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Evaluation of developmental toxicity of β-thujaplicin (hinokitiol) following oral administration during organogenesis in rats

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Abstract

The objective of this study was to evaluate the developmental toxicity of β-thujaplicin (TP) in rats. Pregnant rats were given TP by gastric intubation at 15, 45, or 135 mg/kg on days 6–15 of pregnancy. The maternal body weight gain during administration at 45 and 135 mg/kg and after administration at 136 mg/kg and adjusted weight gain at 45 and 135 mg/kg were significantly reduced. A significant decrease in food consumption during and after administration was found at 45 and 135 mg/kg. A significant increase in the incidence of postimplantation loss was found in pregnant rats given TP at 135 mg/kg. A significantly lower weight was found in female fetuses at 45 and 135 mg/kg and in male fetuses at 135 mg/kg. Although a significantly increased incidence of fetuses with skeletal variations and decreased degree of ossification were found at 135 mg/kg, no significant increase in external, skeletal and internal malformations was detected after administration of TP. The data demonstrated that TP had adverse effects on embryonic/fetal survival and growth only at maternal toxic doses. No adverse effects on morphological development were found in rats fetuses. Based on the significant decreases in maternal body weight gain and weight of female fetuses at 45 mg/kg and higher, it is concluded that the no-observed-adverse-effect levels (NOAELs) of TP for both dams and fetuses are considered to be 15 mg/kg in rats.

Keywords: β-Thujaplicin; Hinokitiol; Developmental toxicity; Teratogenicity; Rat

1. Introduction

β-Thujaplicin (TP; CAS No. 499-44-5; Hinokitiol; 4-isopropyltropolone) is a phenolic component of essential oils extracted from cypress trees. TP has been found to act as an antibacterial agent (Saeki et al., 1989; Osawa et al., 1990; Tonari, 1998) and an antitumor agent (Yamato et al., 1984; Inamori et al., 1993). In addition, it possesses phytogrowth-inhibitory effects (Inamori et al., 1991). TP is used as a natural food preservative in Japan.

Several reports on the toxicity of TP are available. In mutagenicity screening tests of TP, positive results were obtained in a Rec-assay with S9 mix at 1.0 mg/disk and chromosome aberration test in vitro at 0.002–0.003 mg/ml, but not in the Ames test or a micronucleus test in mice (Sofuni et al., 1993). The DNA damaging activity of TP was weak in a spore Rec-assay (Ueno and Ishizaki, 1992). The values of LD50 have been reported to be 504 mg/kg in male ddy mice and 469 mg/kg in female ddy mice after oral gavage of TP (Shimizu et al., 1993). Recently, Ogata et al. (1999) reported a significant increase in the incidence of fetuses with malformations after oral administration of TP at 560 mg/kg and higher on day 9 of pregnancy in ICR mice and that TP induced dysmorphogenicity in cultured mouse embryos at concentrations of 6.25 and 12.5 μg/ml. However, there is no information on the developmental toxicity of TP in rats. Therefore, the present study was conducted to evaluate the potential teratogenicity of TP after administration throughout organogenesis in rats.

Abbreviations: TP, β-thujaplicin; LOAEL, lowest-observed-adverse-effect level; NOAEL, no-observed-adverse-effect level.

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2. Materials and methods

2.1. Animals

Wistar rats (Jcl: Wistar, Clea Co., Ltd., Tokyo, Japan) were used throughout this study. Animals were reared on a basal diet (F-1; Funabashi Farm Co., Funabashi, Japan) and tap water ad libitum and maintained in an air-conditioned room at 24±1 °C, with a relative humidity of 55±5%, under a controlled 12-h light/dark cycle. Virgin female rats, weighing 216–244 g, were mated overnight with male rats. The day when sperm were detected in the vaginal smear was considered to be day 0 of pregnancy. The pregnant rats were distributed on a random basis into four groups of 16–17 rats each and housed individually.

2.2. Chemicals and dosing

The female rats were dosed once daily by gastric intubation with TP (purity >98%, SEIWA Technological Laboratories Ltd., Tokyo, Japan) at a dose of 0 (control), 15, 45, or 135 mg/kg from day 6 through day 15 of pregnancy. The dosage levels were determined based on the results of our range-finding study in which administration of TP by gastric intubation on days 6–15 of pregnancy caused maternal deaths and decreased maternal body weight gain and caused an increase in postimplantation loss and decrease in fetal weight at 125 mg/kg and higher in rats. TP was dissolved in olive oil (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The volume of each dose was adjusted to 5 ml/kg of body weight based on daily body weight. The control rats received olive oil only. The formulations were kept in a cool and dark place for no more than 7 days.

2.3. Observations

The maternal body weight and food consumption were recorded daily. The pregnant rats were euthanized by ether overdose on day 20 of pregnancy. The peritoneal cavity and uterus were opened, and the numbers of live and dead fetuses and of resorptions were counted. The gravid uterus was removed and the dams weighed again. The adjusted weight gain, i.e. maternal weight gain throughout pregnancy corrected for gravid uterine weight, was calculated. To confirm the dam's pregnancy status, the uteri were immersed in 2% sodium hydroxide solution for over 1 h. The uteri were cleared and the implantation traces were seen to be stained yellowish-brown (Yamada et al., 1985). The live fetuses removed from the uterus were sexed, weighed, and inspected for external malformations and malformations within the oral cavity. Approximately one-half of the live fetuses in each litter were randomly selected and fixed in alcohol, stained with alizarin red S (Kawamura et al., 1990) and examined for skeletal malformations. The remaining live fetuses in each litter were fixed in Bouin's solution prior to dissection. To detect internal malformations, fetal heads were examined by the free-hand razor-blade sectioning method of Barrow and Taylor (1969) and the thoracic areas were examined by Nishimura's micro-dissecting method (1974), a modification of Barrow and Taylor's method.

2.4. Data analysis

The litter was considered the experimental unit. The initial body weight, body weight gain and food consumption of pregnant rats, numbers of implantations, postimplantation loss and live fetuses per litter and body weight of live fetuses were evaluated by analysis of variance, followed by Dunnett's multiple comparison test if differences were found. The incidences of post-implantation loss and fetal malformations per litter were analyzed by the Kruskal–Wallis test to assess the overall effects. Whenever a significant trend was noted, pairwise comparisons were made using the Mann–Whitney test. Fisher's exact test was used when the incidence in the control group was zero. The 0.05 level of probability was used as the criterion for significance.

3. Results

Table 1 shows the maternal findings in rats given TP during organogenesis. One pregnant rat was dead on day 20 of pregnancy at 135 mg/kg. The body weight gain on days 6–16 at 45 and 135 mg/kg and on days 16–20 at 135 mg/kg was reduced significantly. The adjusted weight gain, which indicates the net weight gain of pregnant rats, was significantly lower in the 45 and 135 mg/kg groups than in the control group. The food consumption on days 6–16 and days 16–20 was significantly lower in the 45 and 135 mg/kg groups than the control group. These findings indicate that the lowest-observed-adverse-effect level (LOAEL) and no-observed-adverse-effect level (NOAEL) of TP for pregnant rats are 45 and 15 mg/kg, respectively.

Pregnancy outcome in rats given TP during organogenesis are presented in Table 2. Litters totally resorbed were found in three of the 16 pregnant rats at 135 mg/kg. A significant increase in the number of resorptions per litter and incidence of postimplantation loss per litter and a significant decrease in the number of live fetuses per litter were also noted at 135 mg/kg. The weights of live fetuses were significantly decreased at 45 mg/kg and higher in females and at 135 mg/kg in males.

A summary of morphological findings in live fetuses of rats given TP during organogenesis is shown in Table 3. No fetus with external malformations was observed in any group. Skeletal examination revealed

Table 1
Maternal findings in rats given β-thujaplicin (TP) by gastric intubation on days 6–15 of pregnancy

Dose (mg/kg)	0 (control)	15	45	135
No. pregnant rats	16	16	16	17
No. of dead rats	0	0	0	1
Initial body weight	227±8	227±7	227±6	227±6
Body weight gain during pregnancy (g)[a]				
Days 0–6	17±5	17±4	16±2	17±3
Days 6–16	45±4	39±6	32±7*	13±9*
Days 16–20	48±6	48±5	42±6	21±12*
Adjusted weight gain during pregnancy (g)[a,b]	39±7	36±8	28±10*	24±5*
Food consumption during pregnancy (g)[a]				
Days 0–6	105±7	101±6	98±5*	101±5
Days 6–16	157±12	147±13	129±12*	103±11*
Days 16–20	72±5	70±4	63±7*	66±6*

[a] Values are given as mean±S.D.
[b] Adjusted weight gain refers to maternal body weight gain excluding the gravid uterus.
* Significantly different from the control, $P<0.05$.

Table 2
Reproductive findings in rats givenβ-thujaplicin (TP) by gastric intubation on days 6–15 of pregnancy

Dose (mg/kg)	0 (control)	15	45	135
No. litters	16	16	16	16
No. corpora lutea per litter[a]	16.3±1.3	16.3±1.3	15.7±1.4	16.3±0.9
No. implantations per litter[a]	15.4±1.4	15.5±1.2	14.8±1.7	15.6±1.3
No. of litters totally resorbed	0	0	0	3
No. resorptions per litter[a]	1.3±1.4	1.3±1.2	2.0±1.0	9.9±4.6*
No. dead fetuses per litter[a]	0.1±0.3	0	0	0
% Postimplantation loss per litter[b]	8.5	8.0	13.6	63.5*
No. live fetuses per litter[a]	14.1±1.4	14.3±1.5	12.8±1.8	5.7±4.6*
Sex ratio of live fetuses (male/female)	114/111	116/112	107/97	56/35
Body weight of live fetuses (g)[a]				
Male	3.39±0.19	3.26±0.19	3.25±0.18	2.71±0.21*
Female	3.19±0.18	3.13±0.18	3.02±0.19*	2.62±0.11*

[a] Values are given as mean±SD.
[b] (No. resorptions and dead fetuses/No. implantations)×100.
* Significantly different from the control, $P<0.05$.

one fetus with sternoschisis at 135 mg/kg. Skeletal variations in the vertebrae, ribs, and/or sternebrae were found in all groups. The incidences of fetuses with skeletal variations and fetuses with bipartite sternebrae and with rudimentary 14th ribs were significantly higher in the 135 mg/kg group than the control group. The numbers of ossification centers of the caudal vertebrae and of the sternebrae were significantly decreased at 135 mg/kg. Hypoplasia of the spleen occurred in two fetuses in one dam at 135 mg/kg. A few fetuses with thymic remnant in the nick and/or left umbilical artery were found in the control group and TP-treated groups. However, there was no significant difference in the incidence of fetuses with internal malformations and variations between the TP-treated groups and the control group. These findings indicate that the lowest-observed-adverse-effect level (LOAEL) and no-observed-adverse-effect level (NOAEL) of TP for fetal rats are 45 and 15 mg/kg, respectively.

4. Discussion

This study was designed to screen for general developmental toxicity in rats. Doses of TP expected to induce maternal and developmental toxicity, such as a decrease in maternal body weight gain and food consumption and in fetal weight and an increase in postimplantation loss, were given to pregnant rats to characterize the effects of TP on embryonic/fetal development. Maternal toxicity, as evidenced by a significant decrease in body weight gain and food consumption

Table 3
Morphological examinations in fetuses of rats given β-thujaplicin (TP) by gastric intubation on days 6–15 of pregnancy

Dose (mg/kg)	0(control)	15	45	135
External examination				
No. fetuses (litters) examined	225(16)	228(16)	204(16)	91(13)
No. fetuses (litters) with malformations	0	0	0	0
Skeletal examination				
No. fetuses (litters) examined	116(16)	117(16)	105(16)	49(13)
No. fetuses (litters) with malformations	0	0	0	1(1)
Sternoschisis	0	0	0	1(1)
No. of fetuses (litters) with variations	11(7)	8(4)	10(7)	21(11)**
Cervical rib	4(2)	3(1)	3(2)	1(1)
Splitting of thoracic vertebral bodies	0	1(1)	0	0
14th ribs				
Extra	0	0	0	4(3)
Rudimentary	2(1)	1(1)	2(2)	9(7)**
Bipartite sternebrae	1(1)	2(1)	1(1)	9(7)**
Asymmetry of sternebrae	5(5)	1(1)	4(3)	3(3)
Degree of ossification[a]				
No. of ossification centers of caudal vertebrae	3.3±0.4	3.1±0.4	3.2±0.4	2.8±0.3**
No. of sternebrae	4.9±0.4	4.9±0.6	4.8±0.5	3.9±0.7**
Internal examination				
No. fetuses (litters) examined	109(16)	111(16)	99(16)	42(12)
No. fetuses (litters) with malformations	0	0	0	2(1)
Hypoplasia of spleen	0	0	0	2(1)
No. of fetuses (litters) with variations	5(3)	3(3)	2(2)	2(2)
Thymic remnant in neck	4(3)	1(1)	2(2)	2(2)
Left umbilical artery	1(1)	2(2)	0	0

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

during the administration period was found at 45 mg/kg and higher. Although pregnant rats in the 45 mg/kg group recovered with respect to body weight after cessation of administration of TP, such recovery did not occur in the high dose group. This may be due to a lack of conceptuses at 135 mg/kg. However, a significantly low adjusted weight gain at 45 mg/kg and higher may suggest maternal toxicity. These findings indicate that TP exerts maternal toxicity at 45 mg/kg and higher when administered during organogenesis in rats.

Developmental endpoints should include the number and percent of pre-and postimplantation loss, morphological alterations in fetuses, and decreased fetal weight (Kimmel and Price, 1990; Schardein, 2000; OECD, 2001). Schardein (2000) stated that fetal size is an important in the assessment of potential teratogen as an indicator of developmental toxicity, and reduction in size or growth retardation commonly occurs among fetuses of dams given dosages that are toxic to the dam, to the offspring, or both. In the present study, a significant increase in the incidence of postimplantation loss was found at 135 mg/kg and a significantly decreased weight of female fetuses was found at 45 mg/kg and higher. These findings indicated that TP is embryolethal at 135 mg/kg and toxic to fetal growth at 45 mg/kg and higher when administered during the period of organogenesis.

As for morphological examinations in the fetuses of exposed mother, a few fetuses with skeletal or internal malformations were found in the 135 mg/kg group. The malformations observed in the present study are not thought to be due to the administration of TP, because they occurred at a very low incidence and are of types that occur sporadically among control rat fetuses (Kameyama et al., 1980; Morita et al., 1987; Nakatsuka et al., 1997). Several types of skeletal and internal variations were also found in both the control group and TP-treated groups. These variations are frequently observed in fetuses of rats at term (Kimmel and Wilson, 1973; Kameyama et al., 1980; Morita et al., 1987; Nakatsuka et al., 1997). In the 135 mg/kg group, a significant increase in the incidence of fetuses with skeletal variations and fetuses with bipartite sternebrae and with rudimentary 14th ribs, but no extra ribs, and a significant decrease in the degree of ossification were accompanied by a significant decrease in the fetal weight. These findings show a correlation between these morphological alterations and growth retardation in fetuses. Although a skeletal variation, i.e. super-

numerary extra 14th ribs, is a warning sign of possible teratogenicity, the rudimentary 14th ribs, sternebral variations, and bilobed centra of the vertebral column are a normal variation (Kimmel and Wilson, 1973). Chahoud et al. (1999) noted that variations are unlikely to adversely affect survival or health and this might result from a delay in growth or morphologenesis that has otherwise followed a normal pattern of development. Consideration of these findings together suggests that the morphological changes observed in the present study do not indicate a teratogenic response and that TP possesses no teratogenic potential in rats.

In a developmental toxicity study in mice in which a single administration of TP was given at 420, 560, 750, or 1000 mg/kg by gastric intubation on day 9 of pregnancy, maternal deaths, dams with litter totally resorbed, and a significant increase in embryolethality were found at 750 mg/kg and higher (Ogata et al., 1999). A significant increase in the incidence of fetuses with malformations was accompanied by a significant decrease in fetal weight at 560 mg/kg and higher. Two highest doses, 750 and 1000 mg/kg, were maternally lethal, and the dose level of 560 mg/kg was very close to the maternally lethal dose. Thus, fetal malformations occurred after a single administration of TP at high doses in a single species. In other words, TP may be capable to produce fetal malformations under extreme experimental conditions in mice. Studies in additional species would be of great value in evaluating developmental toxicity of TP in conventional experimental conditions. We demonstrated here that TP possesses no adverse effects on morphological development in rat fetuses when administered during the whole period of organogenesis at doses which caused a decreased fetal weight, increased incidence of postimplantation loss, and maternal toxicity.

In conclusion, the administration of TP to pregnant rats throughout organogenesis had adverse effects on maternal rats and embryonic/fetal survival and growth but had no adverse effects on morphological development of fetuses even at maternally toxic and embryolethal doses. The data indicate that TP adversely affected the embryonic/fetal survival and growth only at maternally toxic doses in rats. Based on the significant decreases in maternal body weight gain and weight of female fetuses at 45 mg/kg and higher, it is concluded that the no-observed-adverse-effect levels (NOAELs) of TP for both dams and fetuses are considered to be 15 mg/kg in rats.

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chapter 3

Reproductive and Developmental Toxicity of Organotin Compounds

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Contents

Introduction

Organotin compounds are chemicals widely used in agriculture and industry (Piver 1973, World Health Organization 1980). Tetrasubstituted organotin compounds are mainly used as intermediates in the preparation of other organotin compounds. Trisubstituted organotin compounds have biocidal properties and are used in agriculture as fungicides and acaricides, as rodent repellents, and mulluscicides, and are widely used as antifoulants in ship paints and underwater coatings. Especially, triphenyltins (TPTs) and tributyltins (TBTs) have been used extensively in antifouling products such as algaecides and mulluscicides. Disubstituted organotin compounds are commercially the most important derivatives, and are mainly used in the plastics industry, particularly as heat and light stabilizers for polyvinyl chloride (PVC) plastics to prevent degradation of the polymer during melting and forming of the resin into its final products, as catalysts in the production of polyurethane foams, and as vulcanizing agents for silicone rubbers. Monosubstituted organotin compounds are used as stabilizers in PVC films. Widespread use of organotin compounds has caused increasing amounts to be released into the environment. The most important nonpesticidal route of entry for organotin compounds into the environment is through leaching of organotin-stabilized PVC in water (Quevauviller et al. 1991), and the use in antifouling agents, resulting in the introduction of organotin into the aquatic environment (Maguire 1991). Data are available regarding the detection of butyltin and phenyltin compounds in aquatic marine organisms (Sasaki et al. 1988, Fent and Hunn 1991, Lau 1991) and marine products (Suzuki et al. 1992, Belfroid et al. 2000, Tsuda et al. 1995, Ueno et al. 1999, Toyoda et al. 2000). Food chain bioamplification of butyltin in oysters (Waldock and Thain 1983), mud crabs (Evans and Laughlin 1984), marine mussels (Laughlin et al. 1986), Chinook salmon (Short and Thrower 1986), and dolphin, tuna, and shark (Kannan et al. 1996), and of phenyltin in carp (Tsuda et al. 1987) and horseshoe crab (Kannan et al. 1995) has been reported. These indicate that organotin compounds accumulate in the food chain and are bioconcentrated, and that humans can be exposed to organotin compounds via seafood. The World Health Organization (WHO) reported in 1980 that the estimated mean total daily intake of tin by humans ranged from 200 μg to 17 mg. Recently, Tsuda et al. (1995) reported that the daily intakes in Shiga prefecture in Japan were 0.7 to 5.4 μg in 1991 and 0.7 to 1.3 μg in 1992 for TPT and 4.7 to 6.9 μg in 1991 and 2.2 to 6.7 μg in 1992 for TBT. Toyoda et al. (2000) also showed that the daily intakes in Japanese consumers, based on analysis with the 1998 total diet samples, were 0.09 μg for TPT, 0 μg for diphenyltin (DPT), 1.7 μg for TBT, and 0.45 μg for dibutyltin (DBT). These values are lower than the acceptable daily intake for TPT according to the JMPR (Joint Meetings of the FAO [Food and Agriculture Organization] and World Health Organization

Panel of Experts on Pesticides Residues), 25 μg (World Health Organization 1992), and the guidance value for oral exposure to tributyltin oxide (TBTO), 18 μg (International Programme on Chemical Safety 1999a). Thus, the levels of organotin compounds in seafood are not considered to be sufficiently high to affect human health (Tsuda et al. 1995, Ueno et al. 1999). However, Belfroid et al. (2000) noted that more research on residual TBT levels in seafood is needed before a definitive conclusion on possible health risks can be drawn.

In recent years, adverse effects of environmental chemicals on the reproductive success of wildlife populations have been reported (Colborn et al. 1993). These phenomena may result from interference with the endocrine system. Disturbances of hormonal regulation during pre- and postnatal development may produce deleterious effects on reproduction and development. TPT and TBT are suspected to be endocrine disruptors (Japan Environment Agency 1998). TBT and TPT are known to have strong effects on the development of imposex (imposition of male sex characteristics on females) in the rock shell (Horiguchi et al. 1996, 1997a), and this condition may bring about reproductive failure and a consequent population decline.

Although the toxicity of organotins has been extensively reviewed (World Health Organization 1980, Snoeij et al. 1987, Winship 1988, Boyer 1989, International Programme on Chemical Safety 1999a, b), the reproductive and developmental toxicity of these compounds is not well understood. In this chapter, we summarize the findings of the studies on reproductive and developmental effects of organotin compounds.

Effects on Aquatic Organisms

Imposex on Gastropods

TBT causes reproductive toxic effects in marine gastropods, which were represented by some masculinizing effects including *imposex* or *pseudohermaphodi(ti)sm*. The imposition of male sex organs (a penis and vas deferens) on female mud snails (*Nassarius obsoletus*) was found in near harbors, and the degree of penis development and frequency of imposex were positively correlated to the seawater TBT concentration (Smith 1981a, b). Imposex has been induced experimentally by treatment with 4.5 to 5.5 μg/L of TBT compounds for 60 days. In field studies in southeastern England, imposex has been reported in declining populations of the common dogwhelk (Bryan et al. 1986, 1987, 1989, Gibbs and Bryan 1986, Davies et al. 1987, Gibbs et al. 1987).

Imposex has not just occurred at a regional level, but worldwide on a global scale. Imposex in dogwhelk was not only reported in England, but in Scotland, the Netherlands, and the coastline of the North Sea. Imposex in other whelk species occurred in Canada, West Africa, New Zealand, Australia, Malaysia, Singapole, Indonesia, and Japan (Fent 1996, Horiguchi et al. 1996). Imposex among prosobranchs is known to occur in around 70 species of 50 genera, although some species are less susceptible to TBT compounds (Fioroni et al. 1991, Fent 1996).

TPT also induced imposex in *Thais clavigera* at the same potency as TBT (Horiguchi et al. 1997a). Although, in *Nucwlla lapillus,* TPT did not induce imposex, tripropyltin (TPrT) had a small effect on the development of imposex (Bryan et al. 1988). DBT and monobutyltin (MBT) did not induce imposex in the gastropod species examined. Three trisubstitution compounds (TBT, TPT, TPrT) and monophenyltin (MPT) easily induced imposex in some species, among the eight organotins, i.e., MBT, DBT, TBT, tetrabutyltin (TeBT), MPT, DPT, TPT, and TPrT. (Bryan et al. 1988, Hawkins and Hutchinson 1990, Horiguchi et al. 1997b).

The early studies in the 1980s reached some common conclusions, which are described below (Eisler 2000). Imposex correlated with the body burden of tributyl- and dibutyltin, but not with the tissue concentration of arsenic, cadmium, copper, lead, silver, or zinc. Forty-one percent of females had male characteristics, when the body burden reached to 1.65 mg Sn/kg of dry soft parts, by exposing with 0.02 μg Sn/L for 120 days. Imposex in immature females is caused above the concentration of around 1 ng/L (Sn) in seawater. At higher concentrations of TBT, the oviduct had been blocked, resulting in sterilization. Declining dogwhelk populations could be caused by aborting capsules, sterility, and premature death, which were characterized by a moderate to high degree of imposex, fewer female functions, fewer juveniles, and scarcity of laid egg capsules.

There is also a great variety of gradations of imposex in different species. The intensity is characterized by a classification system, which distinguishes six stages with a few different types, mainly based on a Vas Deferens Sequence (VDS) index (Oehlmann et al. 1991). Imposex development occurred in three variations: (1) a small penis without penis duct, (2) a short distal vas deferens section, or (3) a short proximal vas deferens section (stage 1). At stages 2 and 3 the male sex characteristics of each type are developed continuously. Stage 4 is characterized by a penis with penis duct and a complete vas deferens, and represents the last stage of fertility. The reproductive failure or sterility is induced in later stages. At stage 5 the vagina is replaced with a small prostate gland, the vagina opening is blocked by vas deferens tissue, or the incompletion of the pallial oviduct closure occurs. Abortive egg capsules fill the lumen and vestibulum of the capsule gland and evoke an intense swelling of the gland at stage 6 (Bettin et al. 1996). High TBT exposure in the early stages of life induced gametogenesis or sex changes characterized by a suppression of oogenesis and commencement of spermatogenesis in females (Gibbs et al. 1988, Fioroni et al. 1991, Oehlmann et al. 1991, 1996, Horiguchi et al. 2002). It was thought that the initial phases of imposex corresponding to VDS stages 1 and 2 may be reversible; however, advanced phases of imposex and sterilization with gross morphological changes corresponding to VDS stage 5 and 6 would be irreversible (Fent 1996).

Although many morphological aspects of pseudohermaphodi(ti)sm caused by TBT have been investigated, the biochemical mechanism has been indistinct. It is known that a neurotropic hormone called the penis morphogenic factor (PMF) develops male normal differentiation in mollusks (Féral

and LeGall, 1983). Co-localization of TBT with PMF in ganglia suggested that PMF release through TBT's neurotropic action induced masculinization in females (Bryan et al. 1989). Other studies indicated increased testosterone levels detected in female dogwhelk exposed to TBT, and that testosterone injection without TBT induced penis development in females (Spooner et al. 1991, Stroben et al. 1991). The later studies suggested that TBT disturbed the P-450–dependent aromatization of androgens to estrogen, and a nonsteroidal specific aromatase inhibitor–induced imposex similar to TBT (Bettin et al. 1996). However, the PMF has not been well characterized, and the role of vertebrate sex steroids is not known in gastropods to date. A recent study proposed that the combination of changes in the neuropeptide (APGWamide), which is considered to be a PMF in mud snails, and steroid hormones would lead to imposex induction at extremely low doses of TBT (Oberdörster and McClellan-Green 2002).

Effects on Fish

TBT or TPT exposure in early life stages induces altered embryonic development, and delayed or inhibited hatching in fish. Exposure of TBT or TBT to minnow eggs and larvae at concentrations of 0.2 to 18 µg/L in the water in which the fish lived induced dose-dependent morphological effects on larvae. Marked body axis deformations were observed at more than about 4 µg/L exposure, and incomplete hatching occurred at similar concentrations in 10 to 30% of larvae. At 15.9 µg/L of TPT exposure, hatching was delayed and the hatching rate was reduced significantly (Fent and Meier 1992, 1994). Developmental defects, such as skeletal abnormality and retarded yolk sac resorption, occurred in zebrafish larvae at more than 25 µg/L of triphenyltin acetate (TPTA) exposure, and hatching delay was found at more than 0.5 µg/L (Strmac and Braunbeck 1999). These developmental effects in fish were caused not only by organotin compounds, but also by a variety of contaminants (i.e., heavy metals, chlorinated hydrocarbons, altered pH), suggesting that such alteration would be classified as a nonspecific reaction to organic toxicants (Fent 1996, Strmac and Braunbeck 1999).

Some reproductive effects (i.e., reduced fecundity and sperm counts) in fish were reported. Reproductive success of three-spine stickleback with TBT exposure were examined over a 7-month period; no effects were detected in relation to fecundity, number of hatched fry, or frequency of malformed fry. However, no changes were found in the gonad somatic index (GSI; ovary weight ratio to total body weight); by the 7-month TBT treatment (2 µg/L) despite increasing GSI in controls, which suggested a lack of maturation of egg tissue and consequently a potential reduced fecundity (Holm et al. 1991). In sheepshead minnows, reduction in both total and percent viable eggs was found at more than 1.3 µg/L of TBT exposure, although the reductions were not statistically significant (Manning et al. 1999). TBT exposure to Japanese medaka at 1 mg/kg body weight caused a reduction of the spawning frequency (Nirmala et al. 1999). Additionally, environmentally relevant concentrations of

TBT induced significantly decreased sperm counts in guppies (11.2 to 22.3 ng/L for 21 days), and decreased sperm motility at concentrations less than 1 μg/L (Haubruge et al. 2000, Kime et al. 2001).

Effects on Other Organisms

Despite a great number of studies on imposex in snails and a comparable number of toxicity reports on fish, there is little information on development and reproductive effects on other species by organotin compounds. It was reported that imposex has not only been found in gastropods, but also been induced in Japanese freshwater crabs by TBT (Takahashi et al. 2000). In crabs, imposex has also occurred in males, which is characterized by dual-gender imposex (either a female genital opening or a single ovary occurred in males). Malformations during limb regeneration occurred in fiddler crabs (Weis and Kim 1988) and in axolotl, induced by TBT (Scadding 1990).

Summary of Effects on Aquatic Organisms

TBT or TPT causes the imposition of male sex organs (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, and advanced phases of imposex and sterilization with gross morphological changes are irreversible. The biochemical mechanism studies suggested that the induction of either neurotropic hormone or androgen titer would lead to imposex at an extremely low dose of TBT. Also, TBT or TPT exposure in the early life stages of fish causes altered embryonic development, impaired morphological development, and delayed or inhibited hatching, and reduces fecundity and sperm counts. 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 an important issue in aquatic ecosystems.

Effects on Experimental Animals

Reproductive Toxicity of Phenyltin Compounds

Reproductive Toxicity of Triphenyltins

TPTs have been reported to be insect chemosterilants (Kenaga 1965). Reproductive studies on TPTs are presented in Table 3.1. Several reports on male reproductive toxicity have been published. Male Sharman rats were given a diet containing triphenyltin hydroxide (TPTH) at 50, 100, or 200 ppm and then mated with untreated females repeatedly five times (Gaines and Kimbrough 1968). Reduced fertility, such as decreases in the total number of matings, total number of litters born alive, and ratio of number of litters to number of matings, accompanied by a marked reduction in food consumption

Table 3.1 Reproductive Toxicity of Phenyltin Compounds

Compounds	Animals	Dose	Days of Administration	Route	Reproductive and Developmental Effects	Author(s)
TPTH	Sharman rat	100–200 ppm	64–238 days	Diet	Decreased no. of matings Decreased no. of litters born alive Decreased ratio of no. of litters to no. of matings	Gains and Kimbrough (1968)
TPTA or TPTCl	Holtzman rat	20 mg/kg	19 days	Diet	Decreased tesicular size Change in testicular morphology	Pate and Hays (1968)
TPTA or TPTCl	Holtzman rat	20 mg/kg	20 days	Diet	Impairment of spermatogenic process	Snow and Hays (1983)
TPTA	ICR/Ha Swiss mouse	2.4–12 mg/kg 6 mg/kg	1 day 5 days	ip Gavage	No dominant lethal effect No dominant lethal effect	Epstein et al. (1972)
TPTH		1.3–8.5 mg/kg 11 mg/kg	1 day 5 days	ip Gavage	No dominant lethal effect No dominant lethal effect	
TPTA or TPTCl	Holtzman rat	20 mg/kg	4–24 days	Diet	Decreased no. of mature follicles Increased incidence of atresia in early follicle growth Decreased no.of corpora lutea	Newton and Hays (1968)
TPTCl	Wistar rat	4.7–6.3 mg/kg 12.5–25 mg/kg	Days 0–3 of pregnancy Days 4–6 of pregnancy	Gavage Gavage	Decreased pegnancy rate, decreased fetal wt. Decreased pregnancy rate	Ema et al. (1997a)
TPTCl	Wistar rat	4.7–6.3 mg/kg	Days 0–3 of pseudopregnancy	Gavage	Suppression of uterine decidualization	Ema et al. (1999a)
DPTCl	Wistar rat	16.5–24.8 mg/kg 33.3 mg/kg	Days 0–3 of pregnancy Days 4–7 of pregnancy	Gavage Gavage	Decreased pregnancy rate, preimplatation loss, decreased fetal wt. Effects as above, postimplantation loss	Ema et al. (1999b)
DPTCl	Wistar rat	4.1–24.8 mg/kg	Days 0–3 of	Gavage	Suppression of uterine decidualization	Ema and Miyawaki (2002)

and weight gain, were observed at 100 or 200 ppm for 64 days. At these doses, food consumption later improved, and with it, fertility. Dietary exposure to triphenyltin acetate (TPTA) or triphenyltin chloride (TPTCl) at 20 mg/kg for 19 days produced marked effects on body weight, testicle size, and testicular structure in male Holtzman rats (Pate and Hays 1968). Microscopic examinations revealed degenerative changes, such as a decrease in the number of layers per tubule, a depletion of the more advanced cell forms from the tubules, and a closing of the tubule lumina. Effects were more pronounced in rats treated with TPTA. TPTA or TPTCl at 20 mg/kg in feed for 20 days was reported to cause an impairment of the spermatogenic process in male Holtzman rats; complete recovery of the spermatogenesis was observed after feeding a normal diet for 70 days (Snow and Hays 1983). No mutagenicity was detected in dominant lethal assay in which male ICR/Ha Swiss mice were given a single intraperitoneal injection of TPTA at 2.4 or 12 mg/kg or TPTH at 1.3 or 8.5 mg/kg, or given TPTA at 6mg/kg or TPTH at 11 mg/kg by gavage on 5 successive days and then mated with untreated females, and pregnancy outcome was determined on day 13 of pregnancy (Epstein et al. 1972).

Adverse effects on female reproductive toxicity were also reported. Dietary TPTA and TPTCl at 20 mg/kg for 4 days produced significant changes in the ovarian tissue, including a decreased number of mature follicles, an increased incidence of atresia in early follicle growth, and a pronounced decrease in the number of corpora lutea in female Holtzman rats (Newton and Hays 1968). These effects were regarded as a decrease in ovulation, and thus decreased fertility. The adverse effects of TPTCl on the initiation and maintenance of pregnancy were determined after administration to the mother during early pregnancy (Ema et al. 1997a). Following successful mating, female Wistar rats were given TPTCl by gavage on days 0 to 3 of pregnancy at 3.1, 4.7, or 6.3 mg/kg or on days 4 to 6 of pregnancy at 6.3, 12.5, or 25.0 mg/kg, and pregnancy outcome was determined on day 20 of pregnancy. TPTCl totally prevented implantation in a dose-dependent manner. The pregnancy rate was decreased after administration of TPTCl on days 0 to 3 at 4.7 and 6.3 mg/kg and on days 4 to 6 at 12.5 and 25.0 mg/kg. Preimplantation loss was increased after administration of TPTCl on days 0 to 3 at 4.7 mg/kg and higher. In females having implantations, the numbers of implantations and live fetuses, and the incidences of pre- and postimplantation embryonic loss in the TPTCl-treated groups were comparable to the controls. These results indicate that TPTCl during early pregnancy causes failure in implantation and has greater antiimplantation effects when administered during the preimplantation period than the periimplantation period.

The function of the uterine endometrium is one of the principle factors in embryonic survival. Uterine decidualization is required for normal implantation, placentation, and therefore normal gestation in rats. The uterine growth induced by endometrial trauma in pseudopregnant animals mimics the decidual response of the pregnant uterus that occurs after embryo implantation (Cummings 1990, Kamrin et al. 1994). The decidual cell

response (DCR) is a model for maternal physiological events that are associated with implantation (Cummings 1990). This technique can distinguish between the adverse effects of chemical compounds in the maternal and fetal compartments, and has been used to evaluate the reproductive toxicity of chemical compounds (Spencer and Sing 1982, Bui et al. 1986, Cummings 1990, Kamrin et al. 1994, Ema et al. 1998). The effects of TPTCl on the reproductive capability of the uterus, as a cause of implantation failure, were evaluated using pseudopregnant rats (Ema et al. 1999a). Female Wistar rats were given TPTCl by gastric intubation at 3.1, 4.7, or 6.3 mg/kg on days 0 to 3 of pseudopregnancy. Between 11:00 and 13:00 on day 4 of pseudopregnancy, induction of DCR was performed via midventral laparotomy under ether anesthesia, and experimental decidualization was initiated by scratching the antimesometrial surface of the endometrium with a bent needle. The uterine weight on day 9 of pseudopregnancy served as an index of the uterine decidualization (De Feo 1963). A decrease in the uterine weight, which indicates suppression of the uterine decidualization, was detected at 4.7 and 6.3 mg/kg. TPTCl at 4.7 and 6.3 mg/kg also produced a decrease in the serum progesterone levels in female rats on day 4 and on day 9 of pseudopregnancy. These doses caused an increase in implantation failure (preimplantation embryonic loss) in female rats given TPTCl on days 0 to 3 of pregnancy (Ema et al. 1997a). These results suggest that TPTCl causes the suppression of uterine decidualization correlated with the reduction in serum progesterone levels, and these participate in the induction of implantation failure due to TPTCl. Protective effects of progesterone against suppression of uterine decidualization and implantation failure induced by TPTCl were examined (Ema and Miyawaki 2001). The hormonal regimen, consisting of progesterone and estorone supported decidual development in ovariectomized rats given TPTCl. The pregnancy rate and number of implantations in groups given TPTCl at 4.7 or 6.3 mg/kg in combination with progesterone were higher than those in the groups given TPTCl alone. These results indicate that the TPTCl-induced suppression of uterine decidualization is mediated, at least partially, by ovarian hormones, and that progesterone protects against TPTCl-induced implantation failure.

Reproductive Toxicity of Diphenyltin Compounds

Oral TPT is metabolized to DPT, MPT, and further to inorganic tin in rats (Kimmel et al. 1977, Ohhira and Matsui 1993 a, b). Reproductive toxicity studies on DPTs are also published (Table 3.1). The adverse effects of diphenyltin dichloride (DPTCl) on the initiation and maintenance of pregnancy, and the role of DPT in the implantation failure of TPT were evaluated. Following successful mating, DPTCl was given to Wistar rats by gavage on days 0 to 3 of pregnancy at 4.1, 8.3, 16.5, or 24.8 mg/kg or on days 4 to 7 of pregnancy at 8.3, 16.5, 24.8, or 33.0 mg/kg (Ema et al. 1999b). The pregnancy rate was decreased after administration of DPTCl on days 0 to 3 at 24.8 mg/kg and on days 4 to 7 at 33.0 mg/kg. The incidence of preimplantation loss was increased at 16.5 mg (equivalent to 48 μmol)/kg on days 0 to 3. In

females having implantations, the incidences of pre- and postimplantation embryonic loss in the groups given DPTCl on days 0-3 were comparable to the controls. The incidence of postimplantation embryonic loss was increased after administration of DPTCl on days 4 to 7 at 33.0 mg/kg. These results indicate that DPTCl during early pregnancy causes implantation failure, and that DPTCl has greater effects on reproduction when administered during the preimplantation period rather than the periimplantation period. Following administration on days 0 to 3 of pregnancy, the increased incidence of preimplantation embryonic loss was induced by TPTCl, a parent compound of DPTCl, at 4.7 mg (equivalent to12 μmol)/kg and higher (Ema et al. 1997a), or DPTCl at 16.5 mg (equivalent to 48 μmol)/kg. If, on a mole-equivalent basis, a metabolite is as, or more, effective than the parent compound, this is consistent with the view that the metabolite is the proximate toxicant or at least an intermediate to the proximate toxicant. Thus, it seems unlikely that only DPTCl and/or its further metabolites can be considered the agents responsible for the antiimplantation effects of TPTCl. As for the metabolism of phenyltin, however, Ohhira and Matsui (1993b) showed that TPT compound was formed in the liver of the DPTCl-treated rat by metabolism of DPTCl, and suggested that part of the administered DPT compound has some harmful effect as the TPT compound in rats, and this must be taken into consideration in toxicological research on DPT. Further studies are needed to clarify the difference in the reproductive toxicity induced by TPT and DPT, and to identify the proximate or ultimate toxicant of phenyltins. The effects of DPTCl on the reproductive capability of the uterus were evaluated in pseudopregnant rats according to the procedure described above. Female Wistar rats were given DPTCl by gastric intubation on days 0 to 3 of pseudopregnancy at 4.1, 8.3, 16.5, or 24.8 mg/kg (Ema and Miyawaki 2002). Suppression of uterine decidualization was observed at 16.5 mg/kg and higher. A decrease in the serum progesterone levels in pseudopregnant rats was also found on day 4 and on day 9 of pseudopregnancy at 16.5 mg/kg and higher. These doses induced an increase in preimplantation embryonic loss in female rats given DPTCl on days 0 to 3 of pregnancy (Ema et al. 1999b). No changes in serum estradiol levels in pseudopregnant rats were noted. These results suggest that DPTCl causes the suppression of uterine decidualization correlated with the reduction in serum progesterone levels. These are responsible for the DPTCl-induced implantation failures. The hormonal regimen consisting of progesterone and estrone supported decidual development in ovariectomized rats given DPTCl (Ema and Miyawaki 2002). The pregnancy rate and number of implantations in groups given DPTCl at 16.5 or 24.3 mg/kg in combination with progesterone were higher than those in the groups given DPTCl alone. These results show that the DPTCl-induced suppression of uterine decidualization is mediated, at least partially, by ovarian hormones, and that progesterone protects against the DPTCl-induced implantation failure.

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.