,	Baroncelli et al. (1990)
TBTO	Swiss mouse	10–30 mg/kg	Days 6–15 of pregnancy	Gavage	Decreased litter size, decreased pup wt., changed length of gestation, decreased percentage of dams exhibiting nest-building	Baroncelli et al. (1995)
TBTO	Swiss mouse	5–20 mg/kg	Days 6–15 of pregnancy	Gavage	Nonspecific effects on hematological parameters	Karrer et al (1995)
TBTO	Ha:NMRI mouse	27 mg/kg	Days 6–17 of pregnancy	Gavage	Decreased fetal wt., cleft palate, skeletal malformations	Faqi et al. (1997)
TBTO	Long Evans rat	2.5–16 mg/kg	Days 6–20 of pregnancy	Gavage	Decreased litter size and pup wt., cleft palate, decreased postnatal wt. gain, delayed vaginal opening, decreased brain wt., transient decrease in motor activity	Crofton et al. (1989)
TBTO	THA rat	5–10 mg/kg	Days 6–20 of pregnancy	Gavage	Postnatal death, disruption of learning acquisition	Miyake et al. (1990)
TBTA	Wistar rat	16 mg/kg	Days 7–17 of pregnancy	Gavage	Postimplantation loss, cleft palate, decreased fetal wt.	Noda et al. (1991b)
TBTCl	Wistar rat	5–25 mg/kg	Days 7–15 of pregnancy	Gavage	Postimplantation loss, delayed ossification	Itami et al. (1990)
TBTCl	Wistar rat	25–50 mg/kg	Days 7–9 of pregnancy	Gavage	Postimplantation loss, decreased fetal wt.	Ema et al. (1995a)

Table 3.4 Developmental Toxicity of Butyltin Compounds (continued)

Compounds	Animals	Dose	Days of Administration	Route	Reproductive and Developmental Effects	Author(s)
		20–40 mg/kg	Day 6, 7, 8, or 9 of pregnancy	Gavage	Decreased fetal wt., postimplantation loss after p.o. on day 6, 7, or 8, malformations as above after p.o. on day 7 or 8	
DBTCl	Wistar rat	1–10 mg/kg	Days 6–15 of pregnancy	Gavage	No effects	Farr et al. (2001)
DBTA	Wistar rat	28.1 mg/kg	Day 8 of pregnancy	Gavage	Cleft mandible, cleft lower lip, ankyloglossia, schistoglossia, exencephaly, deformity of ribs and vertebrae	Noda et al. (1993)
DBTCl	Wistar rat	24.3 mg/kg	Day 8 of pregnancy	Gavage	Decreased fetal wt., malformations as above	
DBTM	Wistar rat	27.8 mg/kg	Day 8 of pregnancy	Gavage	Malformations as above	
DBTO	Wistar rat	19.9 mg/kg	Day 8 of pregnancy	Gavage	Malformations as above	
DBTL	Wistar rat	50.0 mg/kg	Day 8 of pregnancy	gavage	Malformations as above	
3-OHDBTL	Wistar rat	100 mg/kg	Day 8 of pregnancy	Gavage	Decreased fetal wt., peaked mandible	
TeBT	Wistar rat	1832 mg/kg	Days 13–15 of pregnancy	Gavage	Cleft palate	Ema et al. (1996a)
TBTCl	Wistar rat	54–108 mg/kg	Days 13–15 of pregnancy	Gavage	Decreased fetal wt., cleft palate	
DBTCl	Wistar rat	50–100 mg/kg	Days 13–15 of pregnancy	Gavage	Decreased fetal wt.	
TBTCl	Wistar rat	40–80 mg/kg	Days 7–8 of pregnancy	Gavage	Postimplantation loss, decreased fetal wt.	Ema et al. (1995b)
DBTCl	Wistar rat	10–15 mg/kg	Days 7–8 of pregnancy	Gavage	Effects as above, malformations as above	
MBTCl	Wistar rat	1000–1500 mg/kg	Days 7–8 of pregnancy	Gavage	Decreased fetal wt.	

Table 3.4 Developmental Toxicity of Butyltin Compounds (continued)

Compounds	Animals	Dose	Days of Administration	Route	Reproductive and Developmental Effects	Author(s)
		50–100 mg/kg	Days 10–12 of pregnancy	Gavage	Effects as above, cleft palate	
		25–100 mg/kg	Days 13–15 of pregnancy	Gavage	Decreased fetal wt., cleft palate	
TBTCl	Wistar rat	100–200 mg/kg	One day during days 7–15 of pregnancy	Gavage	Postimplantation loss, decreased fetal wt., cleft palate after po on day 8, 11, 12, 13, or 14	Ema et al. (1997b)
TBTCl	SD rat	0.25–20 mg/kg	Days 0–19 of pregnancy	Gavage	Postimplantation loss, decreased fetal wt., increased male AGD, delayed ossification, decreased levels of serum thyroxine and triiodothyronine	Adeeko et al. (2003)
		2.5–10 mg/kg	Days 8–19 of pregnancy		Decreased levels of serum thyroxine	
TBTCl	SD rat	0.025–2.5 mg/kg	From day 8 of pregnancy until adulthood	Gavage	Decreased wt of liver, spleen and thymus, reduced serum levels of creatinine, triglyceride, amylase and thyroxine, change in growth profiles	Cooke et al. (2004)
TBTCl	SD rat	0.25–2.5 mg/kg	From day 8 of pregnancy until adulthood	Gavage	Thymus atrophy, increased no. of natural killer cells, increased levels of IgM and IgG, increased no. of immature T lymphocytes, decreased levels of IgG2a	Tryphonas et al. (2004)

was reduced at 10 mg/kg. THA rats were given TBTO by gavage at 5 or 10 mg/kg on days 6 to 20 of pregnancy and allowed to deliver spontaneously, and pups were examined (Miyake et al. 1990). All pups died by PND 3 at 10 mg/kg. In pups at 5 mg/kg, prenatal TBTO disrupted learning acquisition in the Sidman avoidance test and a reversal test in the water E-maze.

Pregnant Wistar rats were given tributyltin acetate (TBTA) by gavage at 1, 2, 4, 8, or 16 mg/kg on days 7 to 17 of pregnancy (Noda et al. 1991b). An increase in incidences of intrauterine deaths, cleft palate, and low fetal weight were found at 16 mg/kg. This dose level also induced severe reductions in maternal weight gain and food consumption, and TBTA at 4 mg/kg and higher lowered maternal thymus weight. Noda et al. (1991b) concluded that the observed teratogenic effects may not be a specific action of TBTA because their results were similar to those of Davis et al. (1987).

No live fetuses were obtained in Wistar rats treated with TBTCl at 25 mg/kg by gavage on days 7 to 15 of pregnancy (Itami et al. 1990). Maternal toxicity at 9 mg/kg and higher, and skeletal retardation in fetuses at 5 mg/kg and higher were observed, but fetal malformations were not found. An increase in placental weight was found at 5 mg/kg and higher. To obtain more precise information on the effects of TBTCl on fetal development, Wistar rats were given TBTCl by gavage at relatively high doses during a shorter period, at 25 or 50 mg/kg on days 7 to 9, at 50 or 100 mg/kg on days 10 to 12, or at 25, 50, or 100 mg/kg on days 13 to 15 of pregnancy (Ema et al. 1995a). A decrease in maternal weight gain was observed in all groups regardless the days of administration. An increase in incidence of postimplantation embryonic loss was found in pregnant rats given TBTCl on days 7 to 9 at 25 mg/kg and higher, and on days 10 to 12 at 100 mg/kg, but not in pregnant rats given TBTCl on days 13 to 15 at up to 100 mg/kg. A lower fetal weight was observed in pregnant rats given TBTCl on days 10 to 12 at 50 and 100 mg/kg, and on days 13 to 15 at 100 mg/kg. An increased incidence of fetuses with malformations was detected after administration of TBTCl on days 10 to 12 at 100 mg/kg, and on days 13 to 15 at 25 mg/kg and higher. The most predominant malformation was cleft palate. These results indicate that the manifestation of abnormal development induced by TBTCl varies with developmental stage at the time of administration, and that TBTCl has teratogenic potential with developmental phase specificity. The most susceptible day to the teratogenicity of TBTCl was determined by a single administration on one of the days during organogenesis (Ema et al. 1997b). An increase in incidence of fetuses with external malformations was detected when TBTCl was given on day 8 at 100 and 200 mg/kg, or on day 11, 12, 13, or 14 at 200 mg/kg, and the most pronounced effect was seen after administration on day 13 of pregnancy. Cleft palate was mainly observed after administration of TBTCl. These findings indicate that TBTCl has a biphasic teratogenicity on day 8 and on days 11 to 14 of pregnancy. Pregnant SD rats were gavaged with TBTCl at 0.25, 2.5, 10, or 20 mg/kg on days 0 to 19 of pregnancy, or at 0.25, 2.5, or 10 mg/kg on days 8 to 19 of pregnancy, and pregnancy outcome was assessed (Adeeko et al. 2003). A

reduced maternal weight gain, decrease in pregnancy rate, increase in postimplantation loss, and decrease in fetal weight were found after administration of TBTCl at 20 mg/kg on days 0 to 19. These findings support the previous results (Harazono et al. 1996, 1998a, b) in which TBTCl during early pregnancy at 12.2 mg/kg and higher caused increases in pre- and postimplantation loss. The incidence of fetal malformations was not increased in any TBTCl-treated groups. An increase in normalized AGD of male fetuses was detected at 0.25 mg/kg and higher on days 0 to 19, but not at any dose on days 8 to 19, even at the highest dose level. Hormonally active agents are known to affect mammalian internal and external genitalia when administered during sex differentiation (i.e., the perinatal period) (Schardein 2000). It is reported that days 16 to 17 of pregnancy were the most sensitive for finasteride-induced feminizing effects, including a decrease in AGD in male rat offspring (Clark et al. 1993), and that the period of days 15 to 17 of pregnancy was the most susceptible for dibutyl phthalate–induced decrease in the AGD of male rat offspring (Ema et al. 2000). There are discrepancies in the effects of TBTCl on the AGD between outcomes after exposure on days 0 to 19 and on days 8 to 19 of pregnancy, and between this study and previous reports in which whole-life exposure to TBTCl caused increase in female AGD in rat two-generation reproductive studies (Ogata et al. 2001). *In vitro* studies showed that TPT and TBT had an ability to activate androgen receptor mediated transcription in mammalian cells (Yamabe et al. 2000). TPTCl, TBTCl, and DBTCl caused aromatase inhibition in the human adenocortical carcinoma cell line (Sanderson et al. 2002). Although TeBT and MBTCl had no effect on either human 5-reductase type 1 or type 2, TBTCl and DBTCl influenced the human 5-reductase isozymes (Doering et al. 2002). DBTCl specifically inhibited brain 5-reductase type 1 with no effect on prostate 5-reductase type 2. TBTCl inhibited both isoenzymes. Doering et al. (2002) noted that the inhibition of the TBTCl inhibited both isoenzymes. Type 2 could potentially disturb normal male physiology. These *in vitro* findings may explain the *in vivo* reproductive and developmental outcomes induced by organotins. Adeeko et al. (2003) also noted that reduced fetal ossification of the sternebrae was found at 10 mg/kg and higher, for which fetal weights at 10 mg/kg were in normal range, and TBTCl at 10 mg/kg and higher during pregnancy decreased maternal circulating thyroid hormone levels and increased the weight of the placenta. They noted that the TBTCl-induced disturbances in maternal thyroid hormone homeostasis could contribute to the reduction in fetal skeletal ossification.

Pregnant SD rats were gavaged with TBTCl at 0.025, 0.25, or 2.5 mg/kg from day 8 of pregnancy until weaning, and offspring were gavaged with the same dose of TBTCl given to their mothers until adulthood (Cooke et al. 2004, Tryphonas et al. 2004). No effects of TBTCl on body weight, food consumption, or histopathological findings in the thyroid, liver, adrenal or colon were observed in maternal rats. No effects of TCBTCl on litter size, sex ratio, postnatal survival rate, or histopathological findings in the liver,

adrenal glan or colon were also found in offspring. Decreased serum levels of creatinine, triglycerides, and magnesium in female offspring and of thyroxine in male offspring were found at 2.5 mg/kg. Decreased weight of the spleen in male offspring and the thymus in female offspring were observed at 0.25 mg/kg. Significant effects on growth profiles in male and female offspring, and decreased liver weights in female offspring were noted even at 0.025 mg/kg (Cooke et al. 2004). Immunotoxic effects of TBTCl were determined in these rat offspring (Tryphonas et al. 2004). Thymus atrophy, an increase in the number of natural killer cells and immunoglobulin M (IgM) levels, a decrease in the IgG2a levels at 2.5 mg/kg, and an increase in the mean percentage immature T lymphocytes and IgG levels at 0.25 mg/kg and higher were observed in offspring. Significant effects were found more frequently at 0.25 mg/kg and higher, and minor effects were observed at 0.025 mg/kg. Tryphonas et al. (2004) concluded that the low levels of TBTCl affected humoral and cell-mediated immunity, and the number and function of cells involved in the hosts immunosurveillance mechanisms against tumors and vital infections in rat offspring.

Postnatal behavioral changes in pups of SD rats that received TBTCl prenatally on days 6 to 20 of pregnancy, at doses not toxic to the mother, were also reported (Gårdlund et al. 1991). An increase in spontaneous activity, such as locomotion, rearing, and total activity, retarded acquisition in radial arm maze performance, and potentiation of d-amphetamine-induced hyperactivity were observed at 1 and 5 mg/kg.1

The adverse effects of DBT, a major metabolite of TBT, on embryonic/fetal development were assessed after maternal administration during organogenesis. Pregnant Wistar rats were given DBTA by gavage at 1.7, 5, or 15 mg/kg during the whole period, on days 0 to 19, of pregnancy (Noda et al. 1988). At 15 mg/kg, a decrease in body weight gain and thymus weight in dams, and a low body weight and increased number of fetal malformations occurred. Administration of DBTA by gavage during the organogenetic period, on days 7 to 17, of pregnancy at 10 mg/kg and higher also caused increased fetal malformations, such as cleft mandible, cleft lower lip, ankyloglossia, schistoglossia, exencephaly, anury, vestigial tail, and deformity of the ribs and vertebrae (Noda et al. 1992a). Decreases in thymus weight and fetal weight at 10 mg/kg and higher, and decreases in maternal weight gain at 15 mg/kg were observed following administration of DBTA on days 7 to 17 of pregnancy. The most susceptible gestational day to teratogenicity of DBTA in rats was day 8 of pregnancy (Noda et al. 1992b). Occurrences of similar types of fetal malformations after administration of DBTA on day 8 of pregnancy were also reported in other papers (Node et al. 1993, 1994, 2001). Teratogenic effects of DBTCl were also studied in Wistar rats. Female rats were given DBTCl by gavage at 2.5, 5.0, or 7.5 mg/kg on days 7 to 15 of pregnancy (Ema et al. 1991). The incidence of fetal malformations was increased and roughly proportional to the dose of DBTCl administered at 5.0 mg/kg and higher. Cleft jaw, ankyloglossia, omphalocele, anomaly of

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	Paulc 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 vacations	
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.

References

Adeeko, A., Li, D., Forsyth, D.S., Casey, V., Cooke, G.M., Barthelemy, J., Cyr, D.G., Trasler, J.M., Robaire, B., and Hales, B.F. (2003) Effects of in utero tributyltin chloride exposure in the rat on pregnancy outcome, *Toxicological Sciences*, 74, 407–15.

Baroncelli, S., Karrer, D., and Turillazzi, P.G. (1990) Embryotoxic evaluation of bis(tri-*n*-butyltin)oxide (TBTO) in mice, *Toxicology Letters*, 50, 257–62.

Baroncelli, S., Karrer, D., and Turillazzi, P.G. (1995) Oral bis(tri-*n*-butyltin) oxide in pregnant mice. I. Potential influence of maternal behavior on postnatal mortality, *Journal of Toxicology and Environmental Health*, 46, 355–67.

Belfoid, A.C., Purperhart, M., and Ariese, F. (2000) Organotin levels in seafood, *Marine Pollution Bulletin*, 40, 226–32.

Bettin, C., Oehlmann, J., and Stroben, E. (1996) YBY-induced imposex in marine neogastropods is mediated by an increasing androgen level, *Helgoländer Meeresunters*, 50, 299–317.

Boyer, I.J. (1989) Toxicity of dibutyltin, tributyltin and other organotin compounds to humans and to experimental animals, *Toxicology*, 55, 253–98.

Bryan, G.W., Gibbs, P.E., Hummerstone, L.G., and Burt, G.R. (1986) The decline of the gastropod *Nucella lapillus* around South-West England: evidence for the effect of tributyltin from antifouling paints, *Journal of the Marine Biological Association of the United Kingdom*, 66, 611–40.

Bryan, G.W., Gibbs, P.E., Burt, G.R., and Hummerstone, L.G. (1987) The effect of tributyltin (TBT) accumulation on adult dog-whelks, *Nucella lapillus*: long-term field and laboratory experiments, *Journal of the Marine Biological Association of the United Kingdom*, 67, 525–44.

Bryan, G.W., Gibbs, P.E., Hummerstone, L.G., and Burt, G.R. (1988) Comparison of the effectiveness of tri-*n*-butyltin chloride and five other organotin compounds in promoting the development of imposex in the dogwhelk *Nucella lapillus*, *Journal of the Marine Biological Association of the United Kingdom*, 68, 733–44.

Bryan, G.W., Gibbs, P.E., Hummerstone, L.G., and Burt, G.R. (1989) Uptake and transformation of 14-C labelled tributyltin chloride by the dog-whelk, *Nucella lapillus*: Importance of absorption from the diet, *Marine Environmental Research*, 28, 241–5.

Bui, Q.Q., Tran, M.B., and West, W.L. (1986) A comparative study of the reproductive effects of methadone and benzo[a]pyrene in the pregnant and pseudopregnant rat, *Toxicology*, 42, 195–204.

Chernoff, N., Setzer, R.W., Miller, D.B., Rosen, M.B., and Rogers, J.M. (1990) Effects of chemically induced maternal toxicity on prenatal development in the rat, *Teratology*, 42, 651–8.

Clark, R.I., Anderson, C.A., Prahalada, S., Robertson, R.T., Lochry, E.A., Leonard, Y.M., Stevens, J.L., and Hoberman, A.M. (1993) Critical developmental periods for effects on male rat genitalia induced by finasteride, a 5 alpha-reductase inhibitor, *Toxicology and Applied Pharmacology*, 119, 34–40.

Colborn, T., vom Saal, F., and Soto, A. (1993) Developmental effects of endocrine-disrupting chemicals in wildlife and humans, *Environmental Health Perspectives*, 101, 378–84.

Cooke, G.M., Tryphonas, H., Pulido, O., Caldwell, D., Bondy, G.S., and Forsyth, D. (2004) Oral (gavage), in utero and postnatal exposure of Sprague-Dawley rats to low doses of tributyltin chloride. Part I: toxicology, histopathology and clinical chemistry, *Food and Chemical Toxicology*, 42, 211–20.

Crofton, K.M., Dean, K.F., Boncek, V.M., Rosen, M.B., Sheets, L.P., Chernoff, N., and Reiter, L.W. (1989) Prenatal or postnatal exposure to bis(tri-*n*-butyltin)oxide in the rat: postnatal evaluation of teratology and behavior, *Toxicology and Applied Pharmacology*, 97, 113–23.

Cummings, A.M. (1990) Toxicological mechanisms of implantation failure, *Fundamental and Applied Toxicology*, 15, 571–9.

Davies, J.M., Bailey, S.K., and Moore, D.C. (1987) Tributyltin in Scottish sea lochs, as indicated by degree of imposex in the dogwhelk, *Nucella lapillus* (L.), *Marine Pollution Bulletin*, 18, 400–4.

Davis, A., Barale, R., Brun, G., Forster, R., Günther, T., Hautefeuille, H., van der Heijden, C.A., Knaap, A.G.A.C., Krowke, R., Kuroki, T., Loprieno, N., Malaveille, C., Merker, H.J., Monaco, M., Mosesso, P., Nuebert, D., Norppa, H., Sorsa, M., Vogel, E., Voogd, C.E., Umeda, M., and Bartsch, H. (1987) Evaluation of the genetic and embryotoxic effects of bis(tri-*n*-butyltin)oxide (TBTO), a broad-spectrum pesticide, in multiple in vivo and in vitro short-term tests, *Mutation Research*, 188, 65–95.

De Feo, V.J. (1963) Temporal aspect of uterine sensitivity in the pseudopregnant or pregnant rat, *Endocrinology*, 72, 305–16.

Doering, D.D., Stechelbroeck, S., Doering, T., and Klingm, D. (2002) Effects of butyltins on human 5-reductase type 1 and type 2 activity, *Steroids*, 67, 859–67.

Eisler, R. (2000) Tin, in *Handbook of Chemical Risk Assessment: Health Hazards to Humans, Plants, and Animals, Volume 1 (Metals)*, Boca Raton, FL, Lewis Publishers, 551–603.

Ema, M. and Harazono, A. (2000) Adverse effects of dibutyltin dichloride on initiation and maintenance of rat pregnancy, *Reproductive Toxicology*, 14, 451–6.

Ema, M. and Harazono, A. (2001) Toxic effects of butyltin trichloride during early pregnancy in rats, *Toxicology Letters*, 125, 99–106.

Ema, M. and Miyawaki, E. (2001) Role of progesterone on suppression of uterine decidualization and implantation failure induced by triphenyltin chloride in rats, *Congenital Anomalies*, 41, 106–11.

Ema, M. and Miyawaki, E. (2002) Suppression of uterine decidualization correlated with reduction in serum progesterone levels as a cause of preimplantation embryonic loss induced by diphenyltin in rats, *Reproductive Toxicology*, 16, 309–17.

Ema, M., Itami, T., and Kawasaki, H. (1991) Teratogenicity of di-*n*-butyltin dichloride in rats, *Toxicology Letters*, 58, 347–56.

Ema, M., Itami, T., and Kawasaki, H. (1992) Susceptible period for the teratogenicity of di-*n*-butyltin dichloride in rats, *Toxicology*, 73, 81–92.

Ema, M., Kurosaka, R., Amano, H., and Ogawa, Y. (1995a) Further evaluation of the developmental toxicity of tributyltin chloride in rats, *Toxicology*, 96, 195–201.

Ema, M., Kurosaka, R., Amano, H., and Ogawa, Y. (1995b) Comparative developmental toxicity of butyltin trichloride, dibutyltin dichloride and tributyltin chloride in rats, *Journal of Applied Toxicology*, 15, 297–302.

Ema, M., Iwase, T., Iwase, Y., and Ogawa, Y. (1995c) Dysmorphogenic effects of di-*n*-butyltin dichloride in cultured rat embryos, *Toxicology in Vitro*, 9, 703–9.

Ema, M., Kurosaka, R., Amano, H., and Ogawa, Y. (1996a) Comparative developmental toxicity of di-, tri- and tetrabutyltin compounds after administration during late organogenesis in rats, *Journal of Applied Toxicology*, 16, 71–6.

Ema, M., Iwase, T., Iwase, Y., Ohyama, N., and Ogawa, Y. (1996b) Change of embryotoxic susceptibility to di-*n*-butyltin dichloride in cultured rat embryos, *Archives of Toxicology*, 70, 742–8.

Ema, M., Miyawaki, E., Harazono, A., and Ogawa, Y. (1997a) Effects of triphenyltin chloride on implantation and pregnancy in rats, *Reproductive Toxicology*, 11, 201–6.

Ema, M., Harazono, A., Miyawaki, E., and Ogawa, Y. (1997b) Effect of the day of administration on the developmental toxicity of tributyltin chloride in rats, *Archives of Environmental Contamination and Toxicology*, 33, 90–6.

Ema, M., Miyawaki, E., Harazono, A., and Ogawa, Y. (1998) Reproductive effects of butyl benzyl phthalate in pregnant and pseudopregnant rats, *Reproductive Toxicology*, 12, 127–32.

Ema, M., Miyawaki, E., and Kawashima, K. (1999a) Suppression of uterine decidualization as a cause of implantation failure induced by triphenyltin chloride in rats, *Archives of Toxicology*, 73, 175–9.

Ema, M., Miyawaki, E., and Kawashima, K. (1999b) Adverse effects of diphenyltin dichloride on initiation and maintenance of pregnancy in rats, *Toxicology Letters*, 108, 17–25.

Ema, M., Miyawaki, E., and Kawashima, K. (1999c) Developmental toxicity of triphenyltin chloride after administration on three consecutive days during organogenesis in rats, *Bulletin of Environmental Contamination and Toxicology*, 62, 363–70.

Ema, M., Miyawaki, E., and Kawashima, K. (2000) Critical period for adverse effects on development of reproductive system in male offspring of rats given di-*n*-butyl phthalate during late pregnancy, *Toxicology Letters*, 111, 271–8.

Ema, M., Harazono, A., Hirose, A., and Kamata, E. (2003) Protective effects of progesterone on implantation failure induced by dibutyltin dichloride in rats, *Toxicology Letters*, 143, 233–8.

Epstein, S.S., Arnold, E., Andrea, J., Bass, W., and Bishop, Y. (1972) Detection of chemical mutagens by the dominant lethal assay in the mouse, *Toxicology and Applied Pharmacology*, 23, 288–325.

Evans, D.W. and Laughlin, R.B., Jr. (1984) Accumulation of bis(tributyltin) oxide by the mud crab, *Rhithropanopeus harrisii*, *Chemosphere*, 13, 213–9.

Faqi, A.S., Schweinfurth, H., and Chahoud, I. (1997) Determination of the no-effect dose of bis(tri-*n*-butyltin)oxide (TBTO) for maternal toxicity and teratogenicity in mice, *Congenital Anomalies*, 37, 251–8.

Faqi, A.S., Schweinfurth, H., and Chahoud, I. (2001) Developmental toxicity of an octyltin stabilizer in NMRI mice, *Reproductive Toxicology*, 15, 117–22.

Farr, C.H., Reinisch, K., Holson, J.F., and Neubert, D. (2001) Potential teratogenicity of di-*n*-butyltin dichloride and other dibutyltin compounds, *Teratogenesis, Carcinogenesis, and Mutagenesis*, 21, 405–15.

Fent, K. (1996) Ecotoxicology of organotin compounds, *Critical Reviews in Toxicology*, 26, 1–117.

Fent, K. and Hunn, J. (1991) Phenyltins in water, sediment, and biota of freshwater marinas, *Environmental Science and Technology*, 25, 956–63.

Fent, K. and Meier, W. (1992) Tributyltin-induced effects on early life stages of minnows *Phoxinus phoxinus*, *Archives of Environmental Contamination and Toxicology*, 22, 428–31.

Fent, K. and Meier, W. (1994) Effects of triphenyltin on fish early life stages, *Archives of Environmental Contamination and Toxicology*, 27, 224–31.

Féral, C. and LeGall, S. (1983) The influence of a pollutant factor (tributyltin) on the neuroendocrine mechanism responsible for the occurrence of a penis in the female of *Ocenebra erinacea*, in *Molluscan neuro-endocrinology*, Lever, J. and Boer, H., Eds., Amsterdam: North Holland Publishing Co., 173–5.

Fioroni, P., Oehlmann, J., and Stroben, E. (1991) The pseudohermaphroditism of prosobranchs; morphological aspects, *Zoologischer Anzeiger*, 226, 1–26.

Fish, R.H., Kimmel, E.C., and Casida, J.E. (1976) Bioorganotin chemistry: reactions of tributyltin derivatives with a cytochrome P-450 dependent monooxygenese enzyme system, *Journal of Organometallic Chemistry*, 118, 41–54.

Gaines, T.B. and Kimbrough, R.D. (1968) Toxicity of fentin hydroxide to rats, *Toxicology and Applied Pharmacology*, 12, 397–403.

Gårdlund, A.T., Archer, T., Danielsson, B., Fredriksson, A., Lindqvist, N.G., Lindstrom, H., and Luthman, J. (1991) Effects of prenatal exposure to tributyltin and trihexyltin on behaviour in rats, *Neurotoxicology and Teratology*, 13, 99–105.

Giavini, E., Prati, M., and Vismara, C. (1980) Effects of triphenyltin acetate on pregnancy in the rat, *Bulletin of Environmental Contamination and Toxicology*, 24, 936–9.

Gibbs, P.E. and Bryan, G.W. (1986) Reproductive failure in populations of the dog-welk, *Nucella lapillus*, caused by imposex induced by tributyltin from antifouling paints, *Journal of the Marine Biological Association of the United Kingdom*, 66, 767–77.

Gibbs, P.E., Bryan, G.W., Pascoe, P.L., and Burt, G.R. (1987) The use of the dog-whelk, *Nucella lapillus*, as an indicator of tributyltin (TBT) contamination, *Journal of the Marine Biological Association of the United Kingdom*, 67, 507–23.

Gibbs, P.E., Pascoe, P.L., and Burt, G.R. (1988) Sex change in the female dog-whelk, *Nucella lapillus*, induced by tributyltin from antifouling paints, *Journal of the Marine Biological Association of the United Kingdom*, 68, 715–31.

Harazono, A. and Ema, M. (2000) Suppression of decidual cell response induced by tributyltin chloride in pseudopregnant rats: as a cause of early embryonic loss, *Archives of Toxicology*, 74, 632–7.

Harazono, A. and Ema, M. (2003) Suppression of decidual cell response induced by dibutyltin dichloride in pseudopregnant rats: As a cause of early embryonic loss, *Reproductive Toxicology*, 17, 393–9

Harazono, A., Ema, M., and Kawashima, K. (1998a) Evaluation of malnutrition as a cause of tributyltin-induced pregnancy failure in rats, *Bulletin of Environmental Contamination and Toxicology*, 61, 224–30.

Harazono, A., Ema, M., and Kawashima, K. (1998b) Evaluation of early embryonic loss induced by tributyltin chloride in rats: Phase- and dose-dependent antifertility effects, *Archives of Environmental Contamination and Toxicology*, 34, 94–9.

Harazono, A., Ema, M., and Ogawa, Y. (1996) Pre-implantation embryonic loss induced by tributyltin chloride in rats, *Toxicology Letters*, 89, 185–90.

Haubruge, E., Petit, F., and Gage, M.J. (2000) Reduced sperm counts in guppies (*Poecilia reticulata*) following exposure to low levels of tributyltin and bisphenol A, *Proceedings of the Royal Society of London. Series B. Biological Sciences*, 267, 2333–7.

Hawkins, L.E. and Hutchinson, S. (1990) Physiological and morphogenetic effects of monophenyltin trichloride on *Ocenebra erinacea* (L.), *Functional Ecology*, 4, 449–54.

Holm, G., Norrgren, L., and Linden, O. (1991) Reprodictive and histophathological effects of long-term experiemental exposure to bis(tributyltin) oxide (TBTO) on the three-spined stickeback, *Gasterosteus aculeatus* Linnaeus, *Journal of Fish Biology*, 38, 373–86.

Horiguchi, T., Shiraishi, H., Shibata, Y., Morita, M., and Shimizu, M. (1996) Imposex and organotin contamination in gastropods after the regulation of organotin use in Japan, *Abstract Book of SETAC 17th Annual Meeting*, 177.

Horiguchi, T., Shiraishi, H., Shimizu, M., and Morita, M. (1997a) Imposex in sea snails, caused by organotin (tributyltin and triphenyltin) pollution in Japan: a survey, *Applied Organometallic Chemistry*, 11, 451–5.

Horiguchi, T., Shiraishi, H., Shimizu, M., and Morita, M. (1997b) Effects of triphenyltin chloride and five other organotin compounds on the development of imposex in the rock shell thais clavigera, *Environmental Pollution*, 95, 85–91.

Horiguchi, T., Kojima, M., Kaya, M., Matsuo, T., Shiraishi, H., Morita, M., and Adachi, Y. (2002) Tributyltin and triphenyltin induce spermatogenesis in ovary of female abalone, Haliotis gigantea, *Marine Environmental Research*, 54, 679–84.

International Programme on Chemical Safety (IPCS) (1999a) *Concise International Chemical Assessment Document, No. 14 Tributyltin Oxide*, IPCS, Geneva, World Health Organization.

International Programme on Chemical Safety (1999b) *Concise International Chemical Assessment Document, No. 13 Triphenyltin Compounds*, Geneva, World Health Organization.

Ishizaka, T., Suzuki, T., and Saito, Y. (1989) Metabolism of dibutyltin dichloride in male rats, *Journal of Agricultural and Food Chemistry*, 37, 1096–101.

Itami, T., Ema, M., Amano, H., Murai, T., and Kawasaki, H. (1990) Teratogenic evaluation of tributyltin chloride in rats following oral exposure, *Drug and Chemical Toxicology*, 13, 283–95.

Iwai, H., Wada, O., and Arakawa, Y. (1981) Determination of tri-, di-, and monobutyltin and inorganic tin in biological materials and some aspects of their metabolism in rats, *Journal of Analytical Toxicology*, 5, 300–6.

Japan Environment Agency (1998) *Strategic Programs on Environmental Endocrine Disruptors '98*, Tokyo, Environmental Health Department.

Kamrin, M.A., Carney, E.W., Chou, K., Cummings, A., Dostal, L.A., Harris, C., Henck, J.W., Loch-Caruso, R., and Miller, R.K. (1994) Female reproductive and developmental toxicology: overview and current approaches, *Toxicology Letters*, 74, 99–119.

Kannan, K., Tanabe, S., and Tatsukawa, R. (1995) Phenyltin residues in horseshoe crabs, *Tachypleus tridentatus* from Japanese coastal waters, *Chemosphere*, 30, 925–32.

Kannan, K., Corsolini, S., Focardi, S., Tanabe, S., and Tatsukawa, R. (1996) Accumulation pattern of butyltin compounds in dolphin, tuna, and shark collected from Italian coastal waters, *Archives of Environmental Contamination and Toxicology*, 31, 19–23.

Karrer, D., Baroncelli, S., and Turillazzi, P.G. (1995) Oral bis(tri-*n*-butyltin) oxide in pregnant mice. II. Alterations in hematological parameters, *Journal of Toxicology and Environmental Health*, 46, 369–77.

Kenaga, E.E. (1965) Triphenyl tin compounds as insect reproduction inhibitors, *Journal of Economic Entomology*, 58, 4–8,

Kime, D.E., Huyskens, G., McAllister, B.G., Ruranguwa, E., Skorkowski, G., and Ollevia, F. (2001) Tributyltin disrupts the vertebrate reproductive system at muliple sites, *Abstracts 11th Annual Meeting of SETAC Europe*, Madrid, May 6–10, 195.

Kimmel, E.C., Fish, R.H., and Casida, J.E. (1977) Bioorganotin chemistry. Metabolism of organotin compounds in microsomal monooxygenase system and in mammals, *Journal of Agricultural and Food Chemistry*, 25, 1–9.

Krowke, R., Bluth, U., and Neubert, D. (1986) In vitro studies on the embryotoxic potential of (bis[tri-*n*-butyltin])oxide in a limb bud organ culture system, *Archives of Toxicology*, 58, 125–9.

Kumasaka, K., Miyazawa, M., Fujimaki, T., Tao, H., Ramaswamy, B.R., Nakazawa, H., Makino, T., and Satoh, S. (2002) Toxicity of the tributyltin compound on the testis in premature mice, *Journal of Reproduction and Development*, 48, 591–7.

Lau, M.M. (1991) Tributyltin antifoulings: a threat to the Hong Kong marine environment, *Archives of Environmental Contamination and Toxicology*, 20, 299–304.

Laughlin, R.B., Jr., French, W., and Guard, H.E. (1986) Accumulation of bis(tributyltin) oxide by the marine mussel *Mytilus edulis*, *Environmental Science and Technology*, 20, 884–90.

Lehotzky, K., Szeberenyi, J.M., Gonda, Z., Horkay, F., and Kiss, A. (1982) Effects of prenatal triphenyl-tin exposure on the development of behavior and conditioned learning in rat pups, *Neurobehavioral Toxicology and Teratology*, 4, 247–50.

Maguire, R.J. (1991) Aquatic environmental aspects of non-pesticidal organotin compounds, *Water Pollution Research Journal of Canada*, 26, 243–360.

Manning, C.S., Lytle, T.F., Walker, W.W., and Lytle, J.S. (1999) Life-cycle toxicity of bis(tributyltin) oxide to the sheepshead minnow (*Cyprinodon variegatus*), *Archives of Environmental Contamination and Toxicology*, 37, 258–66.

Miyake, K., Misawa, T., Aikawa, H., Yoshida, T., and Shigeta, S. (1989) The effects of prenatal trimethyltin exposure on development and learning in the rat, *Japanese Journal of Industrial Health*, 31, 363–71 (in Japanese).

Miyake, K., Misawa, T., and Shigeta, S. (1990) Toxicity of bis (tri-*n*-butyltin) oxide on learning and development in the rat, *Nippon Eiseigaku Zasshi*, 45, 926–34 (in Japanese).

Miyake, K., Misawa, T., and Shigeta, S. (1991) The effects of prenatal triphenyltin exposure on learning and development in the rat, *Nippon Eiseigaku Zasshi*, 46, 769–76 (in Japanese).

Newton, D.W. and Hays, R.L. (1968) Histological studies of ovaries in rats treated with hydroxyurea, triphenyltin acetate, and triphenyltin chloride, *Journal of Economic Entomology*, 61, 1668–9.

Nirmala, K., Oshima, Y., Lee, R., Imada, N., Honjo, T., and Kobayashi, K (1999) Transgenerational toxicity of tributyltin and its combined effects with polychlorinated biphenyls on reproductive processes in Japanese medaka (*Oryzias latipes*), *Environmental Toxicology and Chemistry*, 18, 717–21.

Noda, T. (2001) Maternal and fetal toxicity of dimetyltin in rats, *Journal of Health Science*, 47, 544–51.

Noda, T., Morita, S., and Baba, A. (1993) Teratogenic effects of various di-*n*-butyltins with different anions and butyl(3-hydroxybutyl)tin dilaurate in rats, *Toxicology*, 85, 149–60.

Noda, T., Morita, S., and Baba, A. (1994) Enhanced teratogenic activity of di-*n*-butyltin diacetate by carbon tetrachloride pretreatment in rats, *Food and Chemical Toxicology*, 32, 321–7.

Noda, T., Morita, S., Shimizu, M., Yamamoto, T., and Yamada, A. (1988) Safety evaluation of chemicals for use in house-hold products (VIII)—teratological studies on dibutyltin diacetate in rats, *Annual Report of Osaka City Institute of Public Health and Environ Sciences*, 50, 66–75 (in Japanese).

Noda, T., Morita, S., Yamano, T., Shimizu, M., and Yamada, A. (1991a) Effects of triphenyltin acetate on pregnancy in rats by oral administration, *Toxicology Letters*, 56, 207–12.

Noda, T., Morita, S., Yamano, T., Shimizu, M., Nakamura, T., Saitoh, M., and Yamada, A. (1991b) Teratogenicity study of tri-*n*-butyltin acetate in rats by oral administration, *Toxicology Letters*, 55, 109–15.

Noda, T., Yamano, T., Shimizu, M., Saitoh, M., Nakamura, T., Yamada, A., and Morita, S. (1992a) Comparative teratogenicity of di-*n*-butyltin diacetate with *n*-butyltin trichloride in rats, *Archives of Environmental Contamination and Toxicology*, 23, 216–22.

Noda, T., Nakamura, T., Shimizu, M., Yamano, T., and Morita, S. (1992b) Critical gestational day of teratogenesis by di-*n*-butyltin diacetate in rats, *Bulletin of Environmental Contamination and Toxicology*, 49, 715–22.

Noda, T., Yamano, T., and Shimizu, M. (2001) Effects of maternal age on teratogenicity of di-*n*-butyltin diacetate in rats, *Toxicology*, 167, 181–9.

Noland, E.A., Taylor, D.H., and Bull, R.J. (1982) Monomethyl-and trimethyltin compounds induce learning deficiencies in young rats, *Neurobehavioral Toxicology and Teratology*, 4, 539–44.

Oberdörster, E. and McClellan-Green, P. (2002) Mechanisms of imposex induction in the mud snail, *Ilyanassa obsoleta*: TBT as a neurotoxin and aromatase inhibitor, *Marine Environmental Research*, 54, 715–8.

Oehlmann, J., Stroben, E., and Fioroni, P. (1991) The morphlogical expression of imposex in *Nucella lapillus* (Linnaeus) (Gastropoda: Muricidae), *Journal of Molluscan Studies*, 57, 375–90.

Oehlmann, J., Stroben, E., Fioroni, P., and Markert, B. (1996) Tributyltin (TBT) effects on *Ocinebrina aciculata* (Gastropoda: Muricidae): imposex development, sterilization, sex change and population decline, *The Science of the Total Environment*, 188, 205–23.

Ogata, R., Omura, M., Shimasaki, Y., Kubo, K., Oshima, Y., Aou, S., and Inoue, N. (2001) Two-generation reproductive toxicity study of tributyltin chloride in female rats, *Journal of Toxicology and Environmental Health A*, 63, 127–44.

Ohhira, S. and Matsui, H. (1993a) Gas chromatographic determination of inorganic tin in rat urine after a single oral administration of stannous chloride and mono-, di-, and triphenyltin chloride, *Journal of Chromatography*, 622, 173–8.

Ohhira, S. and Matsui, H. (1993b) Metabolism of diphenyltin compound in rat liver after a single oral administration of diphenyltin dichloride, *Journal of Agricultural and Food Chemistry*, 41, 607–9.

Omura, M., Ogata, R., Kubo, K., Shimasaki, Y., Aou, S., Oshima, Y., Tanaka, A., Hirata, M., Makita, Y., and Inoue, N. (2001) Two-generation reproductive toxicity study of tributyltin chloride in male rats, *Toxicological Sciences*, 64, 224–32.

Pate, B.D. and Hays, R.L. (1968) Histological studies of testes in rats treated with certain insect chemosterilants, *Journal of Economic Entomology*, 61, 32–4.

Paule, M.G., Reuhl, K., Chen, J.J., Ali, S.F., and Slikker, W., Jr. (1986) Developmental toxicology of trimethyltin in the rat, *Toxicology and Applied Pharmacology*, 84, 412–7.

Piver, W.T. (1973) Organotin compounds: industrial applications and biological investigation, *Environmental Health Perspectives*, 4, 61–79.

Quevauviller, P.H., Bruchet, A., and Donard, O.F.X. (1991) Leaching of organotin compounds from poly(vinyl chloride) (PVC) material, *Applied Organometallic Chemistry*, 5, 125–9.

Sanderson, J.T., Boerma, J., Lansbergen, G.W.A., and van den Berg, M. (2002) Induction and inhibition of aromatase (CYP19) activity by various classes of pesticides in H295R human adrenocortical carcinoma cells, *Toxicology and Applied Pharmacology*, 182, 44–54.

Sasaki, K., Ishizaka, T., Suzuki, T., and Saito, Y. (1988) Determination of tri-*n*-butyltin and di-*n*-butyltin compounds in fish by gas chromatography with flame photometric detection, *Journal of the Association of Official Analytical Chemists*, 71, 360–3.

Scadding, S.R. (1990) Effects of tributyltin oxide on the skeletal structures of developing and regenerating limbs of the axolotl larvae, *Ambystoma mexicanum*, *Bulletin of Environmental Contamination and Toxicology*, 45, 574–81.

Schardein, J. (2000) Hormones and hormonal antagonists, in *Chemically Induced Birth Defects*, 3rd ed., revised and expanded, Marcel Dekker, New York.

Short, J.W. and Thrower, F.P. (1986) Accumulation of butyltins in muscle tissue of chinook salmon reared in sea pens treated with tri-*n*-butyltin, *Marine Pollution Bulletin*, 17, 542–5.

Smith, B.S. (1981a). Male characteristics on female mud snails caused by antifouling paints, *Journal of Applied Toxicology*, 1, 22–5.

Smith, B.S. (1981b). Tributyltin compounds induce male characteristics on female mud snails *Nassarius obsoletus* = *Ilyanasa obsoleta*, *Journal of Applied Toxicology*, 1, 141–4.

Snoeij, N.J., Penninks, A.H., and Seinen, W. (1987) Biological activity of organotin compounds—an overview, *Environmental Research*, 44, 335–53.

Snow, R.L. and Hays, R.L. (1983) Phasic distribution of seminiferous tubules in rats treated with triphenyltin compounds, *Bulletin of Environmental Contamination and Toxicology*, 31, 658–65.

Spencer, F. and Sing, L.T. (1982) Reproductive responses to rotenone during decidualized pseudogestation and gestation in rats, *Bulletin of Environmental Contamination and Toxicology*, 28, 360–8.

Spooner, N., Gibbs, P.E., Bryan, G.W., and Goad, L.J. (1991) The effect of tributyltin upon steroid titers in the female dogwhelk, *Nucella lapillus*, and the development of imposex, *Marine Environmental Research*, 32, 37–49.

Strmac, M. and Braunbeck, T. (1999) Effects of triphenyltin acetate on survival, hatching success, and liver ultrastructure of early life stages of zebrafish (*Danio rerio*), *Ecotoxicology and Environmental Safety*, 44, 25–39.

Stroben, E., Oehlmann, J., and Bettin, C. (1991) TBT-induced imposex and role of steroids in marine snails, in *Proceedings Tenth World Meeting of the Organotin Environmental Programme Association*, Berlin, September, 68–73.

Suzuki, T., Matsuda, R., and Saito, Y. (1992) Molecular species of tri-*n*-butyltin compounds in marine products, *Journal of Agricultural and Food Chemistry*, 40, 1437–43.

Takahashi, T., Araki, A., Nomura, Y., Koga M., and Arizono, K. (2000) The occurrence of dual-gender imposex in Japanese freshwater crab, *Journal of Health Science*, 46, 376–9.

Toyoda, M., Sakai, H., Kobayashi, Y., Komatsu, M., Hoshino Y., Horie, M., Saeki, M., Hasegawa, Y., Tsuji, M., Kojima, M., Toyomura, K., Kumano, M., and Tanimura, A. (2000) Daily dietary intake of tributyltin, dibutyltin, triphenyltin and diphenyltin compounds according to a total diet study in Japanese population, *Shokuhin Eiseigaku Zasshi*, 41, 280–6 (in Japanese).

Tryphonas, H., Cooke, G.M., Caldwell, D., Bondy, G., Parenteau, M., Hayward, S., and Pulido, O. (2004) Oral (gavage), in utero and postnatal exposure of Sprague-Dawley rats to low doses of tributyltin chloride. Part II: Effects on the immune system, *Food and Chemical Toxicology*, 42, 221–235.

Tsuda, T., Inoue, T., Kojima, M., and Aoki, S. (1995) Daily intakes of tributyltin and triphenyltin comounds from meals, *Journal of AOAC International*, 78, 941–3.

Tsuda, T., Nakanishi, H., Aoki, S., and Takebayashi, J. (1987) Bioconcentration and metabolism of phenyltin chlorides in carp, *Water Research*, 21, 949–53.

Ueno, S., Susa, N., Furukawa, Y., Komatsu, Y., Koyama, S., and Suzuki, T. (1999) Butyltin and phenyltin compounds in some marine fishery products on the Japanese market, *Archives of Environmental Health*, 54, 20–5.

Waldock, M.J. and Thain, J.E. (1983) Shell thickening in Crassostrea gigas: organotin antifouling or sediment induced? *Marine Pollution Bulletin*, 14, 411–5.

Weis, J.S. and Kim, K. (1988) Tributyltin is a teratogen in producing deformities in limbs of the fiddler crab, *Uca pugilator*, *Archives of Environmental Contamination and Toxicology*, 17, 583–7.

Winek, C.L., Marks, M.J., Jr., Shanor, S.P., and Davis, E.R. (1978) Acute and subacute toxicology and safety evaluation of triphenyl tin hydroxide (Vancide KS), *Clinical Toxicology*, 13, 281–96.

Winship, K.A. (1988) Toxicity of tin and its compounds, *Adverse Drug Reactions and Acute Poisoning Reviews*, 7, 19–38.

World Health Organization (1980) Tin and Organotin Compounds: A Preliminary Review. *Environmental Health Criteria 15*, World Health Organization, Geneva.

World Health Organization (1992) Fentin, in *Pesticide Residues in Food 1991: Evaluations Part II Toxicology*, World Health Organization, Geneva. Online: http://www.inchem.org/documents/jmpr/jmpmono/v91pr11.htm (accessed 16 June 2004).

Yamabe, Y., Hoshino, A., Imura, N., Suzuki, T., and Himeno, S. (2000) Enhancement of androgen-dependent transcription and cell proliferation by tributyltin and triphenyltin in human prostate cancer cells, *Toxicology and Applied Pharmacology*, 169, 177–84.

Yonemoto, J., Shiraishi, H., and Soma, Y. (1993) In vitro assessment of teratogenic potential of organotin compounds using rat embryo limb bud cell cultures, *Toxicology Letters*, 66, 183–91.

Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Mutation Research 588 (2005) 129–135
Genetic Toxicology and Environmental Mutagenesis
www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

In silico assessment of chemical mutagenesis in comparison with results of Salmonella microsome assay on 909 chemicals

Makoto Hayashi [a,*], Eiichi Kamata [b], Akihiko Hirose [b], Mika Takahashi [b], Takeshi Morita [c], Makoto Ema [b]

[a] *Division of Genetics and Mutagenesis, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[b] *Division of Risk Assessment, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[c] *Division of Safety Information on Drug, Food and Chemicals, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*

Received 12 June 2005; received in revised form 20 September 2005; accepted 26 September 2005
Available online 28 October 2005

Abstract

Genotoxicity is one of the important endpoints for risk assessment of environmental chemicals. Many short-term assays to evaluate genotoxicity have been developed and some of them are being used routinely. Although these assays can generally be completed within a short period, their throughput is not sufficient to assess the huge number of chemicals, which exist in our living environment without information on their safety. We have evaluated three commercially available in silico systems, i.e., DEREK, MultiCASE, and ADMEWorks, to assess chemical genotoxicity. We applied these systems to the 703 chemicals that had been evaluated by the Salmonella/microsome assay from CGX database published by Kirkland et al. [1]. We also applied these systems to the 206 existing chemicals in Japan that were recently evaluated using the Salmonella/microsome assay under GLP compliance (ECJ database). Sensitivity (the proportion of the positive in Salmonella/microsome assay correctly identified by the in silico system), specificity (the proportion of the negative in Salmonella/microsome assay correctly identified) and concordance (the proportion of correct identifications of the positive and the negative in Salmonella/microsome assay) were increased when we combined the three in silico systems to make a final decision in mutagenicity, and accordingly we concluded that in silico evaluation could be optimized by combining the evaluations from different systems. We also investigated whether there was any correlation between the Salmonella/microsome assay result and the molecular weight of the chemicals: high molecular weight (>3000) chemicals tended to give negative results. We propose a decision tree to assess chemical genotoxicity using a combination of the three in silico systems after pre-selection according to their molecular weight.

Keywords: In silico; (Quantitative) structure-activity relationship; (Q)SAR; Chemical genotoxicity; Decision tree

1. Introduction

It is said that more than 20,000 chemicals are in use in Japan. Among them, only approximately 10% are thought to have been assessed for human hazard based on data from in vitro and in vivo bioassays. According to the "Law Concerning the Evaluation of Chemical Substances and Regulation of Their Manufacture, etc." [2], the Salmonella/microsome (Ames) assay, in vitro chromosomal aberration assay (or alternatively mouse lymphoma TK assay), and 28-day repeat dose toxicity test in rodents are obligatory to notify new chemicals for production/import at a level of more than 10 t per year.

* Corresponding author.

1383-5718/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.mrgentox.2005.09.009

To screen the remaining 18,000 chemicals for human hazard by application of this three-test battery is not realistic from the time and economical point of view. We need a much higher-throughput system to assess these chemicals, at least for prioritization of those chemicals that should be submitted to biological testing. To assess human hazard for regulatory purposes, in silico systems are now beginning to be used [3]. Here, we evaluated three commercially available in silico (quantitative) structure-activity relationship ((Q)SAR) systems and tried to construct a decision tree for prioritization of which chemicals need in vitro and/or in vivo testing. Also, within the drug discovery process, integrated computational analysis has been proposed to be incorporated as a toxicity prediction tool [4].

Kirkland et al. [1] published a database (CGX database, see http://www.lhasalimited.org/cgx) for nearly 1000 carcinogens and non-carcinogens with results of representative in vitro genotoxicity assays, i.e., Salmonella/microsome assay (Ames), mouse lymphoma TK assay using L5178Y cells (MLA), and in vitro chromosomal aberration assay or in vitro micronucleus assay (CA/MN). We used 703 chemicals that had been assessed in the Ames assay for evaluation of the three in silico systems, i.e., DEREK, MultiCASE (MCase), and ADMEWorks (AWorks). We also used a database (the ECJ database) that we constructed from chemicals existing in Japan that had recently been assessed in the Ames assay, in vitro chromosomal aberration assay, and 28 day repeat dose rodent toxicity test and/or reproductive and developmental toxicity test for their safety evaluation under GLP compliance. The ECJ database consisted of 206 chemicals but only 26 chemicals were positive by the Ames assay. Initially we evaluated both sensitivity and specificity of these three systems using the ECJ database of 206 chemicals [5].

We selected these three in silico systems because of their different modes of analysis. DEREK is a rule-based system [6], MCase [7] is a database/substructure based system, and AWorks is a QSAR. We applied these systems individually to assess gene-mutation induction on the 703 and 206 chemical sets described above and evaluated their sensitivity, specificity, concordance, and applicability (how many chemicals could be assessed), independently.

It is known that high molecular weight polymers tend not to induce gene mutation and chromosomal aberrations mainly because they cannot enter the target cells to react with DNA, or other bio-molecules necessary for genetic stability. We analyzed 194 Ames positive chemicals (confidential source) for the effect of molecular weight.

2. Materials and methods

2.1. Data sources for chemicals assessed

Of about 1000 chemicals, 703 that had been assessed in the Ames test were chosen from the CGX database published by Kirkland et al. [1]. All chemical structures were re-drawn using Chemdraw Ultra (Cambridge Soft Corporation, USA) and converted to MOL files before application to each system. We also used the database of 206 chemicals evaluated in the MHLW project "Safety Examination of Existing Chemicals and Safety Programmes in Japan" (ECJ database). The test summary for each of these chemicals can be seen at http://wwwdb.mhlw.go.jp/ginc/html/db1.html. In addition, we collected 194 Ames positive chemicals from a confidential source and investigated the relationship between gene mutation induction and molecular weight, with identification of any active side chain that might have contributed to the positive result in the Ames assay.

2.2. In silico systems used and definition of positive and negative responses

We used DEREK (Lhasa Ltd., UK) version 8.0.1. When the system gave an evaluation as "certain", "probable" or "plausible" we considered this as "positive", and when the system gave "equivocal", "doubted", "improbable", "impossible", or "no alert" we considered this as "negative". We used MCase (Multicase Co. Ltd.) version mc4pc. When the system gave "active" or "marginal" we considered this as "positive", and when the system gave "Inactive" we considered this as "negative". In the case of AWorks (Fujitsu Kitakyushu, Co. Ltd., version 2.0), we considered as "positive" when system evaluation was "positive", and considered as "negative" when the system evaluation was "negative". We excluded chemicals from further analysis when DEREK or AWorks gave no answer, or the evaluation was "inconclusive" by MCase.

2.3. Definition of sensitivity, specificity, concordance, and applicability

We calculated sensitivity, specificity, concordance, and applicability as follows:

$$\text{sensitivity} = \frac{N_{A+S+}}{N_{A+}} \times 100, \qquad \text{specificity} = \frac{N_{A-S-}}{N_{A-}} \times 100,$$

$$\text{concordance} = \frac{N_{A+S+} + N_{A-S-}}{N_{\text{eval}}} \times 100,$$

$$\text{applicability} = \frac{N_{\text{eval}}}{N_{\text{all}}} \times 100$$

where N_{A+} is number of chemicals revealing positive in Ames assay; N_{A-} is number of chemicals negative in Ames assay; N_{A+S+} is number of chemicals revealing positive by both Ames assay and in silico evaluation; N_{A-S-} is number of chemicals negative in both Ames assay and in silico evaluation; N_{eval} is

Table 1
Performance of in silico systems

	Ames result	+	−	Total	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)
CGX database								
DEREK	+	288	64	352				
	−	69	267	336	81.8	79.5	80.7	97.9
	Total	357	331	688				
MCase	+	235	32	267				
	−	6	249	255	88.0	97.6	92.7	74.3
	Total	241	281	522				
AWorks	+	267	89	356				
	−	149	187	336	75.0	55.7	65.6	98.4
	Total	416	276	692				
ECJ database								
DEREK	+	19	7	26				
	−	21	159	180	73.1	88.3	86.4	100.0
	Total	40	166	206				
MCase	+	13	7	20				
	−	13	133	146	65.0	91.1	88.0	80.6
	Total	26	140	166				
AWorks	+	19	7	26				
	−	54	124	178	73.1	69.7	70.1	99.0
	Total	73	131	204				

MCase: MultiCASE; AWorks: ADMEWorks.

number of chemicals evaluated; and N_{all} is total number of chemicals subjected.

3. Results

Among the set of 703 CGX chemicals with published Ames data, 358 were positive and 345 were negative. The results of the in silico evaluation are summarized in Table 1. The highest sensitivity, specificity, and concordance with Ames assay results was provided by MCase, then followed by DEREK. However, the systems that showed the best applicability were AWorks and (almost the same) DEREK, then followed by MCase. For the database of 206 ECJ chemicals, 26 were positive and 180 were negative. The outcomes of the in silico analyses are summarized in Table 1. The pattern of performance was very similar to that with the 703 chemicals in the CGX database.

Fig. 1 shows the cumulative percent of Ames positive chemicals against molecular weight. It can be seen that 87.1% of those positive chemicals had molecular weights less than 1000, and 96.4% had molecular weights less than 3000; in other words, only 3.6% of the chemicals with a molecular weight >3000 gave a positive response in the Ames assay. Seven of 194 Ames positive chemicals had a molecular weight >3000 and four of these seven polymers had epoxy groups.

When we combined the in silico systems, the performance was different from that when assessed individually (Table 2). If we considered the in silico mutagenicity as positive (or negative) when two or more systems gave positive (or negative) evaluations, 87.8

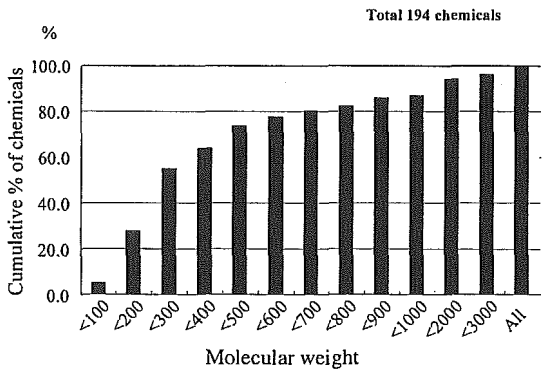

Fig. 1. Cumulative percentage of chemicals based on their molecular weight. 194 Ames positive chemicals were analyzed. 7/194 chemicals were more than 3000 molecular weight and Ames positive and 4/7 contained epoxy groups.

Table 2
Performance of in silico systems after combined

CGX database

In silico / Ames	++ or +++	-- or ---	Total	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)
+	279	40	319				
-	42	249	291	**87.8**	**85.6**	**86.7**	**86.8**
Total	321	289	610				
	+++	---					
+	166	1	167				
-	3	127	130	**99.4**	**97.7**	**98.7**	**42.2**
Total	168	129	297				

ECJ database

In silico / Ames	++ or +++	-- or ---	Total	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)
+	19	7	26				
-	23	147	170	**73.1**	**86.5**	**84.7**	**95.1**
Total	42	154	196				
	+++	---					
+	13	2	15				
-	5	94	99	**86.7**	**94.9**	**93.9**	**55.3**
Total	18	96	114				

Table 3
Performances of DEREK and MCase in several published papers.

Target compounds	In silico system	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)	Reference
394 Drugs	DEREK	52	75	74	94[a]	[11]
	MCase	48	93	90	91[a]	
217 Non-drugs	DEREK	86	50	81	100[a]	[10]
	MCase	91	62	83	100[a]	
520 Drug candidates	DEREK	28	80	72	100	[13]
	MCase	50	86	81	41	
	DEREK + MCase	29	95	88	29	
	DEREK + Mcase + TOPKAT	75	96	95	15	
123 Drug candidates	DEREK	8[b]	31[c]	61	100[d]	[4]
	MCase (A2H)	13[b]	15[c]	72	97[d]	
	Topcat (Ames Mut)	18[b]	15[c]	67	98[d]	
	DEREK + MCase	6[b]	19[c]	75	97[d]	
	DEREK + Mcase + TOPKAT	5[b]	9[c]	86	46[d]	
94 Non-drugs	DEREK	63	81	76	100	[13]
	MCase	40	90	76	75	
	DEREK + MCase	47	100	85	56	
	DEREK + Mcase + TOPKAT	55	100	86	37	
516 Non-drugs	DEREK	6[b]	24[c]	70	100[d]	[4]
	MCase (A2H)	7[b]	12[c]	81	98[d]	
	Topcat (Ames Mut)	25[b]	19[c]	56	97[d]	
	DEREK + MCase	2[b]	16[c]	82	98[d]	
	DEREK + Mcase + TOPKAT	7[b]	10[c]	83	43[d]	

[a] Calculated by us
[b] % False negative.
[c] % False positive.
[d] (1-Indeterminate).

and 73.1% sensitivity, 85.6 and 86.5% specificity, 86.7 and 84.7% concordance, and 86.8 and 95.1% applicability were obtained for the CGX and ECJ databases, respectively. If we considered the in silico mutagenicity as positive (or negative) only when all three systems gave positive (or negative) evaluations, all performance measures (sensitivity, specificity, etc.) increased up to 98.7 and 93.9%. However, applicability decreased to 42.2 and 55.3%, which meant only about half of the chemicals in the CGX and ECJ databases could be evaluated. One chemical, *o*-phenylphenol [90-43-7], was positive in the Ames test but negative by all three in silico systems and three chemicals, carboxymethylnitrosourea [60391-92-6], methidathion [950-37-8], 1-nitroso-3,5-dimethyl-4-benzoylpiperazine [61034-40-0], were negative in the Ames test although all three in silico system gave positive evaluation for mutagenicity in the CGX database. When we used the ECJ database, 2-amino-1-naphthalenesulfonic acid [81-16-3] and 2-vinylpyridine [100-69-6] were positive in the Ames test but negative by all three in silico systems and there was no chemical that was negative in the Ames assay and all positive in in silico system. These exceptional chemicals are listed in Table 3 together with such chemicals taken from literatures.

4. Discussion

It is important to construct a strategy for efficient evaluation of the toxicity of a large number of existing chemicals. Even so-called short-term assays, e.g., Ames assay and in vitro chromosomal aberration assay, can practically assess only 100 chemicals per year according to our experiences in Japan. In this case, it will take 180 years to assess the outstanding 18,000 existing chemicals for genotoxicity, and it will take even longer when repeat dose toxicity tests are also performed, as these are not short-term assays. We therefore need higher-throughput systems to assess chemical safety, or at least to set priorities for those chemicals that should be tested in in vitro and/or in vivo tests. In silico systems have the capability for high throughput but have not yet been well validated for assessment of human hazard, although some regulatory bodies have started to use these methods.

Correlation between the Ames assay result and molecular weight could be explained by the lack of membrane permeability of high molecular weight chemicals, making it more difficult for them to reach target molecules such as DNA and proteins that contribute to the fidelity of cell division. Therefore, only a few chemicals with molecular weight >3000 gave positive responses in the Ames assay. This phenomenon is also true for induction of chromosomal aberrations in vitro (data not shown). The other important issue is the contribution of epoxy group in the polymer. Although of molecular weight >3000, some polymers with an epoxy group gave positive results in both the Ames and chromosomal aberration assays. Epoxy embedding reagents employed in electron microscopy (e.g., epon and araldite) have been reported as mutagenic in the Ames assay [8]. According to these findings, we should include a step to evaluate molecular weight and existence of any epoxy groups in the molecule.

In the present study, we used the CGX database recently published by Kirkland et al. [1] for microbial mutagenicity data on 358 carcinogens and 345 non-carcinogens for validation of three commercially available in silico (Q)SAR systems. When applied individually, MCase gave high sensitivity, specificity, and concordance compared to other two systems. One of the reasons may be because the CGX database contained many results from the U.S. National Toxicology Program (NTP), and the learning dataset of MCase would have used many of the same results. Therefore, some of them were evaluated by direct matching. Moreover, the applicability of MCase was relatively low compared with the other systems in this study (Table 1). MCase judged 119 chemicals as inconclusive and one chemical as marginal, and could not evaluate 67 chemicals. Such selectivity in MCase may contribute to the high concordance. On the other hand, the other systems were not influenced directly by the NTP data. We applied the in silico systems to another dataset, the ECJ database, that does not contain the NTP data and we obtained similar patterns of sensitivity, specificity, etc.

Each in silico system showed different outcomes on some chemicals complimentary by some extent. These different evaluation patterns were mainly due to the different evaluation rules. The DEREK is a rule-based system, AWorks is a discriminant-based system mainly depending on physicochemical descriptors, and MCase is a hybrid system based on a database. Therefore, we concluded that in silico evaluation could be optimized by combining the evaluations from the three systems. Sensitivity, specificity and concordance were increased when we combined the three in silico systems to make a final conclusion of mutagenicity (Table 1). Concordance was much higher after combining but the applicability became poor (42.2%). When two of the in silico systems gave the same evaluations, the applicability (86.8%) was good but the concordance was lower (86.7%) than when all three were combined (98.7).

Recently, several in silico studies for prediction of mutagenicity have been conducted on drugs or non-

Table 4
Exceptional chemicals that showed Ames test gave positive but all three in silico systems (DEREK, MCase, TOPKAT/AWorks) gave negative and Ames test gave negative but all three systems gave positive

Compound	CAS	Ames test	DEREK	MCase	TOPKAT/Aworks	Source[a]
Bupropion	34911-55-2	+	–	–	–	1
Citalopram	59729-33-8	+	–	–	–	1
Naloxone	465-65-6	+	–	–	–	1
Oxcarbazepime	28721-07-5	+	–	–	–	1
Quetiapine	111976-69-7	+	–	–	–	1
Rabeprazole	117976-89-3	+	–	–	–	1
Zolmitriptan	139264-17-8	+	–	–	–	1
2-(2-Methylpropyl) thiazole	18640-74-9	+	–	–	–	2
2-Chloropyridine	109-09-1	+	–	–	–	2
Pyrogallol	87-66-1	+	–	–	–	2
o-Phenylphenol	90-43-7	+	–	–	–	3
2-Amino-1-naphthalenesulfonic acid	81-16-3	+	–	–	–	3
2-Vinylpyridine	100-69-6	+	–	–	–	3
Fosfomycin	23155-02-4	–	+	+	+	1
Toremifene	89778-26-7	–	+	+	+	1
Poly (2-hydroxypropyl methacrylate)	25703-79-1	–	+	+	+	2
Carboxymethylnitrosourea	60391-92-6	–	+	+	+	3
Methidathion	950-37-8	–	+	+	+	3
1-Nitroso-3,5-dimethyl-4-benzoylpiperazine	–	+	+	+	3	

[a] 1: Synder et al. [11] (with TOPKAT), 2: White et al. [13] (with TOPKAT), 3: this study (with AWorks).

drug chemicals with commercially available programs, e.g., DEREK, MCase or TOPKAT, or newly developed computational approaches [4,9–12]. The performances of DEREK and MCase in several of these studies are summarized in Table 4. Generally, similar performance in sensitivity, specificity, concordance, and applicability were shown between DEREK and MCase but with some exceptions, e.g., sensitivity in 520 drug candidates [13], specificity in 516 non-drugs [4], and applicability in 520 pharmaceutical drug candidates and 94 non-drugs [13]. These differences might be due to the chemical class of target compounds in each database. However, there was no remarkable difference in performance whether the chemical was intended for use as a pharmaceutical, agricultural, or industrial agent. Our results on performance of in silico systems showed similarity with the published analyses. With respect to the combination of in silico prediction systems, White et al. [13] reported that combination improved the overall accuracy and specificity, but sensitivity was barely above the 50% level (Table 4). On the other hand, their analysis showed quite low applicability in the combination of three prediction systems, DEREK, MCase and TOPKAT. Our analysis of the combination of DEREK, MCase and AWorks showed good improvements in sensitivity, specificity and concordance, but applicability was low, especially in the 3-system combination.

Exceptional chemicals that gave positive Ames results but were negative in all three in silico systems (DEREK, MCase, TOPKAT/AWorks), and those that were negative in the Ames test but gave positive evaluations in all three systems, are summarized in Table 4. This table, which includes data from Synder et al. [11] and White et al. [13] shows there are 19 exceptional chemicals from both drug and non-drug families. Although it would be unrealistic to expect zero exceptions using this approach, further improvement of the prediction systems is needed. We do not have good reasons to explain the discordance, therefore we will verify the results from both sides, i.e., in silico system and Ames test.

Considering these outcomes, we propose a decision tree (Fig. 2), in order to evaluate chemical induction of gene mutation. We may use the decision tree to prioritize chemicals to be assayed by in vitro and/or in vivo tests. A final goal being that eventually, chemical mutagenicity will be evaluated by in silico systems alone for regulatory use. The decision tree consists of three steps; namely to assess the molecular weight, the existence of epoxy groups, and the in silico evaluation for genotoxicity. Based on the purpose of the in silico evaluation, the tree might be altered by the different final call of the in silico evaluation, i.e., regarding as positive (negative) all three systems show positive (negative). The choice of definition for final call applying to the decision tree should be based on the balance between accuracy of eval-

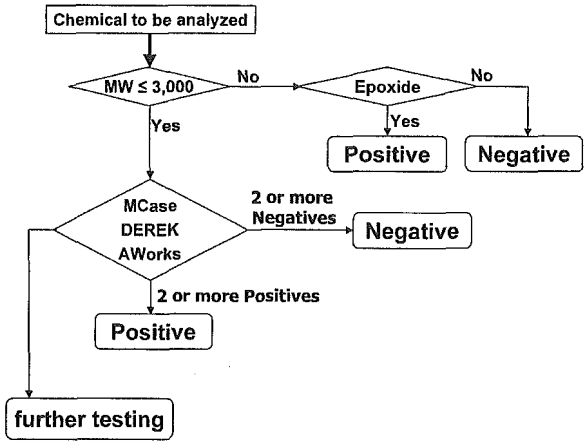

Fig. 2. Decision tree. In in silico evaluation, when two or more give positive then the final call is "positive" and two or more negative then call "negative".

uation and applicability, which are especially important for regulatory purpose. The decision should be made on a case-by-case basis depending upon the purpose of the decisions to be made.

Acknowledgements

The authors thank Dr. D. Kirkland, Covance, for his critical review and kind English edition and Mr. T. Ehara, MHLW, for his invaluable discussion. Authors also want to thank Ms. H. Akiyama (CTC laboratories, Japan), Akamatsu and Naitoh (Charles River Japan, Japan), and Kitajima, Suiroi, and Yuta (Fujitsu Kitakyushu, Japan) for their technical assistance. This work supported by the Health and Labour Sciences Research Grants (H15-Chemistry-003).

References

[1] D. Kirkland, M. Aardema, L. Henderson, L. Müller, Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens. I. Sensitivity, specificity and relative predictivity, Mutat. Res. 584 (2005) 1–256.

[2] Law Concerning the Evaluation of Chemical Substances and Regulation of Their Manufacture, etc., Law No. 117, 16 October 1973 as last amended by Law No.49, 28 May 2003.

[3] M.T.D. Cronin, J.S. Jaworska, J.D. Walker, M.H.I. Comber, C.D. Watts, A.P. Worth, Use of QSARs in international decision-making frameworks to predict health effects of chemical substances, Environ. Health Perspect. 111 (2003) 1391–1401.

[4] G.M. Pearl, S. Livingstone-Carr, S.K. Durham, Integration of computational analysis as sentinel tool in toxicological assessments, Curr. Topoics Med. Chem. 1 (2001) 247–255.

[5] A. Hirose, M. Takahashi, M. Kamata, M. Ema, M. Hayashi, Development of genotoxicity predicting QSAR system for registered and existing industrial chemicals in Japan, Toxicol. Appl. Pharmacol. 197 (2004) 358.

[6] N. Greene, P.N. Judson, J.J. Langowski, C.A. Marchant, Knowledge-based expert systems for toxicity and metabolism prediction: DEREK, StAR and METEOR, SAR QSAR Environ. Res. 10 (1999) 299–314.

[7] H.S. Rosenkranz, A.R. Cunningham, Y.P. Zhang, H.G. Claycamp, O.T. Macina, N.B. Sussman, S.G. Grant, G. Klopman, Development, characterization and application of predictive-toxicology models, SAR QSAR Environ. Res. 10 (1999) 277–298.

[8] M.P. Murray, J.E. Cummins, Mutagenic activity of epoxy embedding reagents employed in electron microscopy, Environ. Mutagen. 1 (1979) 307–313.

[9] N.F. Cariello, J.D. Wilson, B.H. Britt, D.J. Wedd, B. Burlinson, V. Gombar, Comparison of the computer programs DEREK and TOPKAT to predict bacterial mutagenicity. Deductive estimate of risk from existing knowledge. Toxicity prediction by computer assisted technology, Mutagenesis 17 (4) (2002) 321–329.

[10] J.R. Votano, M. Parham, L.H. Hall, L.B. Kier, S. Oloff, A. Tropsha, Q. Xie, W. Tong, Three new consensus QSAR models for the prediction of Ames genotoxicity, Mutagenesis 19 (5) (2004) 365–377.

[11] R.D. Snyder, D.E. Ewing, L.B. Hendry, Evaluation of DNA intercalation potential of pharmaceuticals and other chemicals by cell-based and three-dimensional computational approaches, Environ. Mol. Mutagen. 44 (2) (2004) 163–173.

[12] R.D. Snyder, G.S. Pearl, G. Mandakas, W.N. Choy, F. Goodsaid, I.Y. Rosenblum, Assessment of the sensitivity of the computational programs DEREK, TOPKAT, and MCASE in the prediction of the genotoxicity of pharmaceutical molecules, Environ. Mol. Mutagen. 43 (3) (2004) 143–158.

[13] A.C. White, R.A. Mueller, R.H. Gallavan, S. Aaron, A.G. Wilson, A multiple in silico program approach for the prediction of mutagenicity from chemical structure, Mutat. Res. 539 (1–2) (2003) 77–89.

ORIGINAL ARTICLE

Elevated susceptibility of newborn as compared with young rats to 2-*tert*-butylphenol and 2,4-di-*tert*-butylphenol toxicity

Mutsuko Hirata-Koizumi[1], Masao Hamamura[2], Hiromi Furukawa[2], Naemi Fukuda[3], Yoshihiko Ito[3], Yumi Wako[4], Kotaro Yamashita[4], Mika Takahashi[1], Eiichi Kamata[1], Makoto Ema[1], and Ryuichi Hasegawa[1]

[1]National Institute of Health Sciences, Setagaya-ku, Tokyo; [2]Panapharm Laboratories, Uto-shi, Kumamoto; [3]Research Institute for Animal Science in Biochemistry and Toxicology, Sagamihara-shi, Kanagawa; and [4]Kashima Laboratory, Mitsubishi Chemical Safety Institute Ltd, Ibaraki, Japan

ABSTRACT **In order to determine the susceptibility of newborn rats to 2-*tert*-butylphenol (2TBP) and 2,4-di-*tert*-butylphenol (DTBP) toxicity, studies were conducted with oral administration from postnatal days (PND) 4 to 21 and the findings were compared with results for young rats exposed from 5 or 6 weeks of age for 28 days. In the newborn rats, specific effects on physical and sexual development and reflex ontogeny were not observed. While there were no clear differences in toxicological profiles between newborn and young rats, the no-observed-adverse-effect levels (NOAELs) differed markedly. For 2TBP, clinical signs such as ataxic gait, decrease in locomotor activity and effects on liver, such as increase in organ weight, were observed and the NOAELs were concluded to be 20 and 100 mg/kg/day in newborn and young rats, respectively. Based on hepatic and renal toxicity (histopathological changes and increase in organ weight with blood biochemical changes), the respective NOAELs for DTBP were concluded to be 5 and 20 mg/kg/day. Therefore, the susceptibility of newborn rats to 2TBP and DTBP was found to be 4–5 times higher than that of young rats.**

Key Words: 2, 4-di-*tert*-butylphenol, 2-*tert*-butylphenol, susceptibility of newborn rats

INTRODUCTION

Protection of humans against disease and injury caused by chemicals in the environment is the ultimate goal of risk assessment and risk management (Landrigan *et al.* 2004). However, the focus has long been solely on adult exposure and toxicity and the fetus via maternal transfer, with little consideration given to early childhood. In the past decade, stimulated especially by the 1993 US National Research Council (NRC) report *Pesticides in the Diets of Infants and Children* (NAS 1993), recognition that special consideration is required for children in risk assessment has grown. The NRC report noted that 'children are not little adults', because of their unique patterns of exposures to environmental hazards and their particular vulnerability.

For the susceptibility of children to environmental chemicals, the early postnatal period (the suckling period) is of particular note. During this period, the infant could be exposed to various chemicals not only through mothers' milk, but also directly, by having chemical-contaminated baby food, mouthing toys or household materials, and so on; however, current risk assessment gives no consideration to toxic effects resulting from direct exposure to chemicals. An approach that adequately takes into account the susceptibility of infancy is urgently required. However, because there is no standard testing protocol intended for direct exposure of preweaning animals (newborn animals) to chemicals, and toxicity studies using newborn animals are complicated by practical difficulties regarding grouping, direct dosing, and general and functional observation, there is only limited information on susceptibility of the newborn at the present.

We therefore have established a new protocol for repeated dose toxicity studies using newborn rats (newborn rat studies) (Koizumi *et al.* 2001) for systematic application. Results have been compared with those of 28-day repeated dose toxicity studies using young rats (young rat studies) to provide a basis of analyzing susceptibility. Since young rat studies are routinely conducted as one of a battery of minimum toxicity tests and data are stored for many chemicals, comparative analyzes should provide important information for considering effects of direct exposure to chemicals during the suckling period.

We have already reported analytical results for eight chemicals (4-nitrophenol, 2,4-dinitrophenol, 3-aminophenol, 3-methylphenol, 1,3-dibromopropane, 1,1,2,2-tetrabromoethane, 2,4,6-trinitrophenol, and tetrabromobisphenol A) (Koizumi *et al.* 2001, 2002, 2003; Fukuda *et al.* 2004; Takahashi *et al.* 2004; Hirata-Koizumi *et al.* 2005). The susceptibility of newborn rats to the toxicity of the first four agents was four times higher than that of their young counterparts at a maximum. For 1,3-dibromopropane and 1,1,2,2-tetrabromoethane, while the doses causing clear toxicity were lower in newborn rats, doses at which toxic signs began to appear were paradoxically higher in the newborn case. These six chemicals had no impact on development in the newborn period and showed similar toxicity profiles in both age groups. For the other two chemicals, there were marked differences in toxicity profile between the newborn and young rats. Especially, in the case of tetrabromobisphenol A, a specific rather than enhanced renal toxicity was observed in newborn case.

In the present investigation, two *tert*-butylphenols, 2-*tert*-butylphenol (2TBP), and 2,4-di-*tert*-butylphenol (DTBP), were chosen for comparative toxicity analysis. 2TBP has been used in the production of agricultural chemicals, aroma chemicals, and resins (New Chemical Index 2001), and DTBP in the production of antioxidants and ultraviolet absorbers (Chemical Products' Handbook 2004). For either chemical, there is no available toxicity information on human. Regarding toxicity to experimental animals, results from young rat studies of both chemicals are available in

Correspondence: Mutsuko Hirata-Koizumi, BSc, Division of Medicinal Safety Science, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Email: mkoizumi@nihs.go.jp

Received June 17, 2005; revised and accepted September 2, 2005.

Toxicity Testing Reports of Environmental Chemicals of the Japanese government (MHLW 2001a, 2001b), but no other data have been reported regarding repeated dose toxicity. Since the young rats were only evaluated for toxicity profiles and no-observed-effect levels, we re-evaluated the results for a more practical evaluation index, the no-observed-adverse-effect level (NOAEL), which could serve as the basis for determining tolerable daily intake (TDI) or acceptable daily intake (ADI) for risk assessment, and conducted comparative analyzes with newborn rats.

MATERIALS

2-*tert*-Butylphenol (2TBP, CAS no. 88-18-6, purity: 99.97%) and 2,4-di-*tert*-butylphenol (DTBP, CAS no. 96-76-4, purity: 99.67%), obtained from Dainippon Ink and Chemicals, Incorporated (Tokyo, Japan), were dissolved in olive oil and corn oil, respectively. The test solutions were prepared once a week as stability for eight days had been confirmed. All other reagents used in this study were specific purity grade.

METHODS

All studies were performed under Good Laboratory Practice conditions and in accordance with 'Guidance for Animal Care and Use' of Panapharm Laboratories Co., Ltd, Research Institute for Animal Science in Biochemistry and Toxicology, or Mitsubishi Chemical Safety Institute Ltd.

Animals

In the newborn rat studies of 2TBP and DTBP, pregnant SPF Sprague-Dawley rats [Crj:CD(SD)IGS] were purchased at gestation days 13–15 from Charles River Japan Inc. (Yokohama, Japan), and allowed to deliver spontaneously. All newborn were separated from dams at postnatal day (PND) 3 (the date of birth was defined as PND 0), and pooled according to sex. At the same time, 12 foster mothers were selected among dams, based on the nursing condition. Each foster mother suckled four male and four female newborn, assigned to each of the four dose groups, including the controls, up to weaning on PND 21 (termination of dosing). After weaning, the animals of the recovery-maintenance group (see Study Design) were individually maintained for nine weeks.

In the young rat studies, 4–5 week-old males and females of the same strain were obtained from the same supplier as for the newborn rat studies, and used at ages of 5–6 weeks after acclimation.

All animals were maintained in an environmentally controlled room at 20–26°C with a relative humidity of 40–70%, a ventilation rate of more than ten times per hour, and a 12:12 h light/dark cycle. They were allowed free access to a basal diet (MF: Oriental Yeast Co. Ltd, Tokyo, Japan, or LABO MR Stock: Nihon Nosan Kogyo Inc., Yokohama, Japan) and water (sterile tap water or well water treated with sodium hypochlorite) throughout.

Study design

1. 18-day repeated dose toxicity study in newborn rats (newborn rat study)

Newborn rats (12/sex/dose) were administered the test substances by gastric intubation on PNDs 4–21. On PND 22, six males and six females in each treated group were sacrificed for autopsy (the scheduled-sacrifice group). The remaining animals in all groups (6 rats/sex/dose) were maintained for nine weeks without chemical treatment and then sacrificed at 12 weeks of age (the recovery-maintenance group).

Based on the results of dose-finding studies conducted prior to the main study, the dose, which would show clear toxicity, was selected as the top dose, that without potentially toxic effects as the lowest dose, and the medium dose was set between them. In the dose-finding study for 2TBP (oral administration from PNDs 4–21), some clinical signs and suppressed body weight gain were observed at 200 mg/kg and an increase in relative liver weight at 60 mg/kg and more. For DBTP (oral administration from PNDs 4–17), all of the four males and four females died at 500 mg/kg, and the death of one of the four males, an increase in serum total cholesterol and phospholipid, and increase in relative liver weight were noted in the 100 mg/kg group. Therefore, the doses were set at 0, 20, 60, or 200 mg/kg/day for 2TBP and at 0, 5, 40, or 300 mg/kg/day for DTBP.

During the study, the rats' general condition was observed at least once a day (details of clinical signs noted in this study are described in 'Glossary of terms for toxicity testing' [NIHS 1994]). Body weight and food consumption (only the recovery-maintenance period) was examined once or more a week. As developmental parameters, fur appearance, incisor eruption, pinna detachment and eye opening were assessed for physical development, and testes descent or preputial separation and vaginal opening for sexual development (OECD 2004). In addition, reflex ontogeny, such as visual placing reflex, and surface and mid-air righting reflexes, were also examined (Adams 1986; Jensh & Brent 1988). Urinalysis (color, occult blood, pH, protein, glucose, ketone bodies, bilirubin, urobilinogen, sediment, specific gravity, and volume of the urine) was conducted in the last week of the recovery-maintenance period.

At PNDs 22 and 85, blood was collected from the abdominal aorta under ether anesthesia (for 2TBP) or from the postcaval vein under pentobarbital sodium anesthesia (for DTBP) after overnight starvation for the scheduled-sacrifice and recovery-maintenance groups, respectively. One portion was treated with EDTA-2K and examined for hematological parameters, such as the red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count, platelet count, reticulocyte count and differential leukocyte count. In the recovery-maintenance group, part of the blood was treated with 3.8% sodium citrate, and blood clotting parameters such as prothrombin time (PT) and activated partial thromboplastin time (APTT) were examined. Serum from the remaining portions of blood for both the scheduled-sacrifice and recovery-maintenance groups were analyzed for blood biochemistry (total protein, albumin, albumin-globulin ratio [A/G ratio], glucose, total cholesterol, triglycerides, phospholipid, total bilirubin, urea nitrogen [BUN], creatinine, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, alkaline phosphatase, γ-glutamyl transpeptidase [γ-GTP], calcium, inorganic phosphorus, sodium, potassium, and chlorine). Following collection of blood, all animals were sacrificed by exsanguination, and all organs and tissues were macroscopically examined. Then, the brain, pituitary gland, thymus, thyroids, heart, lungs, liver, spleen, kidneys, adrenals, testes, epididymides, and ovaries were removed and weighed. Histopathological examination was conducted for the control and the highest dose groups. The above-listed organs were fixed in 10% buffered formalin-phosphate (following Bouin's fixation for testes and epididymides), and paraffin sections were routinely prepared and stained with Hematoxylin-Eosin for microscopy. For other groups, organs with macroscopically abnormal findings or in which chemical-related effects were evident on microscopic examination for the highest dose group, were similarly investigated.

2. 28-day repeated dose toxicity study in young rats (young rat study)

Five to six week old rats were given the test substances by gastric intubation daily for 28 days and sacrificed following the last treatment (the scheduled-sacrifice group). Recovery groups were maintained for two weeks without chemical treatment and sacrificed at 11 or 12 weeks of age. The number of animals was six for each sex/dose for both scheduled-sacrificed and recovery cases.

The doses were selected in the same way as the newborn rat studies. In the 12-day dose-finding study for 2TBP, ataxic gait was observed at 300 mg/kg and more, and increase in relative liver and kidney weight at 500 mg/kg. For DTBP, with 14-day administration, the death of one of the four females, various changes in some blood biochemical parameters, increase in relative liver weights and light gray macules on kidneys were found at 500 mg/kg. Increase in serum phospholipid and relative liver weights were also demonstrated in the 100 mg/kg group. Based on the results, the doses were determined at 0, 4, 20, 100, or 500 mg/kg/day for 2TBP and at 0, 5, 20, 75, or 300 mg/kg/day for DTBP. Recovery groups were set at 0, 100, 500 mg/kg/day for 2TBP and 0, 300 mg/kg/day for DTBP.

During the study, rats were examined for general condition, body weight, food consumption, urinalysis, hematology and blood biochemistry, necropsy findings, organ weights, and histopathological findings in compliance with the Test Guideline in the Japanese Chemical Control Act (Official Name: Law Concerning the Examination and Regulation of Manufacture, etc. of Chemical Substances).

Statistical analysis

Data for body weights, food consumption, urinalysis findings (except for the results of qualitative analysis), hematological, blood biochemical findings (except for differential leukocyte count), and organ weights were analyzed by the Bartlett's test (Bartlett 1937) for homogeneity of distribution. When homogeneity was recognized, Dunnett's test (Dunnett 1964) was conducted for comparison between control and individual treatment groups ($P < 0.01$ or 0.05). If not homogeneous or for qualitative urinalysis data and differential leukocyte count, the data were analyzed using Steel's multiple comparison tests (Steel 1959), or tests of the Dunnett type (Hollander & Wolfe 1973) ($P < 0.01$ or 0.05). For reflex ontogeny, and physical and sexual development parameters in the newborn rat studies, the χ^2-test (Fisher 1922) was conducted ($P < 0.01$ or 0.05).

RESULTS

2-*tert*-butylphenol (2TBP)

Newborn rat study

Various clinical signs such as decrease in locomotor activity, ataxic gait, deep respiration, and muscle weakness were observed throughout the dosing period in the 200 mg/kg group, as shown in Table 1. With 60 mg/kg, transient decrease in locomotor activity was noted on the first dosing day limited to only one of 12 males. Body weights were lowered by 8–17% from dosing day 7 through to the end of the dosing period in males and to recovery-maintenance day 14 in females given 200 mg/kg. At the scheduled sacrifice, there were no hematological changes at any dose, but blood biochemical examination of the 200 mg/kg group showed increases in γ-GTP in both sexes and total protein in males. In addition, significant increase in relative liver weights was noted in 9% of the females in the 60 mg/kg group and in 21–23% of both males and females in the 200 mg/kg group. On histopathological examination, slight hypertrophy of centrilobular hepatocytes was found in one female of the 60 mg/kg group, and in four males and three females from the 200 mg/kg group. During the recovery-maintenance period, no clinical signs were observed and the lowered body weights showed a tendency for recovery. In parameters for physical and sexual development and reflex ontogeny, no definitive changes were detected. At the end of the recovery-maintenance period, no chemical-related changes, also in urinalysis data, were found in any dose group.

The results of the newborn rat study of 2TBP are summarized in Table 2. Since clinical signs and histopathological changes in the liver were observed in the 60 mg/kg group, the NOAEL was concluded to be 20 mg/kg/day.

Young rat study

Ataxic gait were observed sporadically during the dosing period in nine males and 12 females, and decrease in locomotor activity in two females from the 500 mg/kg group. During the dosing period, there were no changes in body weight, food consumption, and urinalysis data. At the scheduled sacrifice, hematological and blood biochemical examination also showed no changes. Eighteen to 19% increases were found in relative liver weights of both sexes receiving 500 mg/kg, but no histopathological changes in liver were observed at any dose. No chemical-related changes were noted during and at the end of the recovery period.

Table 1 Clinical signs observed during the dosing period in the newborn rat study of 2-*tert*-butylphenol

	Dose (mg/kg/day)			
	0	20	60	200
No. animals (Male/Female)	12/12	12/12	12/12	12/12
No. animals with clinical signs				
Decrease in locomotor activity	0/0	0/0	1†/0	12/12
Ataxic gait	0/0	0/0	0/0	4/6
Deep respiration	0/0	0/0	0/0	12/12
Tremors	0/0	0/0	0/0	2/4
Muscle weakness	0/0	0/0	0/0	12/12
Emaciation	0/0	0/0	0/0	2/2
Pale skin	0/0	0/0	0/0	4/2

†Observed only on the first dosing day.

Table 2 Summary of the results of the newborn and young rat study of 2-*tert*-butylphenol

Newborn rat study				
Dose (mg/kg/day)	20	60	200	
Clinical signs	–	M: Decrease in locomotor activity	Various†	
Body weight changes	–	–	8–17%↓	
Blood biochemical changes	–	–	GTP↑, M: TP↑	
Changes in relative organ weights	–	F: Liver 9%↑	Liver 21–23%↑	
Histopathological findings in liver				
– Slight centrilobular hypertrophy of hepatocytes	–	M: 0/6, F: 1/6	M: 4/6, F: 3/6	
Young rat study				
Dose (mg/kg/day)	4	20	100	500
Clinical signs	–	–	–	Ataxic gait F: Decrease in locomotor activity
Body weight changes	–	–	–	–
Blood biochemical changes	–	–	–	–
Changes in relative organ weights	–	–	–	Liver 18–19%↑
Histopathological findings	n.d.	n.d.	n.d.	–

Statistically significant increases ($P < 0.05$) in body weights, blood biochemical parameters and relative organ weights are shown as ↑, while decreases are shown as ↓. Data on histopathological findings are given as no. of animals with the findings/no. of animals examined, according to sex. Changes observed only in males or females are shown as 'M' or 'F', respectively, while neither 'M' nor 'F' is mentioned in the case of changes noted in both sexes. No chemical-related changes were observed in developmental parameters (conducted only in newborn rat study), urinalysis (only in young rat study), and hematological parameters. †Decrease in locomotor activity, ataxic gait, deep respiration, tremors, muscle weakness, emaciation, and pale skin were observed, as shown in Table 1. GTP, γ-GTP; TP, total protein; –, no change; n.d., not determined.

A summary of the results of the young rat study of 2TBP is given in Table 2. The NOAEL was concluded to be 100 mg/kg/day, at which no changes were observed.

2,4-di-*tert*-butylphenol (DTBP)

Newborn rat study

Two males and one female of the 300 mg/kg group were found dead on dosing days 3, 4, and 7. In this group, decrease in locomotor activity (12 males and 12 females), bradypnea (10 males and 10 females), and hypothermia (one male) were observed from the first dosing day, but then the incidence decreased, with disappearance after dosing day 7. Body weights of the 300 mg/kg group were lowered by 15–25% in males and by 9–20% in females during the dosing period, compared with the control values. There were no definitive changes in parameters for physical development and reflex ontogeny in any dose group. At the scheduled sacrifice, blood biochemical examination showed an increase in total bilirubin and a decrease in the A/G ratio in both sexes, an increase in γ-GTP in males, and an increase in total protein and BUN in females of the 300 mg/kg group. In the 300 mg/kg group, there was a 39–51% increase in relative liver weights, a 37–41% increase in relative kidney weights in both sexes, and a 24% decrease in relative spleen weights in males. In the 40 mg/kg group, 14% increases in relative weight of liver were found in females. On histopathological examination, various changes were observed in livers and kidneys in the 300 mg/kg group, as shown in Table 3. Furthermore, periportal fatty degeneration of hepatocytes was evident in one female given 40 mg/kg, and basophilic tubules in kidneys in one animal of each sex receiving 40 mg/kg and one control group male. Regarding parameters of sexual development, a slight delay in preputial separation was noted in the 300 mg/kg group (the incidences were 0/5, compared with 2/6 in the control group at PND 42 [recovery-maintenance day 21]; 0/5, 3/6 at PND 43; 2/5, 5/6 at PND 44; 2/5, 6/6 at PND 46; 4/5, 6/6 at PND 47; and 5/5, 6/6 at PND 48). During this observation period, body weights were lowered by approximately 10% in males given 300 mg/kg than control levels, which was not statistically significant. In the last week of the recovery-maintenance period, there were no chemical-related changes on urinalysis in any dose group. At the end of the recovery period, changes noted in the scheduled-sacrifice group were not observed except for histopathological changes in the kidneys, significant in the 300 mg/kg group (Table 3).

A summary of the results of the newborn rat study of DTBP is shown in Table 4. Since fatty degeneration of hepatocytes and increase in liver weight were demonstrated at 40 mg/kg, the NOAEL was concluded to be 5 mg/kg/day.

Young rat study

No chemical-related changes were found in general condition, body weight, and food consumption at any dose. On urinalysis at the fourth week of dosing, an increase in urine volume, and a decrease in specific gravity and osmotic pressure were noted in both sexes of the 300 mg/kg group. At the scheduled sacrifice, hematological examination showed a decrease in hemoglobin and hematocrit, an increase in segmented neutrophils in females, and prolongation of PT and APTT in males at 300 mg/kg. On blood biochemical examination, there was an increase in total bilirubin in males given 300 mg/kg, and an increase in total cholesterol and phospholipid in females given 75 mg/kg and above. For organ weights, there were

Table 3 Histopathological findings for the newborn rat study of 2,4-di-*tert*-butylphenol

		Scheduled-sacrifice group				Recovery-maintenance group†	
Dose (mg/kg/day)	Grade	0	5	40	300	0	300
No. of animals examined (Male/Female)		6/6	6/6	6/6	5/6	6/6	5/5
Liver							
– Fatty degeneration of periportal hepatocytes	+	0/0	0/0	0/1	0/0	0/0	0/0
	++	0/0	0/0	0/0	3/4	0/0	0/0
	+++	0/0	0/0	0/0	2/2	0/0	0/0
Kidneys							
– Basophilic tubules	+	1/0	n.d.	1/1	4/4	0/0	3/0
– Granular casts	+	0/0	n.d.	0/0	4/2	0/0	0/0
– Cystic dilatation of collecting tubules	+	0/0	n.d.	0/0	0/0	0/0	5/4
	++	0/0	n.d.	0/0	3/4	0/0	0/0
	+++	0/0	n.d.	0/0	2/2	0/0	0/0
– Cellular infiltration of neutrophils	+	0/0	n.d.	0/0	2/1	0/0	1/0
	++	0/0	n.d.	0/0	1/1	0/0	1/0
	+++	0/0	n.d.	0/0	1/1	0/0	0/0

†No histopathological examination was conducted at 5 and 40 mg/kg in the recovery-maintenance group. +, mild; ++, moderate; +++, marked; n.d., not determined.

increases in relative liver weights by 40–43% in both sexes given 300 mg/kg, and by 13% in females receiving 75 mg/kg. On histopathological examination, mild to marked changes in livers and kidneys were observed in both sexes from the 300 mg/kg group, as shown in Table 5. At the end of the recovery period, the increase in total cholesterol and phospholipid and renal histopathological changes observed in the scheduled-sacrifice group remained significant in the highest-dose group (Table 5).

The results of the young rat study are summarized in Table 4. Based on increase in the relative liver weights with some changes in blood biochemical parameters in females given 75 mg/kg, the NOAEL was concluded to be 20 mg/kg/day.

DISCUSSION

During development, many rapid and complex biological changes occur, which can have profound consequences on sensitivity to the effects of exogenous chemicals (Scheuplein *et al.* 2002). Although the neonatal body at birth is reasonably well prepared for the abrupt changes associated with parturition, and most functional systems possess a significant portion of their adult capacity (Dourson *et al.* 2002), it is known that the various functions remain immature in early postnatal period and that some organs and tissues, especially in the nervous, immune and reproductive systems, continue to develop after birth (NAS 1993). Therefore, it is important to evaluate toxic effects by exposure to chemicals during the early postnatal period as well as the fetal period for comprehensive risk assessment. However, economic issues and lack of human resources, arising from practical difficulties regarding protocols, have hindered routine implementation of toxicity studies using newborn animals. Our series of comparative analyzes on susceptibility of the newborn are therefore of particular importance for risk assessment.

In the present study on 2TBP and DTBP, there were no clear differences in toxicity profiles between the newborn and young rats in either case. For 2TBP, clinical signs such as a decrease in locomotor activity and ataxic gait, and effects on liver such as an increase in organ weight were observed. In the DTBP case, hepatic and renal toxicity (histopathological changes, increase in organ weight, etc.) were noted. As a characteristic effect of DTBP on male sexual development, slight delay in preputial separation was also observed in the newborn rat study. Preputial separation, an androgen-dependent process which is an early marker of puberty, represents a reliable non-invasive indicator of chemical-induced perturbation of male pubertal development in the rat (Gaytan *et al.* 1988). However, it is known that decreased body weights can result in non-specific delay in puberty (Ashby & Lefevre 2000). Since DTBP lowered body weights in the period of observation of preputial separation and there were no DTBP-related changes in weights or histopathology of the testes and epididymides, well known to be essentially androgen-dependent, no specific effect on male sexual development could be concluded in the present study. As for NOAELs of both chemicals, clear differences were observed between newborn and young rats, with values of 20 and 5 mg/kg/day in newborn rats, and 100 and 20 mg/kg/day in young rats for 2TBP and DTBP, respectively. Therefore, the susceptibility was four- to five-fold higher in newborn than in young rats.

Our previous analysis of 1,3-dibromopropane and 1,1,2,2-tetrabromoethane (Hirata-Koizumi *et al.* 2005) showed dose–response curves to be very different between newborn and young rats. The same was recently reported for the widely used organophosphorus insecticide, chlorpyrifos (Zheng *et al.* 2000), as well as pyrethroid insecticides (Shafer *et al.* 2005). These data showed the importance of estimating unequivocally toxic levels (UETLs), defined for our comparative toxicity analysis as equivalent toxic doses inducing clear toxicity, including death, clinical toxic signs,