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# Molecular Targets of Organotin Compounds in Endocrine Disruption: Do Organotin Compounds Function as Aromatase Inhibitors in Mammals?

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Tributyltin (TBT) and triphenyltin (TPT) cause masculinization in female mollusks. These compounds may act as potential competitive inhibitors of aromatase, which converts androgens to estrogens, although effective concentrations are high. TBT and TPT may, therefore, increase the levels of unconverted androgens in invertebrates and vertebrates. However, at concentrations effective for aromatase inhibition, they are generally toxic to mammalian cells. These compounds markedly enhance aromatase activity and human chorionic gonadotropin (hCG) production, along with their mRNA expression, at very low concentrations in human choriocarcinoma cells. In ovarian granulosa cells, these compounds suppress aromatase gene expression at the same low concentrations. Therefore, it is suspected that, in mammals, these organotin compounds affect target molecules that regulate the gene expressions of aromatase and hCG, rather than functioning as aromatase inhibitors. Recently, it has been demonstrated that TBT and TPT directly bind to the retinoid X receptor (RXR) and the peroxisome proliferator-activated receptor (PPAR) γ with high affinity and function as transcriptional activators. These compounds promoted adipocyte differentiation, which is triggered by the PPAR γ/RXR signaling pathway. They may, therefore, exert their toxic effects through the activation of these pathways in mammals. Here, we review the potential endocrine disruption of organotin compounds via these nuclear receptors in mammals.

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## 1. Introduction

Organotin compounds, such as tributyltin (TBT) and triphenyltin (TPT), have been widely utilized as biocides, agricultural fungicides, wood preservatives, and disinfecting agents in circulating industrial cooling waters and in antifouling paints for marine vessels.[1,2] Human exposure to these organotin compounds may result from the consumption of organotin-contaminated meat and fish products, occupational exposure during the manufacture and formulation of organotin compounds, or the application or removal of organotin-containing paints.[3,4] Potential human exposure to organotins has therefore aroused great concern about potential toxicities. Most of the toxic effects of organotin compounds on sexual development and reproductive function have been documented in mollusks. For example, female neogastropod snails have been observed to suffer from irreversible sex organ alterations, a phenomenon known as "imposex."[5,6] These abnormalities are the result of a masculinization process by which male sex organs develop, notably a penis and a vas deferens. In certain species, the growth of a vas deferens disrupts the structure and function of the oviducts, preventing normal breeding activity and causing population decline. Imposex has now been established as a form of endocrine disruption caused by elevated testosterone titers, leading to masculinization in organotin-exposed females.[7–9] The precise mechanism by which testosterone levels are increased has not been fully elucidated, but the many lines of evidence suggest that organotin compounds act as competitive inhibitors of aromatase activity.[8] Some recent data suggest that organotin compounds may also inhibit the formation of sulfur conjugates of testosterone and active testosterone metabolites.[9] Therefore, it has been theorized that organotin compounds also increase androgen levels through the inhibition of aromatase or the suppression of androgen excretion in mammals.

However, in gastropods, sex steroid receptors and aromatase have not yet been identified, and it remains unclear whether sex steroid hormones are critical factors for sexual development and reproduction. Furthermore, homologues of both the estrogen receptor (ER) and androgen receptor (AR) have not been found in invertebrates[10] and the composition of nuclear receptor family members is very different between vertebrates and invertebrates.[10,11] Therefore, there is some doubt as to whether organotin compounds function as inhibitors of enzymes that metabolize androgens in gastropods, and this doubt led us to suspect that organotin compounds affect other target molecules in mammals.

## 2. Do Organotin Compounds Function as Aromatase Inhibitors in Mammals?

Aromatase, an enzyme complex of the endoplasmic reticulum, catalyzes the biosynthesis of C18-estrogens (17β-estradiol, estrone, and estradiol) from C19-steroids (testosterone, androstenedione, and 16α-hydroxyandrostenedione). This enzyme complex is comprised of the ubiquitous flavoprotein NADPH-cytochrome P450 and a unique form of the cytochrome P450 (P450arom, the product of the *CYP19* gene) that is expressed exclusively in estrogen-producing cells.[13–15] In humans, there appears to be a single *CYP19* gene.[16] Homozygous mutations of the *CYP19* gene result in the virilization of female fetuses in utero and subsequent primary amenorrhea, whereas in males there is continued linear bone growth after puberty, delayed bone age, and a failure of epiphyseal closure.[16] Excessive activity

of aromatase has also been shown to increase the risks of breast and endometrial cancers[17] and endometriosis.[18] Hence, alterations of aromatase function induced by environmental contaminants might cause the above-mentioned failures in the human endocrine system.

As described in the foregoing paragraph, it is believed that organotin compounds are potential aromatase inhibitors. Can organotin compounds inhibit the catalytic activity of mammalian aromatase? The answer to this question seems to be 'yes.' Heidrich *et al.*[19] and Cooke[20] reported that butyltins exhibit a structure-related inhibition of human aromatase activity at concentrations of at least 1 μM. Using microsomes from human choriocarcinoma JAr cells and an NADPH experimental system, Nakanishi *et al.* have also confirmed that both TBT and TPT inhibit human aromatase activity at above 1 μM.[21] However, at concentrations effective for aromatase inhibition, TBT and TPT are generally toxic to mammalian cells because they cause apoptosis or necrosis.[21–23] In the human choriocarcinoma cell lines JAr, JEG-3, and BeWo, exposure to greater than 1 μM TBT or TPT markedly decreases DNA (Fig. 1) and protein syntheses.[21] Concentrations under 1 μM of either organotin compound did not significantly affect aromatase activity in microsomes isolated from JAr cells. These results suggest that we have to consider the toxicities of organotin compounds in distinguishing between the nonspecific toxicity to cells and the specific inhibition of steroidogenic enzymes.

## 3. Organotin Compounds Affect the Gene Expression of Human Aromatase and hCG

In a recent study, Nakanishi *et al.* investigated the effects of organotin compounds on aromatase activity in human choriocarcinoma JAr (Fig. 2), JEG-3, and BeWo cells.[21] In all

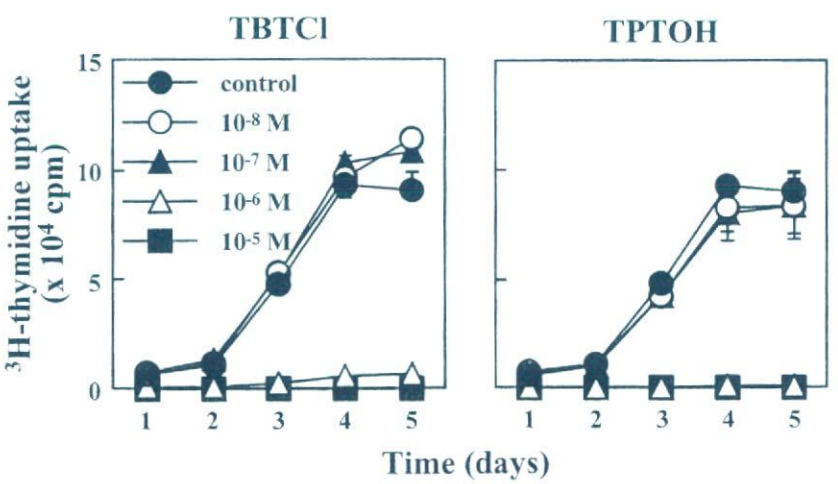


Fig. 1. Effect of organotin compounds on DNA synthesis in JAr cells. Cells ($10^3$ cells/well) were cultured in 96-well microtiter plates. After 24 h of culture, cells were treated with various concentrations of TBT chloride (TBTCl) or TPT hydroxide (TPTOH) for 1 to 5 days. Each culture was pulsed with 20 kBq of $^3$H-labeled thymidine for 2 h before the cells were harvested. The $^3$H-count incorporated into cells was determined by liquid scintillation. Results are expressed as means ± S.D. of triplicate cultures.

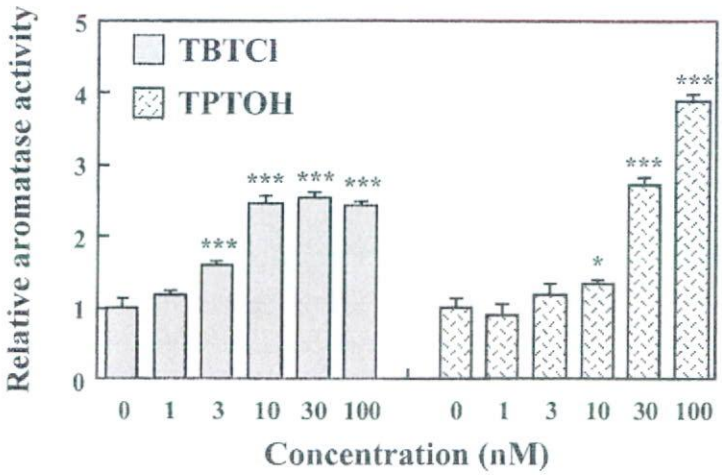


Fig. 2. Effect of organotin compounds on aromatase activity in JAr cells. Cells ($1.5 \times 10^5$ cells/well) were seeded in 12-well plates. After 24 h of culture, cells were treated with various concentrations of TBTCl or TPTOH for 48 h. At the end point of each treatment, cells were washed and aromatase activity was then determined by a tritium release assay, which measures the production of $^3H_2O$ formed as a result of the aromatization of the substrate [1β-$^3$H] androst-4-ene-3,17-dione. Results are expressed as means ± S.D. of triplicate cultures.[21] *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.005$ significantly different from vehicle controls.

cell lines, both TBT and TPT increased aromatase activity and mRNA expression in a dose-dependent fashion following exposure to nontoxic concentration ranges (from 3 to 100 nM). These results indicate that the observed organotin-induced alterations in human choriocarcinoma cells are due to the regulation of aromatase mRNA levels, not the regulation of the aromatase enzyme complex. In addition, these organotin compounds also markedly stimulated hCG production in the same concentration range, along with its mRNA expression (Fig. 3).[21] These results suggest that organotin compounds are potent stimulators of human placental hCG production and aromatase activity *in vitro* and that the placenta represents a potential target organ in pregnant women for organotin compounds, the endocrine-disrupting effects of which might be the result of local changes in hCG and estrogen concentrations.

In contrast to the above results, however, Saitoh *et al.* reported that 20 ng/ml (about 60 nM) TBT and TPT suppressed both the activity and gene expression of aromatase in the ovarian granulose-like cell line KGN.[22] This discrepancy in the action of the organotins on the gene expression of human aromatase is due to the tissue-specific expression of aromatase, which is strictly regulated. Human *CYP19* is a single-copy gene composed of 10 exons (exons II to X encode the aromatase protein), as well as the 3′ untranslated region of mRNA common to all estrogen-producing tissues.[13] A number of variations of exon I exist. These encode the 5′ untranslated regions of various *CYP19* mRNAs, which are selectively expressed in some tissues by alternative splicing.[13,24,25] The tissue-specific expression of *CYP19* in humans appears to be mediated by tissue-specific promoters lying upstream of the respective exon I sequences and by transcription factors binding to specific regions of each promoter. In the placenta, *CYP19* is driven by the placental major promoter (I.1), and the transcript contains exon I.1, located approximately 89 kb upstream from exon

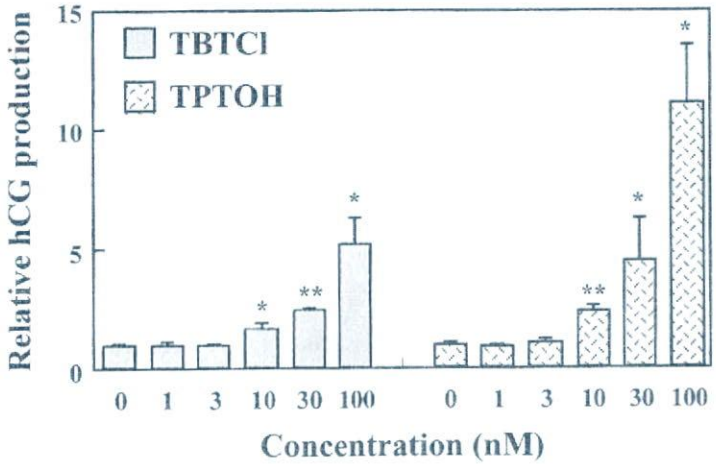


Fig. 3. Effect of organotin compounds on hCG secretion in JAr cells. Cells ($4 \times 10^4$ - 25 cells/well) were seeded in 48-well plates. After 24 h of culture, cells were treated with various concentrations of TBTCl or TPTOH for 48 h. At the end point of each treatment, cells were washed three times and cultured in fresh medium for another 24 h. Culture supernatant was collected, and hCG was determined using ELISA. Results are expressed as means ± S.D. of triplicate cultures.[21] *: $P < 0.05$; **: $P < 0.01$ significantly different from vehicle controls.

II. On the other hand, ovarian transcripts contain a sequence at the 5′-end immediately upstream of the translation start site, because the expression of the gene in the ovary uses a proximal promoter (II). In ovarian granulosa cells, the expression of *CYP19* is strongly regulated by the steroidogenic tissue-specific transcriptional factor Ad4Bp/SF-1, via promoter II. In contrast, Ad4Bp/SF-1 is expressed at very low levels in the human placenta and may not play an important role in the activation of the placental major promoter I.1.[26,27] Saitoh *et al.* suggest that the effects of organotin compounds in KGN cells are caused partly by association with Ad4Bp/SF-1. It is therefore likely that the action of organotin compounds in human placental cells is induced by a pathway clearly different from that in ovarian granulosa cells, giving rise to the promotion of aromatase activity and mRNA expression.

In human placental cells, both hCG production and aromatase activity are controlled by cAMP-dependent intracellular signal pathways. However, neither TBT nor TPT exerted any effect on intracellular cAMP production.[21] In addition, there is little possibility that these organotin compounds affect the cAMP-protein kinase A (PKA) pathway in the human ovary, because the cAMP-PKA pathway stimulates aromatase gene expression in the ovary through promoter II.[28] The possible target of these organotin compounds may be a signaling pathway common to the gene expressions of both hCG and aromatase in the human placenta and ovary.

## 4. Organotin Compounds Function as PPAR γ or RXR Agonists

Nuclear receptors play important roles in the maintenance of the endocrine system, the regulation of organ differentiation, and fetal development. Reproductive abnormalities in

wildlife can be associated with exposure to environmental pollutants capable of mimicking the action of natural hormones. Because the nuclear receptors of intrinsic hormone systems are likely to be the targets of industrial chemicals, information on their ability to bind these chemicals is valuable for environmental risk assessment. Recently, Kanayama *et al.* have reported assay systems for human nuclear receptors to determine whether suspected endocrine disruptors can bind to members of the nuclear receptor family on the basis of the previously described CoA-BAP system.[29,30] Using these systems, they found that TBT and TPT were potential agonists of RXR and PPAR γ.[30] In addition, these compounds also induced the transactivation function of RXR and PPAR γin mammalian culture cells. The effectiveness of each organotin compound was comparable to that of the natural ligand of RXR, 9-cis retinoic acid (9cRA) or the well-known PPAR γ ligand rosiglitazone (Rosi) (Fig. 4).[30] The dose ranges of TBT and TPT that induced the transactivation were 10–100 nM, which do not cause a significant apoptosis or necrosis of mammalian culture cells in general. These results indicate that these organotin compounds function as RXR or PPAR γ agonists in mammalian cells.

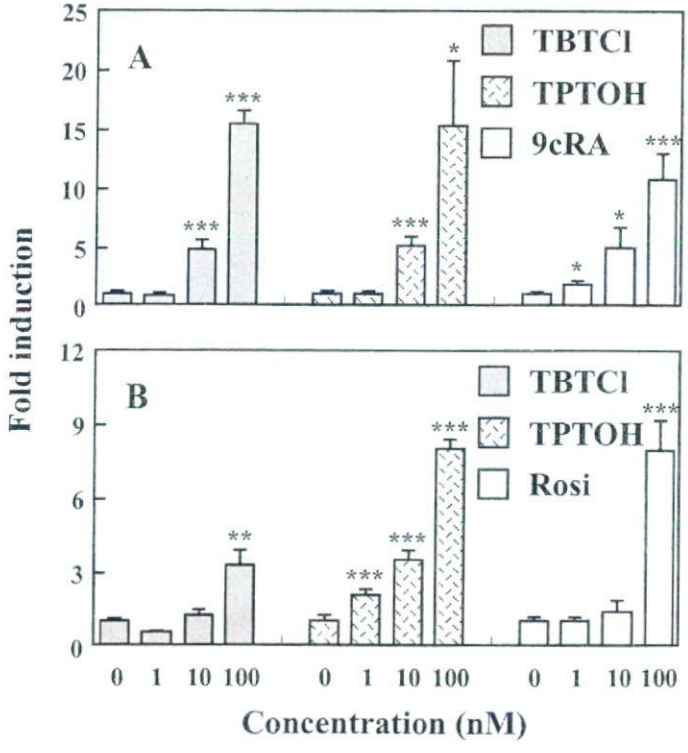


Fig. 4. Organotin compounds induce transcriptional activity through RXR α and PPAR γ. Ligand-dependent transactivation of RXR α and PPAR γ was detected as luciferase activity. (A) JEG-3 cells were cotransfected with a GAL4-DBD-RXR α expression plasmid and a GAL4-responsive reporter plasmid. (B) JEG-3 cells were cotransfected with a GAL4-DBD-PPAR γ1 expression plasmid and a GAL4-responsive reporter plasmid. Luciferase activities relative to Renilla luciferase activity are shown and represent the fold stimulation compared with the activity of the vehicle-only control. Data are shown as means ± S.D. of four independent experiments. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.005$ significantly different from vehicle controls.

The RXR stands out as unique members of the type II nuclear receptor subfamily and play dual roles in nuclear receptor signaling. On one hand, they can bind to their own response element (RXR response element) as a homodimer and activate transcription in response to their ligands, and on the other hand, they serve as partners for other nuclear receptors.[31–33] The existence of three types of heterodimers — fully permissive, conditionally permissive, and nonpermissive — has been described. In the first case, the PPARs/RXR, farnesoid
X-activated receptor/RXR, and liver X receptor/RXR heterodimers exhibit dual ligand permissivity, because they can be activated by the agonists of either RXR or its partner receptor, or both, in a more-than-additive fashion.[34–40] As an example of the second type, the RXR/retinoic acid receptor (RAR) heterodimer exhibits conditional permissivity because a full response to RXR agonists occurs only in the presence of an RAR agonist.[37,41] The third type is the nonpermissive heterodimer, such as the RXR/thyroid hormone receptor (TR) and RXR/vitamin D receptor, which cannot be activated by RXR agonists regardless of the presence (or absence) of the agonist of its partner receptor; the formation of the heterodimer is actually thought to preclude the binding of the ligand to RXR.[42,43] TBT and TPT simulated the transactivations of an RXR homodimer and the PPAR γ/RXR heterodimer at nontoxic concentration ranges (from 10 to 100 nM), whereas they had no effect on the transactivations of RXR/TR and RXR/RAR heterodimers.[44] In particular, these organotin compounds activated PPAR γ/RXR heterodimers more strongly than Rosi, because these compounds may function not only as RXR agonists but also as PPAR γagonists (our unpublished data). Although the effects of organotin compounds on the transactivation of permissive RXR heterodimers other than PPAR γ/RXR have not been determined, it is probably possible to stimulate the transactivation of other heterodimers because these compounds function as RXR agonists.

PPAR γ is activated by a variety of fatty acids and a class of synthetic antidiabetic agents, the thiazolidinediones.[45] PPAR γ serves as an essential regulator of adipocyte differentiation and lipid storage in mature adipocytes.[46] In light of these findings, Kanayama *et al.* evaluated the effects of TPT and TBT on adipogenesis and found that these organotins stimulate the differentiation of preadipocyte 3T3-L1 cells into adipocytes. Taken together, these findings suggest that organotins exert their toxic effects to function as RXR or PPAR γ agonists in mammalian cells.

## 5. Possible Endocrine Disruption by Organotin Compounds through RXR or PPAR γActivation in Mammals

The gene expression of human aromatase is regulated by the activation of PPAR γ or RXR. In the human placenta, a selective RXR ligand, LG69, stimulates aromatase gene expression, whereas a selective PPAR γ ligand, BRL49653 (Rosi), has little or no effect on aromatase gene expression.[47] Unlike in the placenta, both RXR- and PPAR γ-selective ligands suppress aromatase gene expression in the ovary.[48–50] Because the aromatase expression pattern induced in the human placenta and ovary by the activation of PPAR γ or RXR is similar to that induced by organotin compounds, aromatase expression regulated by organotin compounds may involve the activation of PPAR γ or RXR.[21,22] It has already been

found, as supportive evidence, that organotin compounds stimulate the expression of a luciferase reporter construct containing the human placental promoter I.1 sequence of aromatase via a ligand-dependent signaling pathway of RXR.[44]

The exposure of rats *in utero* to TBT induces a sharp increase in the incidence of low-birth-weight fetuses because of maternal hypothyroidism.[51] Furthermore, the RXR agonist bexarotene causes clinically significant hypothyroidism in patients with cutaneous T-cell lymphoma,[52] and the experimental exposure of rats to LG100268 (a selective RXR agonist) induces the acute phase of hypothyroidism.[53] The similarities between the toxicities of TBT and selective RXR agonists suggest to us that at least some of the toxic effects of organotin compounds are mediated by RXR.

Yamabe *et al.* reported that TBT and TPT enhance the proliferation of androgen-dependent human prostate cancer cells and the transactivation of AR.[54] However, the AR antagonist flutamide cannot inhibit organotin-mediated AR transactivation,[54] and these organotin compounds do not function as AR agonists in a yeast two-hybrid system (our unpublished data). Only recently, RXR has been found to function as a novel coregulator for AR, and 9cRA was found to inhibit AR activity through the activation of RXR.[55] It remains unclear whether the coregulators recruited by organotin-activated RXR are different from those recruited by 9cRA, but RXR activation by organotins might be involved in the AR transactivation induced by them.

Taken together, these compounds may be potent endocrine disruptors in mammals through the activation of PPAR $\gamma$ or RXR because of the above-described toxic effects of organotin compounds in human cells and experimental animals.

## 6. Conclusions

Although organotin compounds inhibit the enzymatic activity of aromatase, their effective concentrations are toxic to mammalian cells. In this review, we have proposed the activation of PPAR $\gamma$ or RXR as a novel mechanism for organotin-induced-endocrine disruption in mammals. In addition, Nishikawa *et al.* have recently reported that RXR plays an important role in the development of gastropod imposex, by showing the cloning of an RXR ortholog from a marine gastropod, the binding of organotins to that receptor, and imposex induction by the injection of 9cRA.[56] These findings indicated that RXR activation is also a critical event for the endocrine disruption of organotins in gastropods. However, it is possible that organotin compounds affect target molecules other than PPAR $\gamma$ and RXR. For instance, organotin compounds have been shown to enhance histone acetyltransferase activity.[57] Further studies are needed to clarify the precise mechanism of the action of organotin compounds in mammals in endocrine disruption *in vitro* and *in vivo*, because the toxic mechanisms of organotin compounds appear to be intricate.

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RESEARCH ARTICLE

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# Imposex in marine gastropods may be caused by binding of organotins to retinoid X receptor

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**Abstract** Organotin compounds have been widely used as antifouling paints for ships and fishing nets since the 1960s and have thus been released into marine environments. Aquatic invertebrates, particularly marine gastropods, are extremely sensitive to organotin compounds such as tributyltin (TBT) and triphenyltin (TPT) and undergo changes in sexual identity in response to exposure. This worldwide phenomenon is one of the worst consequences of pollution by man-made chemicals and has led to the ban of such compounds in antifouling paints in a number of countries, although organotin compounds still exist in the environment. So far, very low-concentrations of TBT or TPT have been shown to induce imposex (superimposition of male genitalia on female) in marine gastropods. Although the imposex induction mechanism has been controversial for many years, it was recently reported that TBT and TPT are potent and efficacious activators of retinoid X receptor (RXR), a member of the nuclear receptor superfamily. In this review, I discuss the involvement of RXR in the development of gastropod imposex.

## Introduction

In their book "Our Stolen Future", Colborn et al. (1996) pointed out that a number of environmental chemicals affect hormonal systems and have adverse health effects on wildlife and probably on humans. Such chemicals are referred to as endocrine disruptors, and their effects have emerged as a major environmental issue. The nuclear receptors of intrinsic hormone systems are likely to be targets of endocrine disruptors, because their intrinsic ligands are fat-soluble and low-molecular-weight agents, as are the environmental pollutants. Many synthetic compounds, including the drug diethylstilbestrol (DES), dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCB), and alkylphenols, have been shown to bind nuclear receptors (Sohoni and Sumpter 1998; Blair et al. 2000; Nishihara et al. 2000; Gray et al. 2001). The effects of synthetic chemicals on sex hormone receptors such as the estrogen receptor (ER) and androgen receptor (AR) have attracted much attention, focusing on the reproductive failures observed in wildlife.

Organotin compounds such as tributyltin (TBT) and triphenyltin (TPT) have been used worldwide in antifouling paints for ships and fishing nets since the mid-1960s. Their release into the marine environment has resulted in pollution worldwide. Most marine gastropods in organotin-polluted areas have shown reproductive failure due to oviduct blockage by vas deferens formation, resulting in population decline or mass extinction (Bryan et al. 1986; ten Hallers-Tjabbes et al. 1994). This phenomenon is called "imposex" as an abbreviation of "imposed sexual organs", because male genital organs, such as the penis and vas deferens, are imposed upon female organs (Smith 1971). Approximately 150 species of imposex-affected gastropods have been found in the world (Fent 1996; Matthiessen et al. 1999). Gastropod imposex is reportedly induced by very low concentrations of TBT or TPT and is thought to be one of the mechanisms of endocrine disruption in wildlife (Smith 1971; Bryan et al. 1986, 1987, 1988; Gibbs and Bryan 1986; Gibbs et al. 1987; Axiak et al. 1995; Horiguchi et al. 1997b). Despite several hypotheses on the cause of imposex induction, such as aromatase inhibition, testosterone excretion-inhibition, functional disorder of the female cerebropleural ganglia, and involvement of amidated tetrapeptide Ala-Pro-Gly-Trp-NH2 (APGWamide) (Bettin et al. 1996; Ronis and

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Mason 1996; Oberdörster and McClellan-Green 2000, 2002), the detailed biochemical mechanism behind this phenomenon remains obscure.

It is well known that steroidal sex hormones such as 17$\beta$-estradiol ($E_2$) and 5$\alpha$-dihydrotestosterone (DHT) exert important roles in physiological processes, including sexual development and reproduction in vertebrates. However, homologues of ER and AR have not been found in invertebrates (Escriva et al. 1997). Because gastropods are mollusks, they may not have functional receptors for androgen, suggesting that vertebrate-type sex hormones may not be involved in male sexual development in the gastropods. Recently, it was reported that TBT and TPT are high-affinity ligands for human retinoid X receptor (RXR) and peroxisome proliferator-activated receptor (PPAR) $\gamma$ (Kanayama et al. 2005). In addition, a functional homologue of RXR has been cloned from the rock shell (*Thais clavigera*) and the natural ligand of RXR, 9-*cis* retinoic acid, induces imposex in this species (Nishikawa et al. 2004). These reports suggest that the induction of imposex by organotin compounds may be mediated by RXR.

## Differences in nuclear receptors between invertebrates and vertebrates

Nuclear receptors are structurally related proteins classified into a large superfamily that includes receptors for hydrophobic molecules such as steroid hormones (e.g., estrogens, androgens, progesterone, glucocorticoids, mineralocorticoids), retinoic acids (all-*trans* and 9-*cis* isomers), thyroid hormone, 1,25 $(OH)_2$ vitamin $D_3$, fatty acids. In addition to these receptors, the superfamily also contains a large number of so-called orphan nuclear receptors whose ligands do not exist or have not been identified (Giguére 1999). Nuclear receptors share a common structural organization with a highly conserved DNA-binding domain and a moderately well-conserved ligand-binding domain (LBD) (Fig. 1). Phylogenetic study and extensive polymerase chain reaction (PCR) surveys have revealed that nuclear receptor genes appeared very early on during metazoan evolution, but could not be found in fungi, plants, or unicellular eukaryotes (Escriva et al. 1997, 2000). By virtue of genome projects, we now know that *Homo sapiens*, *Drosophila melanogaster*, and *Caenorhabditis elegans*, respectively, have 48, 21, and 220 kinds of nuclear receptor genes (Maglich et al. 2001). There is a striking difference between vertebrates and invertebrates with respect to their nuclear receptor sets. For instance, receptors for sex and adrenal steroid hormones have not been found in any fully sequenced invertebrate genomes. Although ER-like cDNA was reportedly isolated from the mollusk *Aplysia californica*, it could not bind to estrogens and was a constitutive activated transcription factor like the orphan nuclear receptors (Thornton et al. 2003). So far, functional steroid hormone receptors including AR, ER, progesterone receptor (PR), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR), have not been found in any invertebrate species (Escriva et al. 1997; Laudet 1997).

Reproductive abnormalities in wildlife can be associated with exposure to environmental pollutants capable of mimicking the action of sex hormones. In fact, there are many synthetic chemicals that have been shown to possess estrogenic activity by in vitro binding assay, reporter gene assay, or uterotrophic assay. The typical characteristic of chemicals having estrogenic activity is a phenol with a hydrophobic moiety at the para-position and without bulky groups at the ortho-position (Blair et al. 2000; Nishihara et al. 2000). Although these compounds may have adverse health effects in vertebrates (Colborn et al. 1996), they may not alter the function of the reproductive system through the medium of ER in invertebrates.

## Imposex in marine gastropods

Among the variety of endocrine-disrupting events in marine invertebrates, imposex is one of the most documented. Imposex is induced by TBT at concentrations as low as 1 ng/L of tin (Sn) (Gibbs et al. 1987; Axiak et al. 1995) and is used extensively all over the world as a biomarker to monitor TBT pollution (Gibbs et al. 1987; ten Hallers-Tjabbes et al. 1994; Horiguchi et al. 1997a; Terlizzi et al. 1998, 2004). Not only TBT but also TPT has been shown to have a strong effect on the development of imposex in *T. clavigera* (Horiguchi et al. 1997b). So far, several hypotheses have been proposed to explain imposex induction. The first is that TBT increases androgen levels by inhibiting the enzyme activity that metabolizes testosterone. An aromatase enzyme complex is responsible for converting androgenic to estrogenic steroids. This enzyme complex consists of the microsomal CYP19 enzyme and the flavoprotein nicotinamide adenine dinucleotide phosphate reduced-form reductase. The latter is responsible for transferring reducing equivalents to CYP19 within the membrane of the endoplasmic reticulum. Bettin et al. (1996) reported that TBT increases androgen levels through inhibition of aromatase activity in marine neogastropods at relatively high doses. The TBT also inhibits the catalytic activity of human aromatase from transfected cells or a granulosa cell-like tumor cell line (Cooke 2002; Heidrich et al. 2001; Saitoh et al. 2001). However, it is doubtful whether the inhibitory effect of TBT on aromatase activity is a cause of the imposex, because the role of vertebrate sex steroids is unclear in invertebrates (LeBlanc et al. 1999). The second hypothesis is that TBT acts as a neurotoxin to abnormally release the peptide hormone termed penis morphogenic factor (PMF) (Féral and Le Gall 1983). The peptide hormone APGWamide has been proposed as the putative PMF, because injection of APGWamide significantly induces imposex in the mud snail *Ilyanassa obsoleta* (Oberdörster and McClellan-Green 2000,

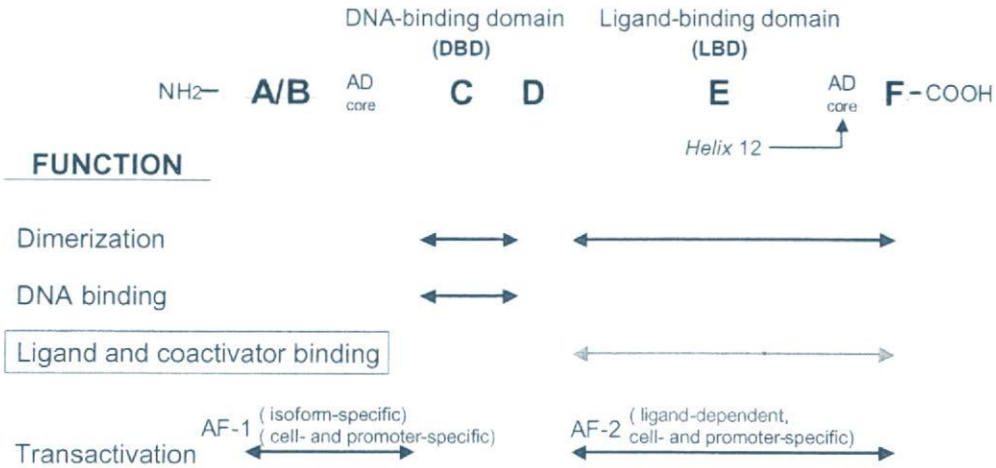


**Fig. 1** Typical structure and functional domain of a nuclear receptor. Nuclear receptors are highly structurally related and share a common structural organization with a variable amino-terminal domain (**a**/**b**); a central, well-conserved DNA-binding domain (**c**); a non-conserved hinge domain (**d**); and a carboxyl-terminal, moderately conserved ligand binding domain (**e**). The ligand-independent transactivation function (**af**-1) is contained within the **a**/**b** region, and the ligand-dependent transactivation function (**af**-2) is within the **e** region

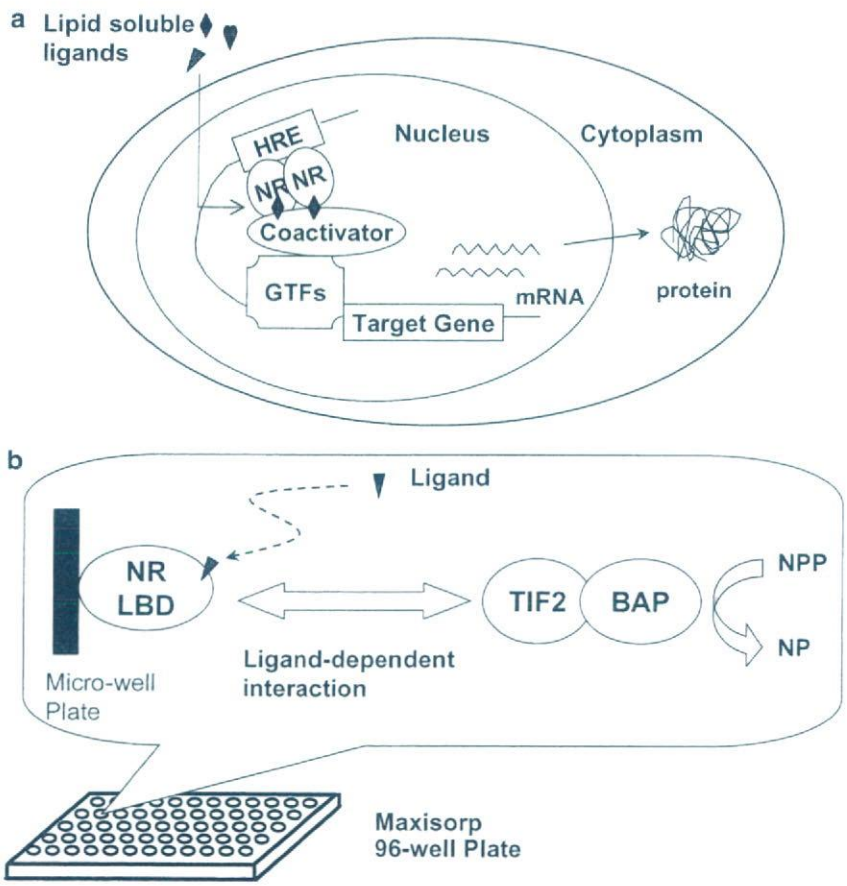


**Fig. 2** **a** Nuclear receptors act as ligand-activated transcription factors by directly interacting with DNA-response elements of target genes as homodimers, heterodimers, or monomers. The effects of nuclear receptors on transcription are mediated through recruitment of co-regulators. Upon ligand binding, the receptors undergo a conformational change that allows the recruitment of coactivator complex. Recruitment of coactivator complex to the target promoter causes chromatin decomposition and transcriptional activation through interaction with general transcription factors (*GTFs*). **b** Principle of the screening method for nuclear receptor ligand. Nuclear receptor ligand-binding domain (*NRLBD*) is immobilized on the surface of a 96-well microplate. Coactivator TIF2 is prepared as a fusion protein with bacterial alkaline phosphatase (*BAP*). Test chemicals are added to the well with TIF2-BAP fusion protein. If the test chemical works as a ligand, it induces conformational change in NRLBD and recruits the TIF2-BAP on the plate surface. *p*-Nitrophenyl phosphoric acid (*NPP*) is used as a substrate for BAP. The BAP converts NPP to *p*-nitrophenol (*NP*), which appears *yellow*

2002). They proposed that PMF causes the development of male sex characteristics following an external stimulus such as TBT exposure. However, PMF cannot be the primary factor in the induction of imposex symptoms by TBT. There must be something other factor that directly interacts with TBT in the initial step of imposex induction.

## Tributyltin and Triphenyltin as high-affinity ligands for nuclear receptors

Recently, Kanayama et al. (2005), by comprehensive screening of suspected endocrine disruptors versus human nuclear receptors, reported that TBT and TPT are high-affinity ligands for RXR and PPAR$\gamma$. Their screening method was based on the ligand-dependent interaction between nuclear receptors and coactivators (Kanayama et al. 2003). In the initiation step of transcriptional activation, cognate ligands change the three-dimensional conformations of nuclear receptors (Bourguet et al. 1995; Renaud et al. 1995; Brzozowski et al. 1997; Agostini et al. 1998; Nolte et al. 1998). Next, a coactivator is exclusively recruited to its ligand-bound form of the receptor, but not to the ligand-free form (Fig. 2a). Kanayama et al. developed an in vitro detection method for ligand-dependent interaction between coactivator and nuclear receptors and applied it to the high-throughput screening (Fig. 2b). Using this system, they found that several suspected endocrine disruptors affected multiple nuclear receptors simultaneously. Among them, the effects of organotin compounds on RXR and PPAR$\gamma$ were most obvious. The agonistic effect of TBT on RXR was as strong as that of its endogenous ligand, 9-*cis* retinoic acid, and the effect of TPT on PPAR$\gamma$ was as strong as that of its well-known ligand, rosiglitazone. They also showed that TBT and TPT induced the transactivation function of RXR and PPAR$\gamma$ in mammalian culture cells (Kanayama et al. 2005). The dose range of TBT or TPT that induced transcriptional activation was 10–100 nM; this is almost pharmacologically relevant to the range reported to induce imposex in gastropods.

In mammals, PPAR$\gamma$ binds to DNA as a heterodimer with RXR and plays a central role in adipocyte gene expression and differentiation (Tontonoz et al. 1994). The PPAR$\gamma$ is abundantly expressed in adipocytes, and its ligands induce the efficient conversion of fibroblastic cells to adipocytes, as measured by induction of adipocyte-specific genes and lipid accumulation (Lehmann et al. 1995). In fact, TBT or TPT promotes differentiation of mouse preadipocyte 3T3-L1 cells to adipocytes (Kanayama et al. 2005). Therefore, organotin compounds may have adverse health effects on mammals by disturbing the endocrine processes mediated by the PPAR$\gamma$/RXR pathway.

However, as I mentioned before, the composition of members of the nuclear receptor superfamily is quite different between vertebrates and invertebrates. The subgroup members of thyroid hormone receptor (TR), retinoic acid receptor (RAR), vitamin D receptor (VDR), and PPAR appear to have been late acquisitions during the evolution of the nuclear receptor superfamily (Escriva et al. 1997; Laudet 1997). Therefore, PPAR$\gamma$ might not be present in marine gastropods (Fig. 3). In contrast, RXR is special among the nuclear receptor superfamily. It is widely conserved in the evolutionary tree and its homologue, called ultraspiracle (USP), is found even in arthropods (Laudet 1997).

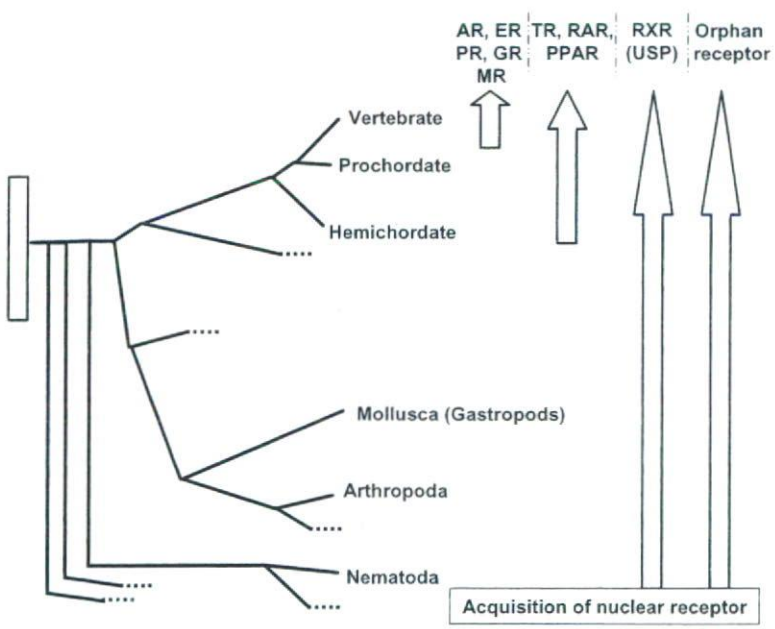


**Fig. 3** Evolutionary tree and acquisition of nuclear receptors. Steroid hormone receptors (*AR* androgen receptor, *ER* estrogen receptor, *PR* progesterone receptor, *GR* glucocorticoid receptor, *MR* mineralocorticoid receptor) exist only in vertebrates. The subfamily of TR, RAR, and PPAR are present in vertebrates to hemichordates. In contrast, RXR or its homologue USP exist even in insects and nematodes

## Characteristics of gastropod retinoid X receptor

Retinoid X receptor homologue has been cloned from *T. clavigera* (Nishikawa et al. 2004). Gastropod RXR has a DNA binding domain (DBD) composed of two $C_2C_2$-type zinc finger motifs and a putative LBD in the C-terminal region (Fig. 4a). The highest similarity with other species is in the DBD, where 85–90% of the amino acids residues are identical (Fig. 4b). The LBD of gastropod RXR also shows considerable similarity with that of vertebrate RXRs but has much less similarity with USP, the RXR homologue first found in *D. melanogaster*. Although RXR binds 9-*cis* retinoic acids in organisms ranging from cnidarians (*Tripedalia cystophora*) to vertebrates, USP from arthropods is unable to do so (Heyman et al. 1992; Mangelsdorf et al. 1992; Henrich and Brown 1995; Kostrouch et al. 1998). As expected by the similarity of a gastropod homologue to vertebrate RXR, the binding of gastropod RXR to 9-*cis* retinoic acid has been confirmed experimentally (Nishikawa et al. 2004). The dissociation constant in the binding of 9-*cis* retinoic acid to gastropod RXR is 15.2 nM, which is similar to the values reported for vertebrate RXRs (1–10 nM)

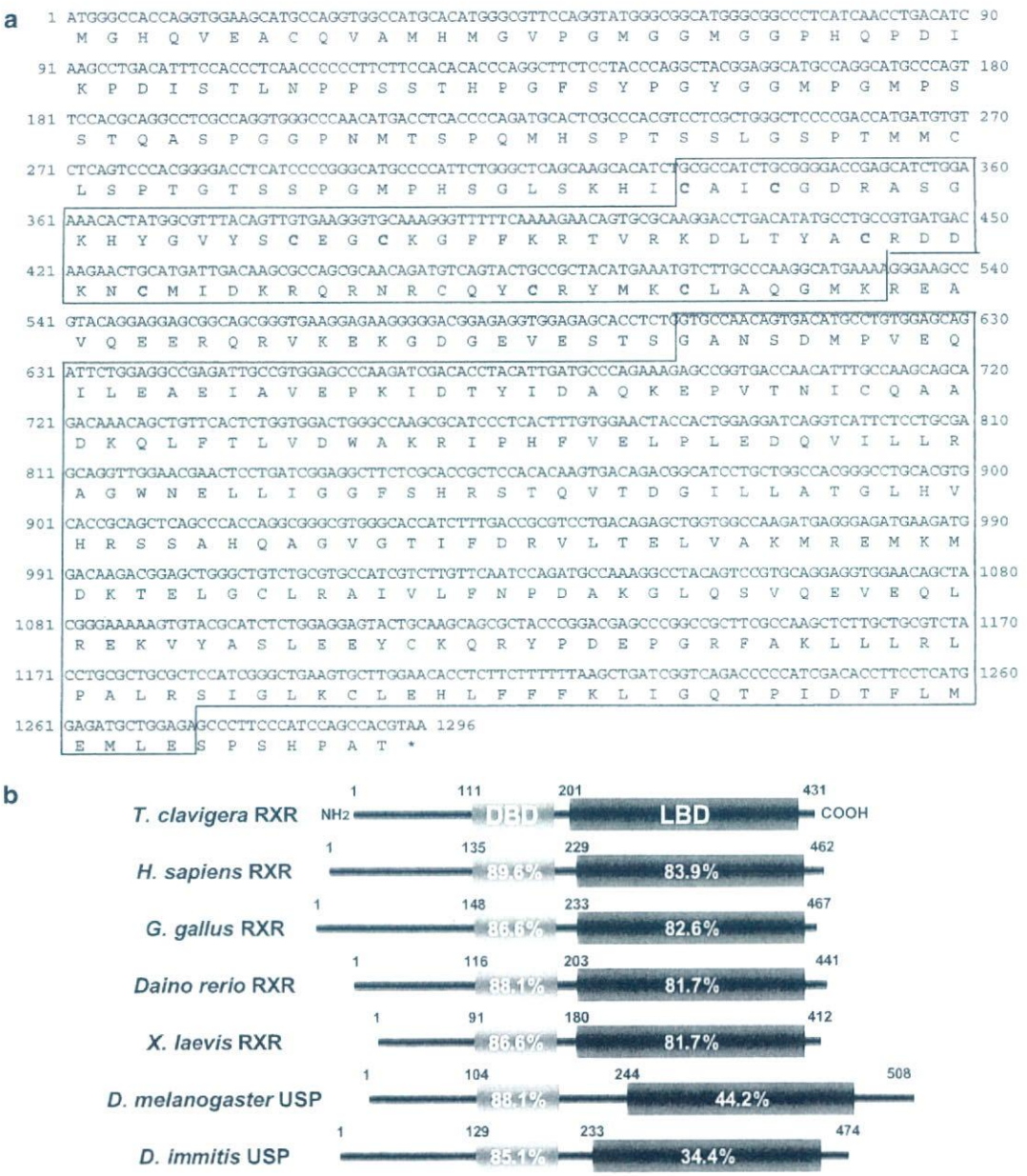


**Fig. 4** **a** The entire coding sequence of gastropod RXR. The DNA and LBDs are *boxed*. The database accession number for the sequence is AY704160. **b** A schematic representation of RXR from various species is shown, along with the percentage of identical amino acid residues shared with those of gastropod RXR

**Table 1** Inhibitory concentrations of 9-*cis* retinoic acid and organotin compounds in binding of radio-labeled 9-*cis* retinoic acid to human or gastropod RXR

| Receptor | Compounds | $IC_{50}$ (μM) |
|---|---|---|
| Human RXRα | 9-*cis* retinoic acid | 0.99 |
| | Tributyltin | 0.99 |
| | Triphenyltin | 0.85 |
| Gastropod RXR | 9-*cis* retinoic acid | 0.81 |
| | Tributyltin | 8.16 |
| | Triphenyltin | 6.49 |

(Heyman et al. 1992). Gastropod RXR also binds to organotin compounds, even though the 50% inhibitory concentration ($IC_{50}$) values are larger than for 9-*cis* retinoic acid (Table 1).

## Development of imposex in *Thais clavigera* by injection of 9-*cis* retinoic acid

Organotin compounds are potent and efficacious agonistic ligands of the vertebrate nuclear receptors RXR and PPARγ (Kanayama et al. 2005). It is worth noting that receptor activation is observed at nanomolar concentrations, whereas other mechanisms of toxicity (e.g., aromatase inhibition) occur in the micromolar range. Furthermore, there is a functional RXR homologue in gastropods that binds to both 9-*cis* retinoic acid and organotin compounds (Nishikawa et al. 2004). These facts suggest that gastropod imposex is mediated by RXR. Consistent with this prediction, Nishikawa et al. observed that 9-*cis* retinoic acid, like TPT, actually induces imposex in female gastropods (Nishikawa et al. 2004).

So far, certain neuropeptides have been considered as sex hormones in mollusks, as opposed to steroid hormones in vertebrates (LeBlanc et al. 1999). Oberdörster and McClellan-Green have demonstrated that only APGWamide, out of four neuropeptides, induces imposex in the mud snail (Oberdörster and McClellan-Green 2000). They put forward the model that the peptide hormones control the release of fat-soluble hormones, similar to the feedback control of the hypothalamic-pituitary axis in vertebrates (Oberdörster and McClellan-Green 2002). It is possible that APGWamide regulates the expression of some cytochrome P450 (CYP) that catalyzes the transformation of retinoids into active forms such as 9-*cis* retinoic acid.

## Perspectives

This review has explored the concept that organotin-induced imposex in marine gastropods is mediated by nuclear receptors. This can be understood in terms of the molecules and mechanisms that regulate male sexual development in mollusks. Vertebrate-type steroid hormone receptors, including AR, are absent in invertebrates, suggesting that androgens may not act as

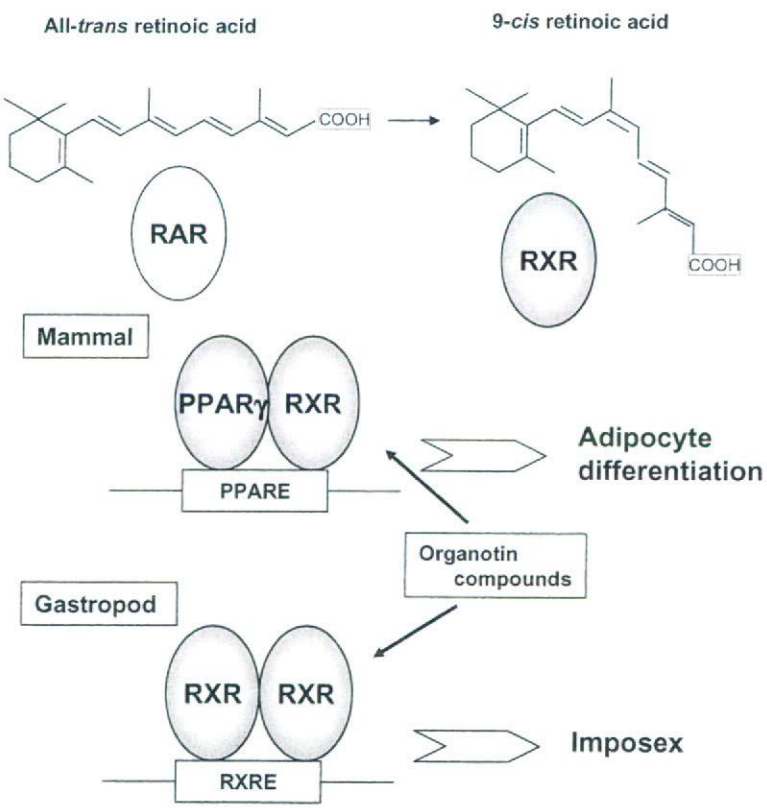


**Fig. 5** Effects of organotin compounds in mammals and gastropods via RXR. In mammals, organotin compounds are ligands for both PPARγ and RXR and affect adipocyte differentiation. In gastropods, organotin compounds induce imposex by binding to RXR

male sex hormones. The TBT or TPT stimulates the development of the male genital tract in female gastropods. The TBT and TPT are high-affinity ligands for RXR. Gastropods have a functional homologue of RXR. The 9-*cis* retinoic acid, a natural ligand of RXR, significantly caused the development of imposex in female rock shells. These results suggest that RXR plays an important role in the induction, differentiation, and growth of male genital organs in female gastropods (Fig. 5).

In mammals, RXR is known to act both as a ligand-dependent transcription factor and as a common heterodimer partner for many non-steroid nuclear receptors (Mangelsdorf and Evans 1995). In the cases of some heterodimers, RXR is not activated by its own ligand (Mukherjee et al. 1997). In contrast, synthetic RXR-selective ligands activate RXR homodimer-dependent transcription (Boehm et al. 1995). Because 9-*cis* retinoic acid effectively induces imposex, RXR may function as a homodimer in gastropods. Meanwhile, we do not know whether gastropods inherently possess a pathway for the biosynthesis of retinoic acid. Therefore, we do not know whether 9-*cis* retinoic acid is a real hormone or whether similar derivatives are. We need to identify the active compound responsible for male sexual development in gastropods. Even in mammals, 9-*cis* retinoic acid is difficult to detect in vivo and its action remains obscure (Horton and Maden 1995). The study of retinoids in gastropod imposex may provide some insight into the physiological function of 9-*cis* retinoic acid.

Knock-out mice have provided important information on the physiological functions of these receptors. There are three subtypes of RXR in mammals. RXR$\alpha$ null mice die at embryonic days 12.5–16.5 and exhibit a hypoplastic ventricular myocardium as well as conotruncal and ocular abnormalities (Kastner et al. 1994; Sucov et al. 1994). Approximately 50% of RXR$\beta$ null mice die before or at birth, and the remaining male null mutants are sterile, owing to the aberrance of lipid metabolism in the Sertoli cells (Kastner et al. 1996). RXR$\gamma$ null mice are viable and do not display any abnormalies (Krezel et al. 1996). Dysfunction study using RNAi or homologous recombination in gastropods will be needed to determine the role of RXR in the imposex development.

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